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PRE- AND POSTNATAL NUTRITION – TARGET FOR ALLERGY RISK REDUCTION

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ABSTRACT

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Pre- and postnatal nutrition – target for allergy risk reduction

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A rapid increase in allergic diseases in Western societies has led to the conclusion that our modern lifestyle is a risk factor for immune dysregulation. Potential culprits and benefactors are searched among early dietary and microbial exposures, which may act to program later allergic disease. The aim of this thesis was to investigate the role of early maternal and child nutrition in reducing the risk of child allergy.

The study population comprised of 256 mother – child pairs from families with a history of allergy participating in a randomized controlled dietary counseling and probiotic intervention (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) study from early pregnancy onwards. The dietary counseling aimed for a diet complying with dietary recommendations for pregnant and lactating women, with special attention to fat quality.

Maternal dietary counseling was reflected in cord blood fatty acids suggesting better essential fatty acid status in infants in the counseling group. Dietary counseling with probiotics or placebo had no effect on child allergy risk, but associations between maternal diet during pregnancy and breastfeeding and child allergic outcomes were found in secondary analyses. During pregnancy, milk intake was related to decreased and cheese intake to increased risk of child atopic eczema. During breastfeeding, intake of vitamin C was related to increased risk of asthma and intake of egg was related to decreased risk of atopic eczema. The timing of introduction of complementary foods to infant's diet was not associated with risk of atopic eczema, when adjusted with parental opinion of child allergic symptoms (i.e., potential reverse causality).

In conclusion, the results demonstrate that infant fatty acid supply can be modified via maternal dietary changes. In addition, interesting associations of maternal diet with child allergy risk were discovered. However, no difference in the incidence of allergic diseases with dietary counseling was observed. This suggests that more potent dietary interventions might be necessitated to induce clinical risk reduction of allergy. High-risk families can safely adhere to dietary recommendations for pregnant and lactating women, and the results support the current conception that no additional benefit is gained with delaying introduction of complementary feeding.

Key words: Allergic diseases, asthma, atopic eczema, breastfeeding, complementary feeding, dietary counseling, fatty acids, maternal diet, nutrition, pregnancy, probiotics, programming

TIIVISTELMÄ

Katri Niinivirta-Joutsa

Varhainen ravitsemus ja allergian ehkäisy

Lastentautien oppiaine, kliininen laitos, lääketieteellinen tiedekunta ja Turun yliopiston tutkijakoulu, kliininen tohtoriohjelma, Turun yliopisto sekä lasten ja nuorten klinikka, Turun yliopistollinen keskussairaala, Turku, Suomi.

Allergiset sairaudet ovat lisääntyneet länsimaisissa yhteiskunnissa nopeasti, mikä on johtanut päätelmään, että elämäntapamme on riskitekijä immuunijärjestelmän häiriöille. Varhainen ravitsemus ja mikrobialtistus ovat nousseet esille mahdollisina riski- ja suojatekijöinä, jotka voivat vaikuttaa myöhempään riskiin sairastua allergiaan.

Tämän satunnaistetun, kontrolloidun tutkimuksen tarkoituksena oli selvittää äidin ja lapsen varhaisen ravitsemuksen merkitystä lapsen allergiariskin vähentämisessä. Tutkimukseen osallistui 256 äiti-lapsi-paria allergisista riskiperheistä. Kahdelle ryhmälle alettiin varhaisraskauden aikana antaa yksilöllistä ravitsemusohjausta sekä joko probioottivalmistetta (*Lactobacillus rhamnosus* GG ja *Bifidobacterium lactis* Bb12) tai lumevalmistetta. Kontrolliryhmä sai lumevalmistetta ja ohjeita neuvolasta tavalliseen tapaan. Ravitsemusohjauksella tavoiteltiin suositusten mukaista ruokavaliota, ja erityisesti rasvan laatuun kiinnitettiin huomiota.

Äidille annettu ravitsemusohjaus näkyi napaveren rasvahappoprofiilissa, mikä viittaa ravitsemusohjausryhmän parempaan välttämättömien rasvahappojen saantiin. Ravitsemusohjaus, johon liittyi joko probiootti- tai lumevalmiste, ei vaikuttanut lapsen allergiariskiin, mutta toissijaisissa analyyseissä paljastui yhteyksiä äidin ruokavalion ja lapsen allergisten sairauksien välillä. Raskaudenaikainen maidon kulutus oli yhteydessä lapsen pienempään atooppisen ekseeman riskiin, kun taas juuston kulutus oli yhteydessä suurempaan riskiin. Imetyksenaikainen C-vitamiinin saanti oli yhteydessä suurempaan astman riskiin, ja kananmunan kulutus pienempään atooppisen ekseeman riskiin. Lisäruokien aloitusikä ei ollut yhteydessä atooppisen ekseeman riskiin, silloin kun otettiin huomioon vanhempien käsitys lapsen mahdollisista allergisista oireista.

Yhteenvetona voidaan todeta, että äidin ruokavaliolla voidaan vaikuttaa lapsen rasvahappojen saantiin. Lisäksi äidin ruokavaliolla sekä allergisten sairauksien riskillä vaikuttaa olevan yhteys. Koska ravitsemusohjauksella ei ollut vaikutusta allergiariskiin, tämä voisi viitata siihen, että ravitsemusinterventioiden pitäisi olla voimakkaampia toimiakseen. Allergiset riskiperheet voivat turvallisesti noudattaa ravitsemussuosituksia, ja tutkimuksen tulokset vahvistavat käsitystä siitä, ettei lisäruokien viivyttäminen auta allergian ehkäisyssä.

Avainsanat: Allergiset sairaudet, astma, atooppinen ekseema, imetys, lisäruokinta, ravitsemusohjaus, rasvahapot, äidin ruokavalio, ravitsemus, raskaus, probiootit, ohjelmoituminen

TABLE OF CONTENTS

ABSTRACT **4**

TIIVISTELMÄ **5**

TABLE OF CONTENTS **6**

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **9**

1. INTRODUCTION **10**

2. REVIEW OF THE LITERATURE **12**

2.1. Developmental origins of disease 12
2.1.1. Programming 12
2.1.2. Mechanisms of programming 14
2.2. Allergic diseases 15
2.2.1. Definitions 15
2.2.2. Current understanding of risk and protective factors 16
2.2.2.1 Genetic inheritance 17
2.2.2.2 Environmental toxins and pollutants 17
2.2.2.3 Hygiene hypothesis 17
2.2.2.4 Diet 19
2.2.2.5 Breastfeeding 24
2.2.2.6 Pro- and prebiotics 25
2.2.2.7 Summary 29

3. OBJECTIVES OF THE STUDY **30**

4. MATERIALS AND METHODS **31**

4.1. Subjects and the study design 31
4.1.1. Maternal dietary counseling and probiotic intervention 31
4.1.2. Study visits 32
4.2. Evaluation of maternal and child diet 34
4.3. Evaluation of child atopic disease and maternal atopy 35
4.4. Fatty acid analyses 35
4.5. Outcomes 36
4.6. Statistical methods 36
4.7. Ethical aspects 38

5. RESULTS **39**

5.1. Clinical characteristics of the study participants 39

5.2. Allergic diseases during the study 40
5.3. Effects of the dietary counseling and probiotic intervention 42
5.3.1. Maternal diet 42
5.3.2. Child fatty acid profiles (I and II) 42
5.3.3. Allergic manifestations in the child (III) 47
5.4. Dietary exposures defining child allergy 47
5.4.1. Maternal diet during pregnancy (III) 47
5.4.2. Maternal diet during breastfeeding 48
5.4.3. Timing of complementary feeding (IV) 49

6. DISCUSSION 51

6.1. Modification of infant fatty acid status by dietary counseling (I and II) 51
6.2. Nutrition to prevent allergic diseases 52
6.2.1. Probiotic supplementation (III) 53
6.2.2. Diet in pregnancy (III) 54
6.2.3. Diet during breastfeeding 56
6.2.4. Introduction of complementary feeding (IV) 58
6.3. Methodological aspects 59
6.4. Considerations regarding the present results and future studies 61

7. SUMMARY AND CONCLUSIONS 64

ACKNOWLEDGEMENTS 66

REFERENCES 68

APPENDIX 86

ABBREVIATIONS

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
CFU	Colony-forming unit
CI	Confidence interval
E%	% of total daily energy intake
FA	Fatty acid(s)
IL	Interleukin
LGG	*Lactobacillus Rhamnosus* GG
MJ	Mega Joule
MUFA	Monounsaturated fatty acid(s)
NAMI	Nutrition, Allergy, Mucosal immunology and Intestinal microbiota research group
OR	Odds ratio
PUFA	Polyunsaturated fatty acid(s)
SD	Standard deviation
SFA	Saturated fatty acid(s)
Th	Helper T cell
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–IV. The original publications have been reproduced with the kind permission of the copyright holders.

I Niinivirta K, Isolauri E, Laakso P, Linderborg K, Laitinen K. Dietary counseling to improve fat quality during pregnancy alters maternal fat intake and infant essential fatty acid status. *J. Nutr. 141: 1281–1285, 2011.*

II Niinivirta K, Laakso P, Linderborg K, Poussa T, Isolauri E, Laitinen K. Maternal dietary counseling during pregnancy and infant fatty acid profiles. *Int J Food Sci Nutr. 2013 Nov 13 [Epub ahead of print]*

III Niinivirta K, Nermes M, Ilmonen J, Poussa T, Isolauri E, Laitinen K. Relation of maternal diet during pregnancy to the risk of atopic eczema and asthma in the offspring: a 4-year follow-up study. *Submitted.*

IV Niinivirta K, Isolauri E, Nermes M, Laitinen K. Early introduction of solid foods does not increase risk of atopic eczema by 4 years of age in children with family history of allergy. *Acta Paediatr. 103(2):168-173, 2014.*

1. INTRODUCTION

During the last few decades, an allergy epidemic is sweeping over the developed world, with the tides already reaching the developing countries. Allergic diseases, however, are not new to the world. Perhaps the earliest report of allergy is approximately between 3640 and 3300 BC, when King Menses of Egypt succumbed to death after a lethal allergic reaction caused by a wasp sting (Simons, 1994). Another ancient report of the hazards of allergy tells the unfortunate fate of Britannicus, the son of Roman Emperor Claudius, whose allergy to horses prevented him from riding as he couldn't see where he was going (Simons, 1994). Thus, Claudius's adopted son Nero was honored to ride at the head of the young patricians. Later, Nero allegedly massacred Christians and murdered poor Britannicus. Although the contemporary Roman philosopher Lucretius had observed exaggerated responses to common substances and wrote "what is food for some may be fierce poisons for others", the modern era of understanding allergies began only when hay fever was described in the 1800's (Simons, 1994). In 1906, Austrian pediatrician Clemens von Pirquet introduced the word "allergy" to describe "different immunological reaction" (von Pirquet, 1906). The word "allergy" draws from the Greek words "allos" denoting changed or an altered state and "ergon" representing reaction or reactivity, and indeed is an allergic reaction a result of the body's inappropriate reaction to a substance normally tolerated.

Today, it has been estimated that approximately 30 percent to 40 percent of the world's population suffer from one or more allergic diseases (allergic asthma, atopic eczema, allergic rhinoconjunctivitis or food allergy) (Pawankar *et al.*, 2011) and allergy has thus become one of the major health challenges of the 21st century. Paralleling the "true increase" in allergic diseases is no doubt the increased awareness amongst both laymen and medical professionals, but the allergy epidemic is indisputable (Asher *et al.*, 2006). Fortunately, some recent studies have indicated that the increase in asthma and allergy has reached a plateau at least in some countries (Akinbami & Schoendorf, 2002; von Hertzen & Haahtela, 2005), but such reverse has not yet been evident in Finland (Latvala *et al.*, 2005) nor at global level (Anandan *et al.*, 2010). A population study in 2003 revealed that over 40 percent of Finnish pre-school children are sensitized to one or more common environmental antigens (von Hertzen *et al.*, 2006). The rising economic costs to health care and the considerable personal and social burden associated with the increase of allergic diseases has brought about the launch of a Finnish Allergy Programme. Previous Finnish Asthma Programme demonstrated encouraging results in reducing the morbidity of asthma (Haahtela *et al.*, 2006), thus the Allergy Programme aims to reduce the harms and costs inflicted by allergic diseases and to shed some light on the reasons behind allergic diseases (Haahtela *et al.*, 2008).

The causes of allergic sensitization represent complex relationships between genetic inheritance and environmental influences. The increased prevalence of allergic disease

cannot be accounted for a genetic change in the population, as the time span for the increase is relatively short. Additionally, the rise in allergy has been especially rapid in developed countries, thus leading to the conclusion that the westernized life-style might be responsible for this unprecedented surge in allergies (Devereux, 2006). This effect of western lifestyle to promote an allergic phenotype irrespective of the genotype is confirmed in reports of a rapid increase in the prevalence of allergic sensitization amongst children from developing countries after migration to developed countries (Waite *et al.*, 1980) and in the increased prevalence of hay fever and allergic sensitization in children from the former East Germany within a few years of the re-unification of Germany (von Mutius *et al.*, 1998). Therefore most of the recent epidemiological research has focused on identifying which factors in our lifestyle might be associated with the increased prevalence of allergies and to find out the appropriate time frame when to target these factors.

Substantive evidence supports that early events during gestation and postnatal life are critical on the development of the immune system and thus, later susceptibility to certain chronic diseases like allergy (Prescott, 2003; Gluckman *et al.*, 2008; Hanson & Gluckman, 2011). Environment-gene interactions may even cause trans-generational changes in gene expression increasing risk of allergy over generations. Early life is an important time for risk and opportunity, and therefore has gained major scientific interest as a target for the ultimate goal, allergy prevention. Potential environmental factors that can influence early immune maturation include dietary (Palmer *et al.*, 2012a) and microbial (Bisgaard *et al.*, 2011; Hooper *et al.*, 2012) patterns and environmental pollutants (Nakamura *et al.*, 2008; Accordini *et al.*, 2012), all of which have undergone substantial changes during the recent century, but are also available for us to further modify in order to reduce the risk of subsequent disease.

The prevention strategies applied so far, such as prolonged exclusive breastfeeding and delayed introduction of complementary feeding, have not been successful and thus have recently been questioned (Halken, 2004; Palmer *et al.*, 2012a). Accordingly, novel innovative strategies for means of allergy prevention and risk reduction are warranted. In this study, we aimed to explore the relevance of pre- and postnatal nutrition and microbial environment to the risk of allergic diseases in a population genetically prone to allergy.

2. REVIEW OF THE LITERATURE

2.1. Developmental origins of disease

2.1.1. Programming

The concept that "stimuli or insults during critical or sensitive periods in early life can have lifetime consequences" on later health is termed "programming" (Lucas, 1991). The first epidemiological evidence from programming effect on man were described by Dr. Barker in his landmark study in 1989 (Barker *et al.*, 1989); he stated that "the intrauterine environment influences blood pressure during adult life" after noting that systolic blood pressure was inversely related to birth weight while the geographical differences in average blood pressure and mortality from cardiovascular disease reflected past differences in undernutrition at the population level. Next year, he proposed the theory of fetal and infant origins of adult disease (Barker, 1990), later referred to also as the developmental origins or Barker's hypothesis. His idea although was not new. The effect of early nutrition to later physiology was already known from animal experiments in the 1960's (McCance, 1962) and the term "programming" was hence introduced to describe this phenomenon (Barker, 1990; Lucas, 1991).

The Dutch famine triggered thought provoking evidence of developmental programming: children of mothers who endured malnutrition perinatally were later discovered to be more obese (van Abeelen *et al.*, 2012b), have higher cholesterol levels (Lumey *et al.*, 2009) and to be more prone to type II diabetes (van Abeelen *et al.*, 2012a), that is, to have several of the risks for cardiovascular disease, another epidemic of our times. Since Barker's popular study, his findings were replicated extensively (Barker *et al.*, 1993; Hovi *et al.*, 2007) and extended to apply also early postnatal nutrition (Singhal *et al.*, 2001; Singhal *et al.*, 2002; Singhal *et al.*, 2004a; Singhal *et al.*, 2004b). Only recently have researches awaken to notice that malnutrition in terms of quantity is not the only aspect to concern oneself about, also the quality of our daily diet is of utmost importance to development and later health (Calder *et al.*, 2010). The developmental programming theory is since extended from cardiovascular diseases to apply also to other modern maladies, such as allergic diseases. Multiple possible environmental factors, like dietary and microbial environment, affect early life and mold one's future health (**Figure 1**).

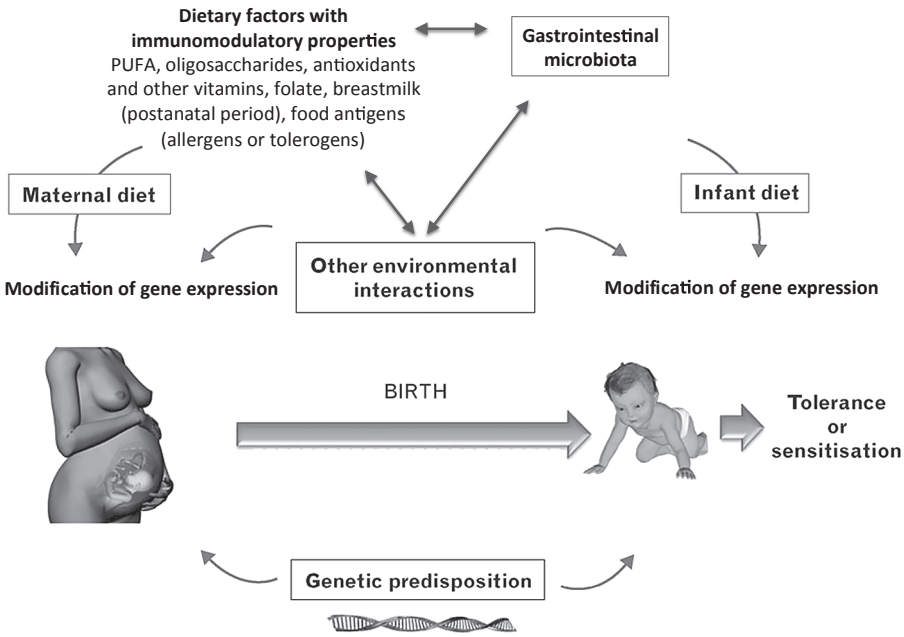

Figure 1. Interactions between environmental and genetic factors in modifying developing immune system (modified from West *et al.*, 2010).

The programming effect is triggered when the fetus or infant struggles to ensure survival in the future environment and thus must adapt its development to match forthcoming challenges. This adaptation, developmental plasticity, begins in the early days of conception and continues at least until the first year of life, a period when the individual is most sensitive to external stimuli, hence called "the window of opportunity". Pregnancy and the immediate postnatal period with exclusive breastfeeding present a very special period, when the mother and child interact to guarantee the growth and development of the child. Both pregnancy and breastfeeding period can be considered as a three-compartment model (Cetin & Cardellichio, 2010), where the placenta or the breast milk is a key player between the mother and child.

In the first trimester of pregnancy, maternal metabolism is switched to an anabolic status, when hyperphagia and increased insulin sensitivity induce fat storage in adipose tissue and an increase in maternal net body weight (Cetin *et al.*, 2005). As fetal growth and nutritional requirements increase rapidly during the third trimester, maternal metabolism turns catabolic in order to meet the growing needs: hormonal changes lead to decreased insulin responsiveness and release of free fatty acids from maternal fat stores (Cousins, 1991; Herrera, 2002; Catov *et al.*, 2007). The placenta is more than a membrane between the mother and child. It has the capacity to regulate the quantity and quality of the nutrient flow to the fetus via active transport systems and metabolic activity (Cetin *et al.*, 2005; Cetin & Koletzko, 2008; Cetin & Alvino, 2009). The fetus itself is not just an inactive recipient, but is also able to modify its endocrine status and metabolism in order to try to adapt to altered nutrient flow (Fowden & Forhead, 2004; Ortega-Senovilla *et*

al., 2009). However, when compensatory mechanisms are insufficient, adverse reactions such as growth restriction or overgrowth may occur. During the neonatal period, the first dietary exposure is usually breast milk, which provides the infant a wide range of health-promoting and immune-modulating compounds (Goldman & Smith, 1973; Heikkilä & Saris, 2003; Penttila, 2010; Walker, 2010). Thus, through thousands of years of product development, breast milk is a very unique mixture of components created by the lactating mother to fit the needs of the infant.

In summary, an increasing body of literature points to the early moments of life as a period of programming, hence providing an opportunity to steer the developing immune system into a tolerant direction. Nutrition is a key player in programming and thus the role of the mother is substantive, as the composition of the nutrient cocktail received by the fetus/child via placenta or breast milk depends on various maternal factors: genetics, diet, body composition, endocrine status and metabolism (Emmett & Rogers, 1997; Picciano, 2001; Belkacemi *et al.*, 2010; Eriksson *et al.*, 2011; Winder *et al.*, 2011).

2.1.2. Mechanisms of programming

The fetus depends on the mother on the supply of nutrients and other vital substances, thus adverse conditions may result from disturbances in mother's diet, metabolic condition or placental function. A unbalanced availability of nutrients, hypoxia and maternal stress influence fetal growth and might lead to growth restriction or later chronic disease. The developing fetus/infant is under a constant flow of cues about the surrounding environment, and depending on the nature, timing and size of these cues different outcomes may occur. The mechanisms under the developmental plasticity are still somewhat obscure, but data on the matter is accumulating.

Firstly, as fetal organogenesis occurs in several different subsequent critical periods during gestation and early postnatal life, the timing of an environmental exposure is crucial. Depending on the timing, the effects of an exposure may vary from no damage to irreversible changes in development. Thus, if a specific exposure were to occur during such critical period, it may generate permanent structural changes in tissue and organ morphology and physiological function in later life (Bateson *et al.*, 2004; Gluckman & Hanson, 2004; Gluckman *et al.*, 2005).

Secondly, although structural and gene expression changes are interrelated, epigenetic changes are indicated as one important mechanism operating behind the allergy epidemic. Epigenetic mechanisms are able to modify patterns of gene expression and thus may cause long-lasting, even trans-generational, changes in phenotype and function. These mechanisms act to regulate the expression of genes without changing the underlying DNA sequence via DNA methylation, post-translational modification to histone tails and regulation through non-coding RNAs (Waterland & Michels, 2007). Thus, the following changes lead to either enhanced or suppressed gene expression, accordingly altering the phenotype depending on the affected genes. Environmental exposures like nutrition

may therefore result in altered epigenetic regulation of immune gene expression and further changes in immune function and allergic phenotype (Hollingsworth *et al.*, 2008). The concept of epigenetic programming offers a comprehensible way by which dietary exposures may contribute to the increasing risk of allergy over generations.

Thirdly, the newborn child receives bacteria from the maternal vaginal and gastrointestinal tract at birth as well as from the surrounding environment. Traditionally, newborns are considered sterile, but recent evidence suggests that exposure to microbial compounds may start already during gestation (Jiménez *et al.*, 2008; Satokari *et al.*, 2009; Hong *et al.*, 2010). The adult body harbors at least 10^4 microbes, most of them residing in the gut. These microbes residing in and on the human body, collectively known as microbiota, are responsible for maintaining normal homeostasis in the host, involving energy harvest and storage, a variety of metabolic functions (Gill *et al.*, 2006) and more importantly, interactions with the immune system (Chow *et al.*, 2010). By the end of the first year of life, the bacterial composition shifts towards an adult–like microbiota, resembling adults by 2.5 years of age (Koenig *et al.*, 2011). Once developed, the composition of the microbiota is relatively stable (Costello *et al.*, 2009). Recently, deviations from the normal colonization pattern associate with the development of several inflammatory conditions (Manichanh *et al.*, 2006; Kalliomäki *et al.*, 2008; Vaahtovuo *et al.*, 2008; Turnbaugh *et al.*, 2009), including allergic diseases (Martinez & Holt, 1999; Strachan, 2000), thus emphasizing the hazards of early life perturbations in the colonization process.

To conclude, although there are many known and alleged mechanisms involved in programming of the development of fetus/infant, the exact connection to development of specific diseases remains unclear. Further, most diseases are probably multifactorial, that is, their development requires more than one environmental exposure in addition of genetic predisposition. These elaborate interactions between genetics and environment are only just beginning to be revealed.

2.2. Allergic diseases

2.2.1. Definitions

Allergic diseases are considered to include atopic eczema, asthma, allergic rhinoconjuntivitis and food allergies. As very different causes may induce similar clinical outcomes, the underlying mechanisms of disease are important to be discriminated. *Hypersensitivity* describes "objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons" (Johansson *et al.*, 2004). The term *allergy* again, refers to "a hypersensitivity reaction initiated by specific immunologic mechanisms" (Johansson *et al.*, 2004). Allergic reactions may be either cell- or antibody-mediated, in the latter case, the antibodies typically belong to the IgE isotype, hence producing the term "IgE-mediated allergy". In this connection, IgE denotes IgE antibody to an allergen, which is an antigen causing allergic disease.

Traditionally, *atopy* is defined as "a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins" (Johansson *et al.*, 2004). Thus, this term should not be used unless an excessive IgE response, i.e. sensitization, has been demonstrated by IgE antibodies in serum or by a positive skin prick test. Atopic individuals are prone to develop typical symptoms of allergic diseases, whereupon these diseases may be referred to as atopic, as with atopic eczema. On the contrary, mere IgE sensitization, like a positive skin prick test, without the presence of clinical symptoms, does not justify the use of the term "allergy".

When asthma is the consequence of immunological reactions, typically instigated by IgE antibodies, it should be referred to as *allergic asthma* (Johansson *et al.*, 2004). Amongst childhood asthma, over 80 percent are of allergic origin (Haahtela *et al.*, 1980). In pre-school children, eczema is a very common condition without any tendency to IgE overproduction (Schäfer *et al.*, 2000; Möhrenschlager *et al.*, 2006). Furthermore, the prognosis of eczema seems to differ depending on the underlying mechanism, thus emphasizing the need to differentiate *atopic eczema* from eczema, in general. However, lately the role of IgE in atopic diseases is questioned and it is suggested that non-atopic and atopic forms are different phases of the same disease (Novak & Bieber, 2003; Bieber, 2008). In this study, we retain the traditional nomenclature and refer to IgE-associated disease as atopic and non-IgE-associated disease as non-atopic.

2.2.2. Current understanding of risk and protective factors

A person's susceptibility to develop an allergic phenotype depends on a multitude of factors. During the window of opportunity, there are various recognized and suspected risk factors, and thus several prevention strategies have been applied and studied (**Figure 2**).

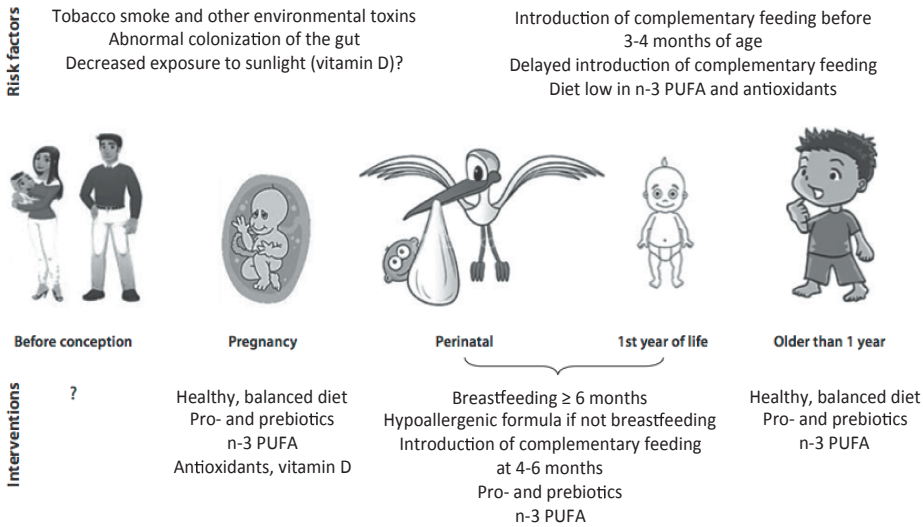

Figure 2. Possible environmental risk and protective factors triggering the development of allergic disease (modified from Prescott & Nowak-Węgrzyn, 2011).

2.2.2.1 Genetic inheritance

The strongest predictor for allergic disease is a positive family history of allergy, thus genetic mechanisms are a strong force and are thoroughly investigated. Children with a family history of allergy have a greater risk to develop allergic disease than those without (50-80% vs. 20%). If both parents are allergic, the risk is even higher (60-80%) (Kjellman, 1998). Interestingly, maternal allergic sensitization is more significant than paternal, thus an atopic milieu *in utero* might prime the developing immune system toward an allergic phenotype (Reece *et al.*, 2011; Følsgaard *et al.*, 2012). Many candidate genes, predisposing to allergic diseases, are known (Tamari *et al.*, 2013). Gender of the developing offspring seems to be a contributing factor. Observational studies indicate that pre-pubertal boys are more susceptible to asthma, allergic rhinitis and atopic sensitization than girls, although the mechanism is not clear (Almqvist *et al.*, 2008). Genetic inheritance might work as an effect modifier, thus different prevention strategies might be in order to at-risk versus normal populations.

2.2.2.2 Environmental toxins and pollutants

There is a significant body of literature establishing a relationship between fetal exposure to maternal cigarette smoking and other respiratory pollutants during pregnancy with asthma and other allergic diseases (Burke *et al.*, 2012). Maternal smoking during pregnancy has adverse effects on the offspring, such as compromised fetal lung growth (Gilliland *et al.*, 2000) and immune development (Noakes *et al.*, 2003). Later in childhood, the risk for early-onset wheezing and reduced lung function is increased (Magnusson *et al.*, 2005). Likewise, there is a relationship between prenatal air pollution exposure and the development of asthma symptoms in children (Peters *et al.*, 2009; Kelly & Fussell, 2011; Proietti *et al.*, 2013). Further, some studies suggest that increased prenatal exposure to metals and chemicals may increase the risk of sensitization and asthma (Linneberg *et al.*, 2004; Peters *et al.*, 2013). Epigenetic modulation of the fetal genome may account for these associations between toxins and allergy (Breton *et al.*, 2009; Perera *et al.*, 2009).

2.2.2.3 Hygiene hypothesis

The original hygiene hypothesis was formulated over two decades ago, when the inverse association between the number of siblings and hay fever was discovered (Strachan, 1989). This preventive effect was originally postulated to be due to growing number of infections transmitted from other children. Certain pathogens, such as *Helicobacter pylori* and *Mycoplasma tuberculosis* and viral infections such as *Epstein-Barr virus*, *Cytomegalovirus* and herpes infections, associate with a reduced risk of allergic diseases (Matricardi *et al.*, 2002; Linneberg *et al.*, 2003; Nilsson *et al.*, 2005; Janson *et al.*, 2007). On the contrary, some early respiratory viral infections, such as RSV, associate with an increased incidence of allergic asthma (Sigurs *et al.*, 1995; Illi *et al.*, 2001). Recent evidence suggests that the overall microbial burden in early life is more important in

protecting against the development of allergies than any specific infection. Lately, this hypothesis has been further extended to apply reduced contact of people with natural environment and biodiversity (Hanski *et al.*, 2012).

The period during and immediately after birth and the early neonatal period are particularly critical, as it has become increasingly evident that the normal maturation of the immune system and development of oral tolerance is subject to stimuli provided by the host's microbial milieu. As suggested already almost two decades ago (Björkstén, 1994; Holt, 1995), the revised hygiene hypothesis (or the microbiota hypothesis of allergy) states that a lack of exposure to microbial stimuli results in Th2 dominant responses and subsequent development of allergic disease (Wills-Karp *et al.*, 2001; Prescott & Björkstén, 2007). Consistent with this theory, reduced bacterial diversity during the early life is linked to the development of allergic sensitization (Stsepetova *et al.*, 2007; Sjögren *et al.*, 2009; Bisgaard *et al.*, 2011) and allergic diseases (Forno *et al.*, 2008; Wang *et al.*, 2008; Bisgaard *et al.*, 2011; Abrahamsson *et al.*, 2012). Further, researchers have discovered differences in the microbiota composition between healthy and atopic children (Böttcher *et al.*, 2000; Björkstén *et al.*, 2001; Watanabe *et al.*, 2003) and these differences manifest years before clinical disease (Björkstén *et al.*, 2001; Kalliomäki *et al.*, 2001; Penders *et al.*, 2007; Sjögren *et al.*, 2009). There is some evidence that infants with later atopic sensitization have a reduced ratio of *Bifidobacteria* to *Clostridia* and distinct *Bifidobacterial* microbiota strains (Kalliomäki *et al.*, 2001), although the results have not always been consistent (Penders *et al.*, 2006a; Adlerberth *et al.*, 2007) and there is variation between the candidate bacterial species associated. Potential reasons for the development of aberrant microbiota are multiple, and interaction between genetic factors, birth mode, type of feeding, environment, and medication are probable.

Children born by caesarian section have markedly different early microbiota than those born vaginally, as they harbor microbes from the skin and hospital environment as opposed to vaginally delivered children having bacteria resembling the microbial communities from their mother's vaginal and gastrointestinal tract (Adlerberth & Wold, 2009; Dominguez-Bello *et al.*, 2010). These differences in early colonization are still evident even at seven–years-of-age (Salminen *et al.*, 2004). In line with the reported microbiota differences, the risk for allergic diseases is higher in children born by caesarian section compared with those born vaginally (Kero *et al.*, 2002; Thavagnanam *et al.*, 2008; van Nimwegen *et al.*, 2011).

After the initial perinatal colonization process, the infant feeding type is the most important determinant of further microbiota development. Breast milk naturally contains live *Bifidobacteria* and *Lactobacilli* strains (Grönlund *et al.*, 2007; Gueimonde *et al.*, 2007), thus these predominate in the gut of full-term breast-fed infants (Favier *et al.*, 2002). Further, breast milk serves as a source for indigestible oligosaccharides that actively stimulate proliferation of the colonizing bacteria and promote the growth of *Bifidobacteria* (Newburg, 1999). Interestingly, allergic and overweight mothers provide

less *Bifidobacteria* to their infants than healthy and normal-weight mothers (Grönlund *et al.*, 2007).

Growing up in a farming environment is consistently reported to protect against allergic diseases (von Mutius & Vercelli, 2010; Fuchs *et al.*, 2012). Independent exposures associated with this protective effect are attributed to the contact with livestock, animal feed, and the consumption of raw cow's milk (Von Ehrenstein *et al.*, 2000; Riedler *et al.*, 2001; Ege *et al.*, 2007). Thus, this protective effect is hypothesized to confer through exposure to increased microbial diversity in the farming as opposed to urban environments (Ege *et al.*, 2011). There is some evidence that similar effects may be achieved with pet keeping in urban environments (Nermes *et al.*, 2013; Pelucchi *et al.*, 2013). Interestingly, the strongest protective effect of farm exposure against allergic diseases is achieved during pregnancy and the first years of child life (Ege *et al.*, 2006; Douwes *et al.*, 2008), again supporting the importance of this window of opportunity for timing of possible interventions to reduce the risk of allergy.

Antibiotic use has a considerable effect on the microbiota (Jernberg *et al.*, 2007), thus early use might lead to major alterations and even aberrant development of the infant's immune system (Celedón & Weiss, 2004). There is intriguing data to suggest an increased risk of atopy and allergic diseases after maternal antibiotic use before and during pregnancy (McKeever *et al.*, 2002; Metsälä *et al.*, 2013), but again others have found no association with maternal antibiotic use and infant microbiota changes (Penders *et al.*, 2006b).

In conclusion, the microbiota is a strong force in the development of immunity and early microbial stimuli are crucial in modulating and activating the immune system. Nonetheless, the evidence is not unambiguous in which specific microbes or microbial colonization patterns would confer risk or protection from allergy.

2.2.2.4 Diet

Early nutrition of the newborn provides antigens to which the immune system has to become tolerant and already the fetus may be exposed directly to antigens crossing the placental barrier (Warner *et al.*, 2000; Peters *et al.*, 2009). Previously, reducing the dietary allergen load of high-risk children was considered a key factor in allergy prevention, thus elimination diets in both mothers and children were recommended for decades. These practices however, were not based on robust scientific evidence (Kramer & Kakuma, 2003) and are widely abandoned. The current expert recommendations for families with history of allergy are reviewed in **Table 1**.

Table 1. Expert dietary recommendations for high-risk families to reduce the risk of allergy.

Expert organization	Maternal diet during pregnancy and breast-feeding	Duration of exclusive breast-feeding	Use of hypoallergenic formulas	Introduction of complementary feeding	Introduction of potentially allergenic foods (eggs, fish, nuts, seafood)
American Academy of Allergy, Asthma & Immunology (Fleischer *et al.*, 2013)	Normal diet[1]	4 – 6 months	First 4 – 6 months if not breastfed	4 – 6 months	4 – 6 months, however not as one of the first foods
American Academy of Pediatrics (Greer *et al.*, 2008)	Normal diet[1]	4 – 6 months	First 4 – 6 months if not breastfed	4 – 6 months	4 – 6 months
American College of Allergy, Asthma and Immunology (Fiocchi *et al.*, 2006)	NA[2]	6 months	NA[2]	6 months	Dairy products 12 months, eggs 24 months, nuts and seafood 3 years
European Academy of Allergy and Clinical Immunology (Høst *et al.*, 2008)	Normal diet[1]	4 – 6 months	First 4 months if not breastfed	4 – 6 months	4 – 6 months
European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (Agostoni *et al.*, 2008)	Normal diet[1]	6 months	NA[2]	17 – 26 weeks	17 – 26 weeks
Australasian Society of Clinical Immunology and Allergy (Prescott *et al.*, 2005)	Normal diet[1]	4 – 6 months	First 4 – 6 months if not breastfed	4 – 6 months	Peanut, nut and shellfish 2- 4 years

[1]Normal diet = what is recommended for pregnant and breastfeeding women. [2]NA = not assessed in the recommendation.

At present, diet is viewed more as a source of active compounds, with factors that have independent immune-modulating properties and factors that act via modulating intestinal microbiota and thus influence antigen exposure, immune maturation or immune responses. Several nutrients possess immune-modulating properties (**Figure 1**) and observational studies show that changes in dietary habits have preceded or paralleled the increase of allergic diseases in Western countries (Seaton *et al.*, 1994; Black & Sharpe, 1997). Specifically, a change in dietary fat quality and fruit and vegetable consumption attract much attention and thus two of the most studied dietary hypotheses are the lipid and the antioxidant hypothesis (Devereux & Seaton, 2005).

In an attempt to reduce the risks of cardiovascular diseases, the public health guidelines have resulted in decreased consumption of saturated fats and increased consumption of margarines and vegetable oils rich in n-6 polyunsaturated fatty acids (PUFA) in many western countries (Burdge & Calder, 2005). Simultaneously, the intake of n-3 PUFA present in especially oily fish or fish products has remained same or decreased. The most abundant dietary PUFA are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), both of which cannot be synthesized by the human body but have to be received in diet, and thus they are referred to as essential FA. Popular corn, soybean,

safflower and sunflower oils widely used in westernized countries are rich in n-6 PUFA with over 50% LA (Calder *et al.*, 2006). Average LA:ALA ratio in western societies is 10:1 or higher, compared to 3:1 at the beginning of the last century, although intakes vary widely (Innis, 2007). In Finland, popular rapeseed oil has LA:ALA ratio of 2:1 (THL, 2014a) and according to national survey the average dietary LA:ALA ratio is 3,6:1 (Helldán *et al.*, 2013). LA and ALA act as precursors to longer-chain (LC) PUFA, **Figure 3**.

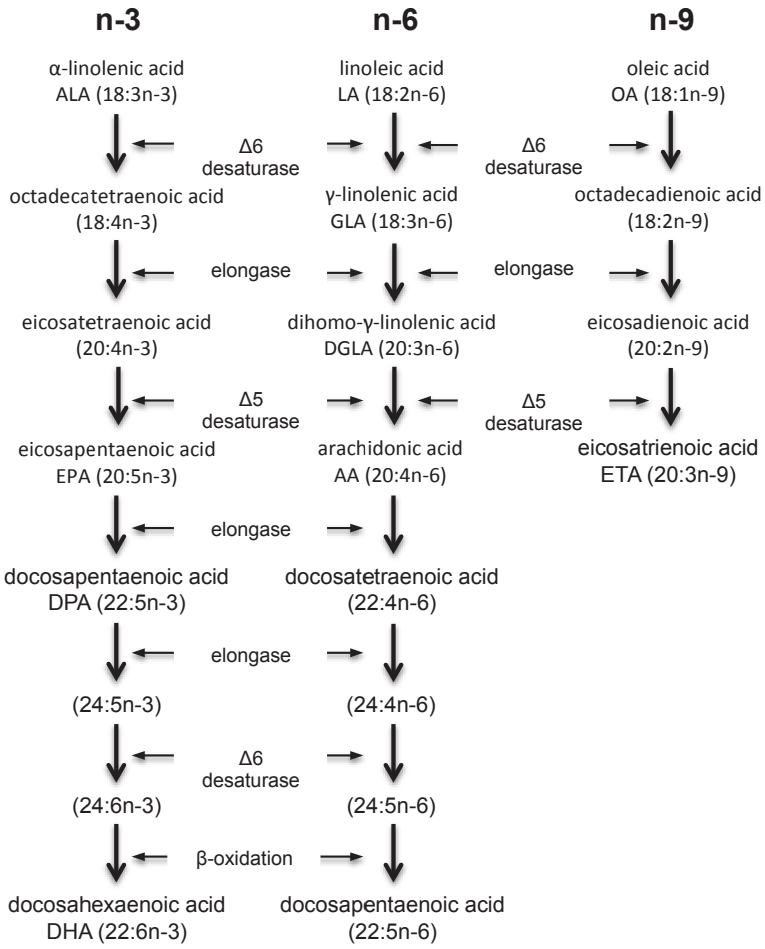

Figure 3. The biosynthesis of n-3, n-6 and n-9 polyunsaturated fatty acids from 18 carbon precursors. The essential FA, LA and ALA are further metabolized to form AA and EPA, respectively. N-3, n-6 and n-9 PUFA are thought to be metabolized by the same desaturase and elongase enzymes, thus this can result in competition between LA and ALA as well as inhibition of the enzyme pathway by products of the same and other series FA. The first step enzyme Δ6-desaturase shows *In vitro* preference in the order ALA > LA > oleic acid (Innis, 1991). EFA deficiency results in an increased production of eicosatrienoic acid (20:3n-9) from oleic acid (Mead, 1958).

LA is converted into arachidonic acid (AA, 20:4n-6), a substrate for the synthesis of eicosanoids such as 2-series prostaglandins, thromboxanes and 4-series leukotrienes, which are involved in regulation of many physiological cell and tissue functions (Lewis

et al., 1990). It is thought that the 4-series leukotrienes promote allergic inflammation by inducing vascular permeability, leucocyte chemotaxis and production of inflammatory cytokines while the 2-series prostaglandin E2 (PGE2) decreases the production of Th1-type cytokines IFN-γ and IL-2, increases the production of Th2-type cytokines IL-4 and -5 and enhances IgE synthesis from B-cells (Tilley *et al.*, 2001). Thus, increasing dietary intake of n-6 LA might increase the risk of atopic Th2 sensitization and allergic disease (Calder, 2003). In support of this hypothesis, some epidemiological studies show a connection between high n-6 PUFA intake and allergic diseases (Pöysä *et al.*, 1991; Bolte *et al.*, 2001; Trak-Fellermeier *et al.*, 2004; Bolte *et al.*, 2005; Sausenthaler *et al.*, 2006). Conversely, the intake of ALA results in the incorporation of its metabolic derivatives, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) into immune cells at the expense of AA, thus decreasing the production of 2-series prostaglandins and 4-series leukotrienes (Calder, 2003). Further, metabolites of EPA and DHA, termed docosatriens, resolvins and neuroprotectins, have potent inflammation resolving functions (Serhan, 2005). Fatty acids and eicosanoids also regulate gene expression through activation or changes in the availability of transcription

Table 2. Randomized controlled studies with fatty acid supplementation during pregnancy and lactation reporting allergy related outcomes in the offspring.

Study	FA used	Placebo used	Subjects	Child allergy risk
Dunstan *et al.*, 2003b; a Australia	n-3 PUFA total 3.7 g/day; 2070 mg DHA + 1025 mg EPA	Olive oil 4g/day; 2664 mg OA, < 1% n-3 PUFA	98 mothers randomized, 83 infants completed the study	High; all mothers had allergic disease with IgE-sensitization
Lauritzen *et al.*, 2005 Denmark	Fish oil; n-3 PUFA total 1.5 g/day	Olive oil	122 mothers randomized, 72 children completed 30 months follow-up	Low
Krauss-Etschmann *et al.*, 2008 multicenter Spain, Hungary, Germany	500 mg DHA + 150 mg EPA OR folic acid 0.4 g/day OR both, all in milk-based supplement	Milk-based supplement without EPA or DHA	315 mothers randomized, 220 CB samples obtained	Low
Olsen *et al.*, 2008 Denmark	Fish oil 1 g/day; 320 mg EPA and 230 mg DHA + 2mg/ml α-tocopherol	Olive oil 1 g/day; 720 mg OA and 120 mg LA	533 mothers randomized, 528 children identified in registry	Low
Furuhjelm *et al.*, 2009; Furuhjelm *et al.*, 2011 Sweden	1.1 g/day DHA + 1.6 g/day EPA + 23 mg α-tocopherol	Soy oil; 2.5 g/day LA + 0.28 g/day ALA + 36 mg α-tocopherol	145 mothers randomized, 117 children completed the follow-up	High; family history of allergy
Palmer *et al.*, 2012b; 2013 Australia	Fish oil; 800 mg DHA + 100 mg EPA	1500 mg vegetable oil without n-3 PUFA	706 mothers randomized, 681 infants completed 12 mo and 638 3 yr follow-up	High; family history of allergy

CB = cord blood. OR = odds ratio. HR = hazard ratio. RR = risk ratio. All group comparisons are intervention (I) vs. control (C) group. NS = no significant differences between groups. EPA = eicosapentaenoic acid (20:5n-3), DHA = docosahexaenoic acid (22:6n-3), OA = oleic acid (18:1n-9), LA = linoleic acid (18:2n-6), ALA = α-linolenic acid (18:3n-3).

factors (Kliewer *et al.*, 1997). In addition, maternal n-3 FA intake is demonstrated to alter offspring microbiome and immune responses in mice (Myles *et al.*, 2014). Therefore, a protective effect of n-3 PUFA towards allergic diseases has been postulated (Hodge *et al.*, 1994; Hodge *et al.*, 1996; Black & Sharpe, 1997) and evidence to support this hypothesis is provided by studies associating higher blood total and LC n-3 PUFA concentrations with lower risk of allergic diseases (Hoff *et al.*, 2005; Montes *et al.*, 2013). However, RCTs aiming to modify child inflammatory responses and risk of allergic diseases with n-3 LC-PUFA supplementation during pregnancy and lactation are few so far. The methods used in these studies vary significantly in the amount of n-3 LC-PUFA supplemented (from 0.65 to 3.7 g/day), quality of placebo (olive, soy and vegetable oils and milk), timing of intervention (4 studies prenatal, 1 postnatal, 1 both), duration of follow up (delivery to 16 years) and allergy risk of the target population (**Table 2**). However, it appears that n-3 LC-PUFA supplementation during pregnancy modulates neonatal cord blood immune responses, and the effects on incidence of later child allergic diseases are encouraging but any conclusions cannot be drawn due to methodological differences.

Intervention period	Duration of follow-up	Outcomes	Main findings
From 20 weeks of gestation until delivery	12 months	Primary: CB cytokines and IgE Secondary: IgE-sensitization, eczema, scorad > 25, asthma, food allergy	Weaker cytokine (IL-5, IL-13, IL-10, and IFN-γ) responses to allergens in I vs. C group, IgE-levels NS IgE-sensitization to egg OR 0.34 (95% CI, 0.11, 1.02), P = 0.55; scorad OR 0.09 (0.01, 0.94), P = 0.045; eczema, asthma and food allergy NS
First 4 months of lactation	30 months	Primary: Plasma cytokines and IgE Secondary: eczema, asthma, food allergy (self-reported)	IFN-γ levels increased in I vs. C group, IgE NS Eczema, asthma and food allergy NS
From 20-22 weeks of gestation until delivery	Delivery	CB cytokines	Decreased levels of IL-4, IL-13, and CCR4 and with decreased frequencies of cord blood NK cells and CCR31CD81 T cells in fish oil groups
From 30 weeks of gestation until delivery	16 years	Asthma (registry) Allergic asthma (registry)	Asthma HR 0.37 (0.15, 0.92), P= 0.03 Allergic asthma HR 0.13 (0.03, 0.60), P = 0.01
From 25 weeks of gestation until end of lactation (mean 30.9 weeks)	12 months	Atopic eczema IgE- sensitization Food allergy	Atopic eczema OR 0.22 (0.06, 0.81), P < 0.05; IgE-sensitization OR 0.36 (0.14, 0.95), P < 0.05; food allergy OR 0.09 (0.01, 0.74), P < 0.05 (In mothers with allergic diseases all NS; in mothers without allergic diseases all significant)
From 21 weeks of gestation until delivery	12 months, follow-up at 3 years	Atopic eczema IgE- sensitization Food allergy Allergic asthma and rhinitis (3 yr)	12 months: atopic eczema RR 0.64 (0.40, 1.03), P = 0.06; IgE-sensitization overall NS, egg sensitization RR 0.62 (0.41, 0.93), P = 0.02; food allergy NS 3 years all NS

Fruits and vegetables are a source for many important vitamins and trace elements, with some of these possessing antioxidant functions. Maternal intakes of fruits and vegetables influence fetal antioxidant status, which might have implications for immuno-programming. Therefore, the increase in asthma and other allergic diseases might be due to low antioxidant intakes (Devereux & Seaton, 2005) causing compromised lung antioxidant defenses and subsequent inflammation. In contrast, an alternative hypothesis suggests that the increase in allergies is caused by increased intakes of antioxidants from functional and antioxidant enriched foods (Murr *et al.*, 2005). This is because *in vitro* observations show that antioxidant rich foods suppress cytokine secretion from Th1-cells (Neurauter *et al.*, 2004; Allan *et al.*, 2010), and are thus hypothesized to promote the development of Th2 dominant phenotype. Although the literature is inconsistent, the majority of published observational studies suggest beneficial associations between intakes of antioxidants and established allergic disease, especially asthma (Allan *et al.*, 2010). Further, animal studies suggest that antioxidant supplementation before allergen sensitization has potential to reduce the risk for sensitization (Zheng *et al.*, 1999; Okamoto *et al.*, 2006). In addition of antioxidants, other nutrients associate with allergic disease. Vitamin D has immunomodulatory potential on B- and T-cell functions (Vassallo & Camargo, 2010) and folate is a known methyl donor with potential to induce effects on gene expression via epigenetic mechanisms (West *et al.*, 2011).

In summary, although data is somewhat complex, they would imply that diet is able to modify immune responses, but the associations of specific nutrients with allergic diseases is far from understood. The associations of fatty acids and antioxidants with development of allergies need to be replicated in further studies.

2.2.2.5 Breastfeeding

The current consensus is that breast milk provides the best source of nutrition to the newborn infant, but the optimal quality of breast milk and duration of breastfeeding is not clear. The quality of breast milk varies from one individual to another and in the same individual with time. Breast milk provides the infant essential nutrition with several immune-modulating factors (e.g., immunoglobulin A, lactoferrin, nucleotides, glutamine, oligosaccharides, fatty acids, antioxidants and *Bifidobacteria* and *Lactobacilli*) (Gueimonde *et al.*, 2007; Mihrshahi *et al.*, 2007). In addition, several food or environment-derived antigens that have been modified or degraded in the maternal gut are transferred to the newborn via breast milk (Vance *et al.*, 2005). The quality of breast milk depends greatly on the mother's immunological status, weight, microbiota and nutritional status. The composition of the breast milk is related to maternal diet especially regarding fat content (Rocquelin *et al.*, 1998; Kelishadi *et al.*, 2012; Maru *et al.*, 2013), and thus is also fairly easily modified by conscious dietary choices during breastfeeding (Urwin *et al.*, 2012).

The World Health Organization (WHO) recommends that the duration of exclusive breastfeeding should be for at least 6 months, but this recommendation is based on

general nutritional aspects and prevention of infections, as well as prevention of allergic diseases (WHO, 2001). However, in a recent Cochrane review there was no difference in long-term impact of exclusive breastfeeding for 6 months versus three to four months on general child health (Kramer & Kakuma, 2012). When it comes to solely preventing allergic diseases, the optimal duration of breastfeeding is controversial. For this purpose, most experts recommend exclusive breastfeeding for 4-6 months (Prescott *et al.*, 2005; Fiocchi *et al.*, 2006; Agostoni *et al.*, 2008; Greer *et al.*, 2008), as a protective effect for this duration of breastfeeding on the risk of allergic diseases is reported in previous studies (Saarinen & Kajosaari, 1995; Oddy *et al.*, 1999) and two meta-analyses (Gdalevich *et al.*, 2001; van Odijk *et al.*, 2003), particularly in children with a family predisposition for allergies. On the contrary, a few studies have found no evidence of protective effect of breastfeeding over six-months on allergies (Kramer *et al.*, 2007; Mihrshahi *et al.*, 2007), instead, the risk may even increase with prolonged breastfeeding (Wright *et al.*, 2001; Sears *et al.*, 2002; Pesonen *et al.*, 2006; Matheson *et al.*, 2007). Further, the protective effects of breast milk might be modified by maternal allergy status, as some studies suggest that atopic mothers might actually increase the risk of child atopy by breastfeeding, most likely through the milk's qualitative changes (Wright *et al.*, 2000; Snijders *et al.*, 2008). If exclusive breastfeeding of high-risk infants is not possible during the first months, the use of a hydrolyzed formula is recommended over a conventional cow's milk-based formula to reduce the risk of cow's milk allergy and atopic eczema (Osborn & Sinn, 2006; von Berg *et al.*, 2013). However, no advantage over breastfeeding is documented in allergy prevention (von Berg *et al.*, 2008). Thus, although breastfeeding is highly recommended for other health promoting reasons, it is challenging to arrive at an explicit conclusion on the significance of breastfeeding in preventing allergic diseases in the child.

2.2.2.6 Pro- and prebiotics

WHO defines probiotics as: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Prebiotics refer to "a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro *et al.*, 2008). Thus, probiotics contribute to intestinal microbiota, whereas most prebiotics are nondigestible oligosaccharides that stimulate the growth and/or activity of lactic acid bacteria (Salminen *et al.*, 1998). Considering the hygiene hypothesis of allergy and the findings of microbiota diversity and quality differences between atopic and non-atopic individuals, pre- and probiotics have become popular as possible tools in reducing the risk of allergic diseases.

The mechanisms of actions of pro- and prebiotics are elucidated to act at different levels. They directly modulate the composition of the microbiota and hence affect enzymatic activities of the gut, they promote the mucosal barrier function and stimulate the intestinal immune system (Rijkers *et al.*, 2010). Prenatal administration of probiotics shapes maternal vaginal and intestinal microbiota and, accordingly, may set its own pattern on

neonatal gut colonization. Moreover, there is evidence of more elaborate mechanisms; maternal probiotic supplementation modulates placental and fetal gut immune gene expression (Rautava *et al.*, 2012a) and immunologic properties of breast milk (Rautava *et al.*, 2002; Huurre *et al.*, 2008).

To date, 16 randomized controlled trials for primary prevention of allergy exists, 11 of them using *Lactobacillus rhamnosus* strains, **Table 3.** Taken together, these data suggest that *Lactobacillus rhamnosus* provides best protection from eczema with combined pre- and postnatal supplementation. In addition, variable results from 5 RCT studies are published using both pre- and postnatal dosage with *L. reuteri* (Abrahamsson *et al.*, 2007), combined *L.acidophilus*, *B. bifidum* and *B. lactis* (Kim *et al.*, 2010) and combined *L. lactis*, *B. bifidum* and *B. lactis* (Niers *et al.*, 2009), and postnatal use alone with *L. acidophilus* (Taylor *et al.*, 2007) and *L. paracasei* (West *et al.*, 2009). Several reviews and meta-analyses have been published in recent years, although comparison of different studies is challenging due to variation in the number and age of participants, eczema severity, and diagnostic criteria and variation of probiotic strains and dosages with differing treatment periods across the studies. A Cochrane review from 2007 included five trials and concluded that the relative risk for probiotic use was 0.82 (95% CI 0.70-0.95) for eczema and 0.8 (0.62-1.02) for atopic eczema (Osborn & Sinn, 2007). A Chinese meta-analysis included only trials using lactic acid bacteria either alone or in combination and reported RR 0.80 (95% CI 0.70-0.90) for eczema and 0.78 (0.64-0.97) for atopic eczema (Zhu *et al.*, 2010). A recent meta-analysis from 2012, including every probiotic strain studied reported RR 0.79 (95% CI 0.71-0.88) for eczema and 0.80 (0.66-0.96) for atopic eczema, corresponding to a reduction of about 20 percent in the incidence of eczema and atopic eczema (Pelucchi *et al.*, 2012). No consistent evidence yet exists for other allergic diseases (Azad *et al.*, 2013). Prebiotics supplementation in infant feeding may prevent eczema, but the evidence it weak (Osborn & Sinn, 2013). In conclusion, there is accumulating evidence that probiotic supplementation is a potential way for primary prevention of eczema and atopic eczema, but no recommendations for routine use can yet be given.

Table 3. Randomized controlled studies using *Lactobacillus rhamnosus* –strains in primary prevention of allergy.

Study	Probiotic type(s) +- prebiotics	Subjects	Child allergy risk	Intervention period	Duration of follow-up	Outcomes	Main findings
Kalliomaki *et al.*, 2001, Kalliomaki *et al.*, 2003, Kalliomäki *et al.*, 2007 Finland	*Lactobacillus rhamnosus* GG 2 x 10^{10} CFU daily	159 mothers randomized; 132 infants completed the study at 2 yr, of those, 107 and 116 children participated in follow-up at 4 and 7 yr, respectively	High; family history of allergy	From 2-4 weeks before birth to 6 months post-partum to breastfeeding mothers or directly to infants if not breastfed	2 years of age, follow-up at 4 and 7 years of age	Eczema IgE-sensitization	2 years: eczema RR 0.51 (95% CI 0.32, 0.84) for I vs. C group; IgE-sensitization NS 4 years: eczema RR 0.57 (0.33, 0.97); IgE-sensitization NS 7 years: eczema cumulative RR 0.64 (0.45, 0.92), IgE-sensitization NS
Rautava *et al.*, 2006 Finland	*L. rhamnosus* GG and *Bifidobacterium lactis* Bb12 1 x 10^{10} CFU daily each	81 infants randomized, 72 completed the study	Low	Capsules mixed in infant formula from starting formula feeding before 2 months of age to 12 months of age	1 year of age	Eczema IgE-sensitization	Eczema frequency 4/32 (13%) in the I group vs. 8/40 (20%) in the C group, P = 0.40 IgE-sensitization NS
Kukkonen *et al.*, 2007, Kuitunen *et al.*, 2009 Finland	*L. rhamnosus* GG 5 x 10^9, *L. rhamnosus* LC705 5 x 10^9, *B. breve* Bb99 2 x 10^8, *Propionibacterium freudenreichii* 2 x 10^9 CFU; all twice daily	1223 mothers randomized, 925 and 891 children completed the study at 2 and 5 yr, respectively	High; family history of allergy	From 36 weeks of gestation (mother) to 6 months post-partum directly to infants	2 years of age, follow-up at 5 years of age	Eczema Atopic eczema IgE-sensitization Asthma Allergic rhinitis	2 years: eczema OR 0.69 (0.52, 0.93), P = 0.015; atopic eczema OR 0.61 (0.42, 0.90), P = 0 .012; IgE-sensitization NS 5 years: all NS In caesarian-delivered children: atopic eczema OR 0.43 (0.19, 0.95), IgE-sensitization to any food antigen 0.33 (0.12, 0.85) for I vs. C group
Huurre *et al.*, 2008 Finland	*L. rhamnosus* GG and *B. lactis* Bb12 1 x 10^{10} CFU daily each	171 mothers enrolled, 140 infants completed the study	High; family history of allergy	From the first trimester of pregnancy to the end of breastfeeding (mother), maximum of 6 months postpartum	1 year of age	Atopic eczema	NS In children of IgE-positive mothers, the frequency of atopic eczema was lower in the I group 10 % vs. 18 % in the C group
Kopp *et al.*, 2008 Germany	*L. rhamnosus* GG 5 x 10^9 CFU daily	105 mothers randomized, 94 infants completed the study	High; family history of allergy	From 4-6 weeks before delivery to 3 months post-partum to breastfeeding mothers or directly to child if not breastfed, then 3 months directly to all children	2 years of age	Eczema IgE-sensitization (inhalant allergens only)	All NS

Study	Probiotic type(s) +- prebiotics	Subjects	Child allergy risk	Intervention period	Duration of follow-up	Outcomes	Main findings
Wickens *et al.*, 2008, Wickens *et al.*, 2012, Wickens *et al.*, 2013 New Zealand	*L. rhamnosus* HN001 6 x 10^9 CFU/day OR *B. animalis lactis* HN019 9 x 10^9 CFU/day	512 mothers randomized, 411, 425 and 374 completed the study at 2, 4 and 6 years, respectively	High; family history of allergy	From 35 weeks gestation to the end of breastfeeding, maximum of 6 months postpartum. Infants started the capsules between 2 and 16 days postpartum, continuing until age 2 years	2 years of age, follow-up at 4 and 6 years of age	Eczema Atopic eczema IgE-sensitization Allergic rhinitis	2 years: AE LR HR 0.51 (0.27, 0.97), P = 0.04, BA NS; sensitization and eczema NS 4 years: eczema LR cumulative HR 0.57 (0.39, 0.83); allergic rhinitis LR HR 0.38 (0.18-0.83); AE and sensitization LR NS; BA all NS 6 years: eczema LR cumulative HR 0.56 (0.39, 0.80); sensitization LR cumulative HR 0.69 (0.48-0.99); AE and allergic rhinitis LR NS; BA all NS
Soh *et al.*, 2009 Singapore	*L. rhamnosus* LPR and *B. longum* BL999 at least 2.8 x 10^8 CFU/day	253 infants randomized, 245 completed the study	High; family history of allergy	From birth to 6 months of age directly to children	1 year of age	Eczema Atopic eczema	NS
Dotterud *et al.*, 2010 Norway	*L. rhamnosus* GG 5 x 10^{10}, *L. acidophilus* La-5 5 x 10^9 and *B. animalis subsp lactis* Bb12 5 x 10^{10} CFU daily	415 mothers randomized, 278 infants completed the study	Low	From 36 weeks of gestation to 3 months postpartum to breastfeeding mothers	2 years o f age	Eczema Atopic eczema IgE-sensitization Asthma Allergic rhinitis	Eczema: OR 0.43 (0.23, 0.83) Atopic eczema, asthma or sensitization NS, allergic rhinitis not analyzed
Boyle *et al.*, 2011 Australia	*L. rhamnosus* GG 1.8 x 10^{10} CFU daily	250 mothers randomized, 210 infants completed the study	High; family history of allergy	From 36 weeks of gestation until delivery	1 year of age	Eczema Atopic eczema IgE-sensitization	All NS
Ou *et al.*, 2012 Taiwan	*L. rhamnosus* GG 1 x 10^{10} CFU daily	191 mothers randomized, 128 infants completed the study	High; all mothers had allergic disease with IgE-sensitization	From 24 weeks of gestation until delivery, then to breastfeeding mothers or to non-breastfeeding neonates with water for 6 months	3 years of age	Eczema by parental questionnaire, IgE-sensitization	All NS
Rautava *et al.*, 2012b Finland	*L. rhamnosus* LPR and *B. longum* BL999 OR *L. paracasei* ST11 and *B. longum* BL999, 1 x 10^9 CFU each	241 mothers randomized, 205 infants completed the study	High; all mothers had allergic disease with IgE-sensitization	From 2 months before the expected day of delivery until 2 months postpartum to breastfeeding mothers	2 years of age	Eczema, IgE-sensitization	Eczema: LPR + BL999 OR 0.17 (0.08, 0.35), P < 0.001 and ST11 + BL999 0.16 (0.08, 0.35), P < 0.001 Sensitization: LPR + BL999 OR 0.81 (0.38, 1.76), P = 0.60 and ST11 + BL999 0.99 (0.46, 2.13), P = 0.99

All group comparisons are intervention (I) vs. control (C) group. RR = risk ratio. OR = odds ratio. HR = hazard ratio. NS = no significant differences between groups. AE = atopic eczema. LR = *L. rhamnosus*. BA = *B. animalis lactis*.

2.2.2.7 Summary

The development of immunological tolerance to environmental antigens constantly encountered after birth is an immensely complex phenomenon, starting already during pregnancy with lifelong continuance. This delicate process is susceptible to several external distractions, leading to unbalanced responses and thus allergic manifestations. Moreover, there is not enough data to draw any firm conclusions about the role of specific protective factors. The key to understanding successful tolerance induction requires resolving the interactions among these factors, including maternal nutrition, colonization, breastfeeding and antigen exposure patterns. Thus, there is a need for more integrative studies, addressing these interactions.

3. OBJECTIVES OF THE STUDY

The overall purpose of the present study was to modify pre- and postnatal nutrition in order to modify infant fatty acid supply and achieve risk reduction of atopic eczema and asthma by 4 years of life.

The specific objectives in this thesis were:

1. To test the hypothesis that dietary counseling during pregnancy and breastfeeding increases infants' n-3 and essential fatty acid supply. (Studies I and II)

2. To test the hypothesis that maternal dietary counseling and probiotic supplementation from early pregnancy onwards reduces the risk of atopic eczema or asthma in the children. (Study III)

3. To investigate the impact of maternal dietary intake during pregnancy (Study III) and breastfeeding (unpublished results) on infants' risk of atopic eczema and asthma.

4. To evaluate the effect of age at introduction of complementary feeding on risk of atopic eczema. (Study IV)

4. MATERIALS AND METHODS

4.1. Subjects and the study design

A randomized, placebo-controlled trial, was conducted in Southwest Finland, where altogether 256 pregnant women were recruited during first trimester of pregnancy between April 2002 and November 2004 (Laitinen *et al.*, 2009). Inclusion criteria were: age over 18 years, pregnancy duration under 17 weeks and the unborn child having a high risk for allergic disease (mother, father or sibling having food allergy, atopic eczema, asthma or allergic rhinoconjunctivitis). The allergic diseases were self-reported. Women with other chronic than allergic diseases were excluded from the study.

Information about the study was distributed in local prenatal clinics and published in local newspapers. Interested women contacted the study nurses who explained the study protocol and assessed eligibility. Before the first study visit, the women were randomly assigned in 3 study groups: (1) dietary counseling with probiotic supplement, (2) dietary counseling with placebo, and (3) a control group with placebo. The random allocation was computer-generated with block-randomization of 6 women independently from the investigators by a statistician. The study was open with respect to the dietary counseling, double-blinded to intervention with probiotics or placebo, and single-blinded to controls with placebo. Information on whether the participant would receive dietary counseling was enclosed in sealed envelopes, which were opened by the study nurse and nutritionist at the first study visit in the presence of each study participant in the order of recruitment. The randomization code was opened after all the infants had completed the 12-month follow-up.

A subsample of 90 women was taken to participate in fatty acid analyses (Studies I and II) at randomization. Forty-five mothers from the dietary counseling group without probiotics and 45 mothers from the control group, i.e. mothers either receiving dietary counseling or not, were included in consecutive order of recruitment. The number of participants was based on previous fatty acid supplementation studies (Velzing-Aarts *et al.*, 2001; Dunstan *et al.*, 2004) and practical resources.

4.1.1. Maternal dietary counseling and probiotic intervention

The dietary counseling and probiotic/placebo intervention started at the first study visit at the first trimester of pregnancy. The probiotic capsules used contained 1 x 10^{10} colony-forming units (CFU) of *Lactobacillus rhamnosus* GG (ATCC 53103, Valio Ltd, Helsinki, Finland) and 1 x 10^{10} CFU of *Bifidobacterium lactis* Bb12 (Chr. Hansen, Hoersholm, Denmark) and the placebo capsules microcrystalline cellulose and dextrose anhydrate (Chr. Hansen, Hoersholm, Denmark). The probiotic and placebo preparations were similar in appearance, taste and smell. Compliance to intervention was good; more than 95 percent of the subjects consumed the capsules daily.

The dietary counseling groups received detailed counseling by a study nutritionist during each study visit, aimed at a diet complying with that recommended for pregnant women during the study years (NNR, 1996; Becker, 2005). Also, all the women were advised not to avoid any foods during pregnancy unless allergic to a particular food. The prime objective of the dietary counseling was to consider the diet as a whole and focus on the quality and quantity of dietary fat and fiber, with the intention of improving the consumption of fiber, increasing the intake of unsaturated fatty acids and reducing that of saturated fatty acids (SFA) (Piirainen *et al.*, 2006). The counseling was provided with layman terms and was adjusted with the women's daily dietary habits. Each session lasted around 30 minutes. The use of vegetables, fruits, berries, whole-grain cereals and bread, vegetable-oil-based spreads and oil was recommended, and the consumption of leaner meat products and low-fat cheese and fat-free/low-fat milk products was encouraged. Fish was recommended twice per week as a main meal. The recommended amounts of foods were planned to result in monounsaturated fatty acids (MUFA) contributing 10–15 percent of total daily energy intake (E%), PUFA contributing 5–10 E%, and SFA contributing 10 E% or less. Total intake of fat was aimed at 30 E%, carbohydrates 55–60 E%, and protein 10–15 E%. To enhance compliance with the recommended diet and to demonstrate sources of ideal fat and fiber content, readily available food products with favorable fat compositions (e.g., low-erucic acid rapeseed oil–based spreads and salad dressings, fiber-enriched pasta, muesli and porridge cereals) were provided for use at home. The Raisio Group, Raisio, Finland, donated the food products. The probiotics/placebo capsules and food products were provided until the cessation of exclusive breastfeeding, however not beyond 6 months *postpartum*, while dietary counseling was provided at each study visit.

4.1.2. Study visits

The study visits were scheduled at the study clinic three times before delivery and 1, 6 and 12 months after delivery. The mothers and infants were invited for further follow-up visits at the infants' ages of 2, 4 and 7 years. At the first visit, serving as the baseline, the mothers' pre-pregnancy weights were recorded from well-baby records and study nurses measured their heights. Background information was collected by interview with the help of semi-structured forms throughout the study. The duration of pregnancy was calculated from the date of the last menstruation. At birth, cord blood samples were collected and the infants were measured in the delivery hospital. Subsequently, at 1 month of age, blood was drawn from the antecubital vein after topical lidocain anesthesia by a trained nursing staff of TYKSLAB (laboratory belonging to the Hospital District of Southwest Finland).

At the 6-month visit, the parents were asked about their perceptions of the child's health; whether their child had had any adverse reactions, and what they considered to be the cause of these reactions. A study physician performed clinical examination of the infants at study visits, except for the 1-month visit when a study nurse met the

family for interview and measured the neonate's weight and length. The continuation of breastfeeding (exclusive, partial or ended) was recorded at home and checked at each visit in conjunction with the interview of the mother. Of the 256 women recruited, 238 of them continued the study throughout pregnancy. Of the 238 children delivered (three b-twins excluded), 219 completed the 6 months', 208 the 12 months', 186 the 24 months' and 126 the 48 months' follow-up (**Figure 4**). In addition, to maximize the number of subjects in analyses, part of the missing data was obtained retrospectively after missed study visits in the following time-point. Thus data was obtained in total from 223 children from the 6 months', 211 from the 12 months', 186 from the 24 months' and 129 from the 48 months' time-points.

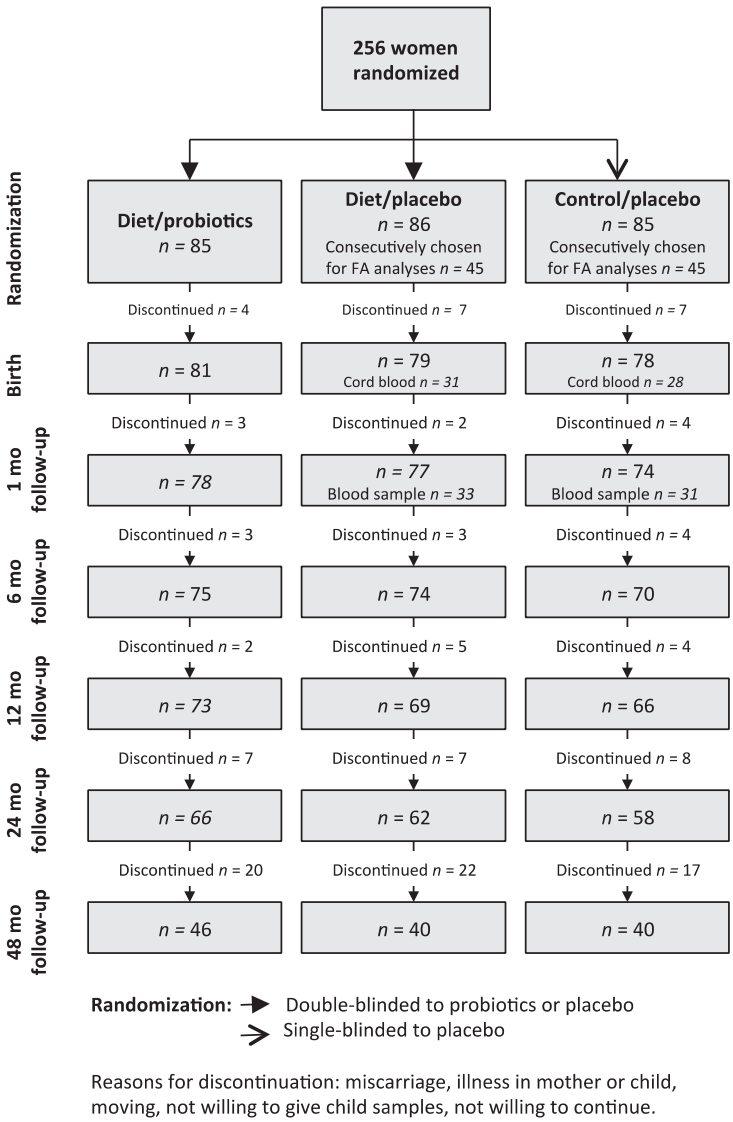

Figure 4. Study design and subject flow.

4.2. Evaluation of maternal and child diet

The mothers received 3-day food diaries to be completed at home before each study visit before and after delivery. The days were self-selected but had to be consecutive and include one weekend day. The women gave information in household measures (e.g., one apple, one glass of water). The diaries were collected at each visit to assess baseline level and the impact of nutrition counseling on food and nutrient intake. A nutritionist verified the diaries with the study subjects with the help of a portion picture booklet and specified the information, if needed. The nutritionist gave verbal feedback based on the food diary and proposed improvements to the diet for those in the dietary counseling groups. After the study visits, written feedback of the intakes of nutrients were sent to all women by post. Daily intakes of energy and nutrients were calculated using a computerized program (Micro-Nutrica version 2.5; Research Centre of the Social Insurance Institution) with a validated database and continuous updates on new commercially available foods (Jaakkola, 2013).

Based on literature search, nutrients and their food sources with possible immunomodulatory potential, were defined from the Micro-Nutrica report: LA, ALA, vitamin C, vitamin D, vitamin E, vitamin A (retinol equivalent comprising retinols and carotenoids), folate, zinc, carbohydrate, fiber, protein, MUFA, PUFA, SFA and fat; fruits/berries, vegetables, fish, meat, egg, cheese, milk, margarine, oil and butter (Hoppu *et al.*, 2000; Martindale *et al.*, 2005; Devereux *et al.*, 2006; Sausenthaler *et al.*, 2006; Devereux *et al.*, 2007; Romieu *et al.*, 2007a; Sausenthaler *et al.*, 2007; Willers *et al.*, 2007; Litonjua, 2008; Whitrow *et al.*, 2009; Lange *et al.*, 2010; Miyake *et al.*, 2010a; b; Saito *et al.*, 2010; Nurmatov *et al.*, 2011). The n-3 and n-6 FA intakes could not be reliably separated from the database used by the Micro-Nutrica computer program as this information was often missing. Nutrient intakes only from diet were included into the analyses; intakes from dietary supplements were not taken into account.

The mothers were asked to fill a free-form diary at home about the introduction of complementary foods. They were asked to record the date for introduction of every new complementary food during the first year; either the name of the food item or the commercial name of a food product introduced. The duration of exclusive and total breastfeeding was recorded from the mother. After the first year, the introduction of missing food groups was asked and recorded at every study visit. Later these foods were categorized into cow's milk (infant formula, infant gruel and cow's milk in food like porridge or plain cow's milk), cereals (wheat, barley, oat and rye), vegetables, fruits and berries, meat (red and white), fish and hen's egg. The timing of introduction was categorized as less than 4 months, 4-6 months or greater than or equal to 7 months for cow's milk and fruits and berries. Cereal, fish, meat and egg were categorized as 0-6 months or greater than or equal to 7 months due to small number of introductions under 4 months of age, and vegetables as less than 4 months or greater than or equal to 4 months due to small number of introductions over 7 months of age.

4.3. Evaluation of child atopic disease and maternal atopy

A physician examined the infants at 6, 12, 24 and 48 months of age. Diagnosis of eczema was confirmed according to the criteria introduced by Hanifin: pruritus, typical morphology and distribution (facial and/or extensor involvement) and chronic relapsing course (Hanifin, 1991). The last criterion was fulfilled at 6- and 12-month study visits if there had been manifest eczema with duration of 1 month or longer. At 24 and 48 months, the criterion was fulfilled only if eczema evinced a chronic relapsing course, i.e. it had occurred at least twice with duration of 1 month each or longer. In addition, atopic eczema was diagnosed in the case of a positive skin prick test to comply with definition for atopy (Johansson *et al.*, 2004).

Diagnosis of asthma was confirmed if a child had been qualified by the Social Insurance Institution of Finland for a special reimbursement for asthma medication up to the age of 4 years (the information was obtained from the national registry of the Social Insurance Institution of Finland).

The children were skin-prick tested to objectively assess atopic sensitization at the ages of 6 months, 1, 2 and 4 years. Skin-prick testing was chosen over serum allergen-specific IgE antibody concentrations for the high sensitivity and equal or superior accuracy it offers in predicting allergic reactions (Caffarelli *et al.*, 1995; Isolauri & Turjanmaa, 1996; Majamaa *et al.*, 1999). A pre-defined panel representing the most common causes of sensitization in Finnish populations was applied. Allergens included cow's milk, raw hen's egg white, wheat and rice flour both diluted 1/10 (w/v) with 0.9 percent (w/v) sodium chloride, gliadin diluted 1 mg/mL with an ethanol/glyceroleum/ALK-diluent (Hospital Pharmacy of University Hospital of Turku, Turku, Finland and ALK, Hoersholm, Denmark) mixture, cod, soya bean, birch, six grasses, cat, dog, *Dermatophagoides pteronyssimus* allergen Der p1 (ALK), latex (Laboratoire Stallergenes, Antony cedex, France) and potato, carrot and banana by prick–prick technique. In addition to the above-mentioned allergens, the mothers were also tested at the third study visit for peanut, hazelnut, alder and mugwort (ALK). Positive control was histamine dihydrochloride 10 mg/ml (ALK). The testing was performed on the volar side of the forearm with a 1-mm, 1-peak lancet (Allergologisk Laboratorium A/S) with a shoulder to prevent deeper penetration. The study nurses recorded the perpendicular diameters of each SPT reaction after 15 minutes. An allergen skin-test reaction with a mean wheal diameter of at least 3 mm more than the negative control (saline solution, ALK) was regarded positive.

4.4. Fatty acid analyses

The fatty acid concentration (mg/L) and composition (proportion of total fatty acids, w/w%) of serum phospholipids (PL), cholesteryl esters (CE), and triacylglycerols (TAG) were analyzed from infants' cord blood (a mixture of venous and arterial blood) and 1-month blood samples. In addition, alike FA analyses were performed from maternal

serum at third trimester and at 1 month postpartum (Hautero *et al.*, 2013). Blood samples were obtained from the infants as depicted in **Figure 4**. At least one sample was available from 81 infants (42 in the dietary counseling/placebo and 39 in the control group) and both samples were available from 42 infants (22 in the dietary counseling/placebo and 20 in the control group). A detailed report of the fatty acid analysis protocol and used internal standards has been described in studies I and II.

4.5. Outcomes

In studies I and II, the FA status of the infants was assessed with focus on PUFA and essential fatty acids (EFA). Detectable levels of eicosatrienoic acid (ETA, 20:3n-9) were taken as a marker for EFA deficiency, because EFA deficiency results in an increased production of ETA from oleic acid (Mead, 1958; Fokkema *et al.*, 2002). When ETA synthesis increases, the triene:tetraene ratio (20:3n-9/20:4n-6), the Holman index, rises with values above 0.2 taken to indicate EFA deficiency (Holman *et al.*, 1979). In addition, DHA sufficiency (22:6n-3/22:5n-6) and deficiency (22:5n-6/22:4n-6) indexes were calculated, which reflect the relative proportions between different classes of FA and thus are considered sensitive markers of functional DHA status (Fokkema *et al.*, 2002).

The primary outcome in studies III and IV was the cumulative incidence of atopic eczema (eczema with skin prick positivity), and in study III, also the cumulative incidence of asthma, in the children by the age of 4 years. A cumulative incidence of atopic eczema was chosen as the primary outcome because the chronic and relapsing nature of the disease may cause point prevalence to be an unreliable measure (Laitinen *et al.*, 2005).

4.6. Statistical methods

The distributions of continuous variables were checked using tests for normality (Shapiro-Wilk) and graphical plots. In variables describing the characteristics of the women and infants, departures from normal distribution were not marked and were thus analyzed using parametric methods. Two-group comparisons of continuous variables were analyzed using either the independent-samples *t* test or the Mann-Whitney U test, as appropriate. The Chi-squared test was used for dichotomous variables. SPSS (SPSS Inc., Chicago, Illinois, USA) version 16.0 (Studies II and III) or 20 (Study I) or SAS for Windows version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) (Study IV) were used for statistical analyses. P-values < 0.05 for a two-sided test were considered statistically significant.

Studies I and II. Fatty acids were analyzed using the analysis of variance for repeated measures (rmANOVA), in which the factors were group (dietary counseling/placebo vs. control), time and group x time interaction. The potential role of maternal skin prick test as an effect modifier was tested with the two-way ANOVA (with interaction

term). Fatty acids are presented as means (SD) with 95% confidence intervals (CI) or as medians and IQR. Intakes of energy and energy-yielding nutrients at the 3rd trimester and at 1 month *postpartum* were adjusted to baseline and ANCOVA for repeated measures was applied to study two-group comparisons. Post hoc tests were not conducted, as the interactions between time and intervention were not significant ($P > 0.10$) for all nutrient variables. Correlations between maternal and child fatty acids were studied with Pearson's correlation coefficient. The Shapiro-Wilk test for normality and Levene's test for homogeneity of variances were used to confirm the assumptions of the tests.

Study III and previously unpublished data about dietary intake during lactation. The associations of the dietary counseling to cumulative incidence of atopic eczema and asthma by the age of 4 years were calculated using binary logistic regression analyses. The contributions of several background variables (parity, smoking during pregnancy, animals at home during pregnancy, maternal positive skin prick test, maternal BMI before pregnancy, method of birth, sex of the child, birth weight, birth length and total breastfeeding over 6 months) to the risk of outcome variables were analyzed using the Chi-squared test. Of all those tested in the initial analyses, only a child being male was a significant predictor. The comparisons of study groups was then conducted with and without age-adjustment and the results are given as unadjusted and age-adjusted odds ratios (OR) with 95% confidence intervals using the control/placebo group as a reference group. The nutrients and foods with possible immunomodulatory potential were analyzed as additional predictors to the risk of outcome variables during pregnancy using the univariate Chi-squared test. Mean intakes during pregnancy (Table 1 in Study III) and lactation (Appendix) were calculated and divided into tertiles (low, middle, and high tertile). Only those mothers who were breastfeeding at both study visits 1 and 6 months postpartum (either partially or exclusively) were included into the analyses regarding dietary intake during lactation. The intakes of nutrients and foods were introduced to the forward stepwise logistic model if $P < 0.10$ in the Chi-squared test (criterion for entry $P < 0.10$), in addition, the intervention and gender were forced in the model. Intakes of nutrients and foods were analyzed in separate models.

Study IV. Kaplan-Meier analysis was applied to study the univariate associations of background factors (study group, parity, animals at home during pregnancy, method of birth, sex of the child, breastfeeding > 6 months and parental perception that the child's possible reactions at 6 month visit might be allergic) and age at the introduction of each food group to atopic eczema. Multivariate Cox's regression analyses were then used to further calculate the associations between age at the introduction of each food group and atopic eczema using those background factors with $P < 0.1$ (male gender and parental perception of allergy) as covariates. In addition, the study group was forced into the model regardless of it not being significant in the Kaplan-Meier analyses. The results were presented as hazard ratios (HRs) with their 95% confidence interval.

4.7. Ethical aspects

The participating mothers received written information about the study and discussed different study phases with the study nurses prior to enrollment. The mothers gave written informed consent. The participants had the right to cancel their consent at any time without any effect on their medical care. All women attended the care provided by the municipal prenatal clinics with standard health dietary counseling for pregnant women according to a national program. The information collected in the study was handled in confidence and kept in locked cabinets. Study visits were recorded in the hospital registry. The subjects were given feedback about their study results and were referred to health care centers or hospital if needed. The study complies with the Declaration of Helsinki as revised in 2000 and the study protocol was assessed ethically acceptable by the Ethics Committee of the Hospital District of Southwest Finland.

5. RESULTS

5.1. Clinical characteristics of the study participants

The clinical characteristics of the mothers and their infants are presented in **Table 4**. In general, the mothers were in good health with approximately 80 percent reporting allergic disease. The children were born healthy with only four pre-term deliveries (range: 30.3-36.9 weeks). The study groups did not have any significant differences in clinical characteristics, and the characteristics of the subgroup of 90 women chosen for FA analyses were comparable to those of the whole study population (**Table 4**). The characteristics of the dropouts did not differ from those who continued the study at 4 years of age, **Table 5**.

Table 4. Clinical characteristics of the participating mothers and their infants by study group.

	Dietary counseling and probiotics	**Dietary counseling and placebo**	*Subgroup[1] of dietary counseling and placebo*	**Controls**	*Subgroup[1] of controls*
	(n = 85)	*(n = 86)*	*(n = 45)*	*(n = 85)*	*(n = 45)*
Mothers					
Age, y	29.7 (4.2)	30.1 (5.2)	30.8 (5.4)	30.2 (5.0)	30.3 (4.4)
College or university degree	65 (78.3)	58 (67.4)	31 (68.9)	67 (78.8)	36 (80.0)
Primigravida	55 (64.7)	44 (51.2)	21 (46.7)	48 (56.5)	25 (55.6)
Allergic disease	67 (78.8)	70 (81.4)	36 (80.0)	65 (76.5)	36 (80.0)
Prick positive	50 (61.7)	43 (55.1)	23 (52.3)	44 (56.4)	24 (54.5)
Prepregnancy BMI, kg/m^2	22.9 (3.2)	24.3 (4.4)	24.2 (4.2)	23.7 (3.5)	23.1 (3.4)
Smoking during pregnancy	1 (1.3)	5 (6.4)	2 (4.4)	5 (6.3)	2 (4.4)
Infants					
Birth at weeks of gestation	40.0 (1.3)	40.0 (1.7)	39.7 (2.1)	40.1 (1.3)	40.0 (1.4)
Caesarian section	13 (17.3)	12 (15.6)	7 (15.9)	11 (14.3)	6 (13.6)
Male gender	42 (51.9)	39 (49.4)	22 (48.9)	43 (55.8)	27 (62.8)
Birth weight, g	3490 (430)	3600 (440)	3610 (480)	3600 (510)	3590 (520)
Birth length, cm	50.7 (1.8)	51.3 (1.7)	51.3 (1.7)	51.0 (2.2)	50.8 (2.2)
Exclusive breastfeeding, mo	3.1 (1.7)	3.3 (2.1)	2.9 (2.3)	3.1 (1.6)	3.0 (1.8)
Total breastfeeding, mo	7.7 (4.4)	9.0 (5.8)	8.4 (5.9)	8.3 (4.5)	7.7 (4.2)

Values are mean (SD) or n (%). No significant differences between the three original study groups (ANOVA) or between subjects included vs. not included to the subgroup (independent samples *t*-test or Chi-squared test). [1]Subgroup = those participating in FA analyses in studies I and II.

Table 5. Clinical characteristics of those continuing in the study at 4 years of age and the dropouts.

	Continuing the study at 4 y *n =126*	**Drop-outs** *n =130*
Study group		
Dietary counseling/probiotics	46 (36.5)	39 (30.0)
Dietary counseling/placebo	40 (31.7)	46 (35.4)
Controls	40 (31.7)	45 (34.6)
Maternal age at baseline, y	30.4 (4.5)	29.6 (5.1)
College or university degree	99 (79.2)	91 (70.5)
Maternal allergic disease	99 (78.6)	103 (79.2)
Maternal pre-pregnancy BMI, kg/m^2	23.4 (3.4)	23.8 (4.1)
Birth at weeks of gestation	40.1 (1.4)	39.9 (1.6)
Total breastfeeding, mo	8.5 (4.6)	7.9 (5.4)
Child prick positive at 6 mo	24 (19.4)	14 (15.9)
Child eczema at 6 mo	21 (16.7)	18 (19.6)

Values are mean (SD) or n (%). No significant differences between groups with independent samples *t*-test or Chi-squared test.

5.2. Allergic diseases during the study

The development of allergic diseases was carefully assessed at each study visit by a study physician. By 4 years of age, altogether 74 of the 219 children with available data (33.8%) were sensitized to one or more of the tested allergens, 86 out of the 219 (39.3%) had eczema, 40 out of the 219 (18.3%) had the diagnosis of atopic eczema and 20 out of the 194 (10.3%) the diagnosis of asthma. The prevalence of atopic sensitization, eczema and atopic eczema during each study visit is shown in **Figure 5**.

During the first 6 months after birth, any adverse reaction in child was reported by 84 (39.4%) of the 213 parents (missing data *n*=6), of whom 69 (82.1%) reported that they suspected these reactions to be allergy-induced. At 6 months of age, 57 of the 213 children (26.8%) were on elimination diet due to suspected allergy (parental report), although at the same time only 20 of the 221 children (9.0%) were diagnosed with atopic eczema by a physician.

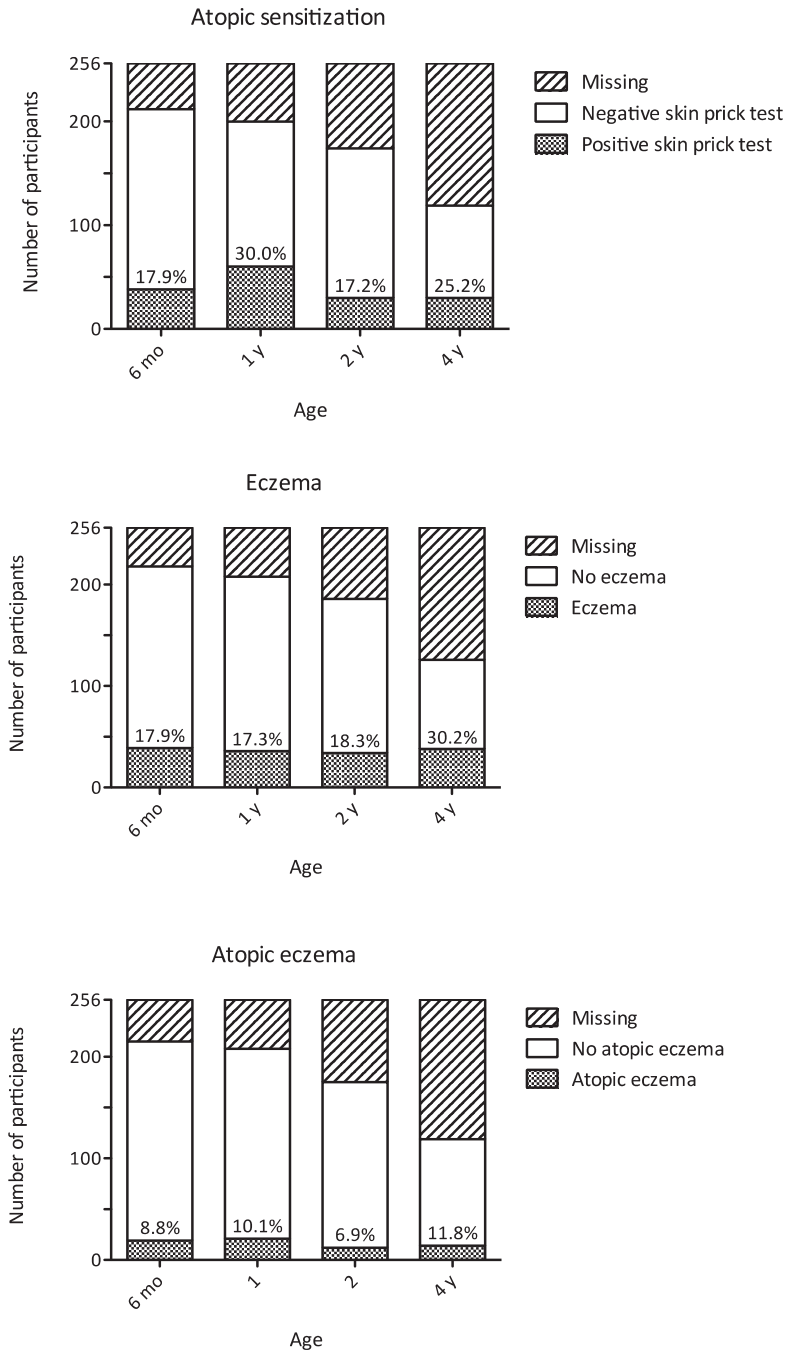

Figure 5. The prevalence of atopic sensitization, eczema (both atopic and nonatopic) and atopic ezcema at each study visit. Percentages indicate subjects with positive skin prick test, eczema or atopic eczema from those present at respective study visit.

5.3. Effects of the dietary counseling and probiotic intervention

5.3.1. Maternal diet

The dietary intakes of the mothers were similar at the first trimester of pregnancy (baseline) in all three groups. There were no significant differences in dietary intakes between mothers in the dietary counseling group with probiotics or placebo. The findings in the subgroup of 90 women participating in the fatty acid study (studies I and II) were similar to the whole study population.

During pregnancy (the average of all three trimesters, whole study population in study III), mothers receiving dietary counseling (n = 171) consumed significantly less SFA [mean 12.1 E% (SD 2.3) vs. 13.1 E% (2.4), P = 0.003] and significantly more PUFA [5.5 E% (1.1) vs. 5.1 E% (1.1), P = 0.001] than controls (n = 85). In addition, the consumption of dietary fiber [21.3 g (5.9) vs. 19.4 g (5.7), P = 0.016)], folate [300 μg (71) vs. 280 μg (64), P = 0.016] and vitamins C [150 mg/day (63) vs. 130 mg/day (49), P = 0.006] and E [10.5 mg/day (2.8) vs. 9.0 mg/day (2.6), P < 0.001] was significantly higher in the dietary counseling groups than in the control group. Total energy intake (kJ) and intakes of total fat, protein, and carbohydrate as a percent of energy and other nutrients were similar between the study groups. The change in dietary habits was evident as greater consumption of combined amount of vegetables, fruits and berries [640 g/day (200) vs. 580 g/day (150), P = 0.007], margarine [18.6 g/day (8.3) vs. 15.8 g/day (8.6), P = 0.015] and lesser consumption of butter [3.9 g/day (4.3) vs. 6.1 g/day (6.8), P = 0.009] in the dietary counseling groups than in the control group. There was no difference between the groups in consumption of other foods, including also vegetable oil, fish and whole-grain products.

Likewise, during the first 6 months after giving birth (the average of 1 and 6 months *postpartum*), the mothers in the dietary counseling groups (n = 154) consumed significantly less SFA [mean 12.0 E% (SD 2.5) vs. 13.6 E% (2.8), P < 0.001] and significantly more PUFA [6.0 (1.2) vs. 5.4 (1.3) E%, P = 0.001], MUFA [11.5 E% (1.9) vs. 10.8 E% (2.3), P = 0.012] and E-vitamin [10.6 mg/day (3.0) vs. 9.3 mg/day (2.4), P = 0.001] than control mothers (n = 73). The use of margarine [22.8 g/day (10.7) vs. 19.1 g/day (9.5), P = 0.011], vegetable oil [12.3 g/day (8.9) vs. 9.0 g/day (6.4), P = 0.002] and combined amount of vegetables, fruits and berries [530 g/day (220) vs. 460 g/day (170), P = 0.015] was greater and the use of butter [2.7 g/day (4.4) vs. 5.4 g/day (7.2), P = 0.004] lesser than in those receiving dietary counseling. No differences were observed in intakes of other nutrients or consumption of other foods.

5.3.2. Child fatty acid profiles (I and II)

Fatty acid profiles in cord blood. In cord blood PL FA, the proportion and concentration of ETA (20:3n-9) and the proportion of docosapentaenoic acid (22:5n-6) were significantly

lower in the dietary counseling/placebo than in the control group, **Table 6**. Further, the EFA deficiency index (the Holman index, 20:3n-9/20:4n-6) was lower [median 0.05 (IQR 0.03-0.06) vs. 0.07 (0.05-0.08), P = 0.004], the DHA sufficiency index (22:6n-3/22:5n-6) was higher [14.2 (9.7-17.0) vs. 9.9 (8.3-13.6), P = 0.009] and the DHA deficiency index (22:5n-6/22:4n-6) was lower [0.83 (0.73-1.13) vs. 1.04 (0.86-1.14), P = 0.048] in the dietary counseling/placebo than in the control group, respectively. In line with these indexes, the proportion of DHA (22:6n-3) in TAG fraction was significantly higher in the counseling versus the control group.

No other significant differences were observed between the study groups in proportions or concentrations of CE or TAG FA, **Tables 6 and 7**.

Table 6. The proportions (percentage of total FA) of serum PL, CE and TAG FA in cord blood.

	Phospholipids			**Cholesteryl esters**			**Triacylglycerols**		
	Diet/placebo (n = 31)	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*	
	Mean (SD)	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]
SFA	48.76 (6.50)	47.32 (0.85)	0.252	24.0 (2.0)	24.2 (1.2)	0.620	34.91 (2.27)	36.22 (2.61)	0.043
MUFA	14.48 (2.51)	15.15 (1.63)	0.231	42.3 (4.7)	42.1 (4.0)	0.857	41.52 (3.62)	42.02 (3.28)	0.582
PUFA	36.77 (4.89)	37.52 (1.71)	0.439	33.7 (4.8)	33.7 (4.2)	0.998	23.58 (3.95)	21.76 (3.53)	0.069
18:2n-6	6.95 (1.38)	7.23 (1.27)	0.420	16.2 (2.0)	16.3 (2.8)	0.877	9.89 (1.79)	9.59 (2.34)	0.585
18:3n-6	0.08 (0.05)	0.08 (0.06)	0.956	0.72 (0.24)	0.78 (0.23)	0.318	0.49 (0.13)	0.50 (0.18)	0.784
20:3n-6	5.23 (0.96)	5.56 (0.80)	0.158	1.45 (0.23)	1.48 (0.43)	0.793	0.83 (0.16)	0.85 (0.31)	0.764
20:4n-6	14.54 (2.22)	14.77 (1.59)	0.867	12.1 (2.8)	12.0 (2.2)	0.841	3.49 (0.99)	3.22 (0.88)	0.281
22:4n-6	0.53 (0.12)	0.56 (0.08)	0.266	0.01 (0.03)	0.02 (0.1)	0.534	0.60 (0.17)	0.57 (0.14)	0.412
22:5n-6	0.49 (0.18)	0.61 (0.19)	0.015*	0.15 (0.17)	0.11 (0.13)	0.393	0.86 (0.26)	0.85 (0.30)	0.880
Sum n-6	27.82 (3.95)	28.81 (2.04)	0.238	30.6 (4.4)	30.6 (4.2)	0.981	16.16 (2.60)	15.58 (2.93)	0.426
18:3n-3	0.08 (0.14)	0.04 (0.04)	0.247	0.28 (0.18)	0.28 (0.16)	0.960	0.65 (0.22)	0.62 (0.23)	0.610
20:5n-3	1.09 (0.71)	1.18 (0.65)	0.624	0.85 (0.40)	0.80 (0.31)	0.560	0.85 (0.45)	0.78 (0.34)	0.540
22:5n-3	0.56 (0.25)	0.47 (0.17)	0.141	0.04 (0.12)	0.01 (0.04)	0.202	0.60 (0.32)	0.47 (0.18)	0.055
22:6n-3	6.47 (1.66)	6.02 (1.03)	0.223	1.47 (0.50)[2]	1.29 (0.37)[2]	0.129	4.44 (1.87)[2]	3.21 (0.99)[2]	0.002*
Sum n-3	8.19 (2.03)	7.71 (1.50)	0.313	2.65 (0.8)	2.38 (0.7)	0.190	6.53 (2.45)	5.08 (1.32)	0.006
n-6/n-3	3.55 (0.87)	3.91 (0.99)	0.137	12.4 (3.1)	13.7 (3.8)	0.141	2.72 (0.81)	3.22 (0.91)	0.029
20:3n-9	0.76 (0.42)	1.01 (0.39)	0.023*	0.46 (0.30)	0.70 (1.22)	0.303	0.89 (0.34)	1.11 (0.34)	0.017*

[1]Data were analyzed by independent-samples *t*-test. [2]Eluted with 24:1 in the analyses. *P < 0.05.

Table 7. The concentrations (mg/L) of serum PL, CE and TAG FA in cord blood.

	Phospholipids			**Cholesteryl esters**			**Triacylglycerols**		
	Diet/placebo (n = 31)	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*	
	Mean (SD)	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]
SFA	42.39 (11.31)	42.77 (11.93)	0.901	16.78 (5.82)	16.57 (5.56)	0.889	9.10 (4.87)	12.22 (11.47)	0.190
MUFA	12.92 (4.51)	13.72 (4.32)	0.491	29.41 (9.97)	28.81 (10.57)	0.823	10.79 (5.68)	13.98 (12.69)	0.230
PUFA	32.75 (10.49)	33.87 (9.38)	0.669	23.31 (8.40)	22.79 (7.22)	0.803	6.29 (3.73)	7.50 (7.14)	0.425
18:2n-6	6.20 (2.11)	6.45 (1.90)	0.635	11.22 (4.04)	10.93 (3.53)	0.768	2.69 (1.69)	3.52 (3.83)	0.296
18:3n-6	0.07 (0.05)	0.06 (0.05)	0.903	0.49 (0.21)	0.52 (0.20)	0.540	0.13 (0.08)	0.16 (0.16)	0.360
20:3n-6	4.63 (1.47)	5.03 (1.52)	0.312	1.00 (0.34)	1.02 (0.42)	0.854	0.22 (0.13)	0.30 (0.36)	0.282
20:4n-6	12.95 (4.29)	13.28 (3.84)	0.757	8.36 (3.44)	8.13 (3.02)	0.794	0.95 (0.68)	1.04 (0.95)	0.656
22:4n-6	0.48 (0.18)	0.51 (0.16)	0.514	0.01 (0.02)	0.02 (0.09)	0.439	0.17 (0.13)	0.20 (0.20)	0.469
22:5n-6	0.44 (0.22)	0.56 (0.27)	0.062	0.01 (0.11)	0.08 (0.09)	0.482	0.23 (0.16)	0.29 (0.29)	0.385
Sum n-6	24.75 (7.85)	25.88 (7.00)	0.564	21.17 (7.71)	20.69 (6.73)	0.804	4.38 (2.76)	5.50 (5.61)	0.344
18:3n-3	0.07 (0.12)	0.04 (0.04)	0.255	0.19 (0.12)	0.19 (0.11)	0.747	0.18 (0.12)	0.22 (0.22)	0.342
20:5n-3	0.97 (0.74)	1.12 (0.74)	0.449	0.59 (0.33)	0.53 (0.23)	0.453	0.20 (0.12)	0.23 (0.15)	0.510
22:5n-3	0.51 (0.30)	0.42 (0.19)	0.219	0.02 (0.06)	0.01 (0.03)	0.226	0.17 (0.13)	0.17 (0.17)	0.868
22:6n-3	5.783 (2.33)	5.46 (1.85)	0.558	1.02 (0.51)[2]	0.88 (0.38)[2]	0.241	1.13 (0.71)[2]	1.03 (0.83)[2]	0.610
Sum n-3	7.33 (3.00)	7.04 (2.58)	0.697	1.83 (0.81)	1.61 (0.62)	0.250	1.68 (1.02)	1.65 (1.33)	0.927
20:3n-9	0.67 (0.38)	0.95 (0.55)	0.026*	0.31 (0.19)	0.95 (0.55)	0.263	0.23 (0.13)	0.35 (0.32)	0.061

[1]Data were analyzed by independent-samples *t*-test. [2]Eluted with 24:1 in the analyses. *P < 0.05.

Fatty acid profiles at 1 month of age. There were no significant differences between the dietary counseling and control group in proportions or concentrations of PL, CE, or TAG fatty acids at 1 month of age, **Tables 8 and 9**.

All FA concentrations increased or decreased significantly from cord blood to 1 month, but in general, the change was not significantly different between the study groups (repeated measures ANOVA, no interaction between time and group). However, a distinction in EPA (20:5n-3) status existed: in TAG and PL, the concentration of EPA increased in children, whose mothers received dietary counseling, but declined in control subjects from birth to 1 month of age (time x group interaction P = 0.009 for TAG and P = 0.044 for PL). When examining cord blood and 1 month time points together using ANOVA for repeated measures, in TAG the proportion of sum of n-3 FA was higher (4.88% vs. 3.89%, *P*=0.012), and consequently also the ratio of sum of n-6 to n-3 FA was lower (3.85 vs. 4.61, P = 0.022) over the study period in children whose mothers were receiving vs. not receiving counseling.

Table 8. The proportions (percentage of total FA) of serum PL, CE and TAG FA at 1 month of age.

	Phospholipids			Cholesteryl esters			Triacylglycerols		
	Diet/placebo (n = 31)	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*	
	Mean (SD)	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]
SFA	44.84 (1.41)	44.73 (1.36)	0.757	19.0 (2.0)	19.4 (1.7)	0.401	35.42 (4.25)	36.10 (3.26)	0.478
MUFA	16.33 (1.35)	16.47 (2.00)	0.746	34.2 (2.9)	34.6 (3.5)	0.630	47.19 (2.69)	46.97 (3.26)	0.769
PUFA	38.83 (1.50)	38.80 (2.03)	0.946	46.8 (3.5)	46.0 (4.2)	0.416	17.39 (2.38)	16.93 (2.97)	0.496
18:2n-6	19.46 (2.91)	19.19 (2.65)	0.690	36.7 (3.6)	36.0 (3.9)	0.471	12.40 (2.27)	12.09 (2.72)	0.620
18:3n-6	0.06 (0.03)	0.06 (0.03)	0.720	0.45 (0.11)	0.48 (0.16)	0.519	0.17 (0.10)	0.17 (0.11)	0.869
20:3n-6	3.14 (0.54)	3.32 (0.43)	0.145	0.78 (0.17)	0.80 (0.16)	0.610	0.35 (0.11)	0.36 (0.12)	0.651
20:4n-6	9.51 (1.85)	9.64 (1.59)	0.773	6.32 (1.73)	6.25 (1.61)	0.866	0.94 (0.32)	0.94 (0.31)	0.933
22:4n-6	0.26 (0.04)	0.27 (0.04)	0.577	0.00 (0.01)	0.00 (0.01)	0.809	0.12 (0.05)	0.14 (0.07)	0.429
22:5n-6	0.20 (0.08)	0.19 (0.07)	0.489	0.09 (0.10)	0.08 (0.09)	0.712	0.08 (0.06)	0.10 (0.10)	0.364
Sum n-6	32.65 (1.70)	32.66 (2.29)	0.974	44.3 (3.5)	43.6 (4.3)	0.463	14.06 (2.10)	13.79 (2.82)	0.665
18:3n-3	0.19 (0.04)	0.17 (0.05)	0.065	0.66 (0.20)	0.58 (0.17)	0.103	1.67 (0.47)	1.48 (0.44)	0.098
20:5n-3	0.72 (0.39)	0.69 (0.39)	0.753	0.70 (0.41)	0.68 (0.35)	0.856	0.29 (0.20)	0.25 (0.23)	0.442
22:5n-3	0.73 (0.20)	0.72 (0.13)	0.924	0.03 (0.03)	0.02 (0.04)	0.570	0.26 (0.14)	0.26 (0.11)	0.911
22:6n-3	4.10 (1.09)	4.07 (0.93)	0.907	0.70 (0.23)[2]	0.70 (0.21)[2]	0.935	0.78 (0.45)[2]	0.76 (0.47)[2]	0.840
Sum n-3	5.74 (1.56)	5.66 (1.23)	0.811	2.08 (0.73)	1.98 (0.50)	0.509	3.01 (1.02)	2.75 (0.83)	0.270
n-6/n-3	6.35 (2.89)	6.09 (1.58)	0.668	24.4 (10.6)	23.5 (6.5)	0.684	5.29 (2.33)	5.44 (1.90)	0.778
20:3n-9	0.44 (0.21)	0.48 (0.20)	0.472	0.39 (0.30)	0.42 (0.27)	0.625	0.33 (0.17)	0.40 (0.23)	0.174

[1]Data were analyzed by independent-samples *t*-test. [2]Eluted with 24:1 in the analyses. *P < 0.05.

Table 9. The concentrations (mg/L) of serum PL, CE and TAG FA at 1 month of age.

	Phospholipids			Cholesteryl esters			Triacylglycerols		
	Diet/placebo (n = 31)	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*		*Diet/placebo (n = 31)*	*Control (n = 28)*	
	Mean (SD)	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]	*Mean (SD)*	*Mean (SD)*	*P*[1]
SFA	70.83 (20.54)	74.66 (15.55)	0.407	24.86 (8.44)	27.00 (7.72)	0.295	34.25 (16.57)	32.73 (13.88)	0.693
MUFA	25.66 (7.54)	27.22 (5.58)	0.352	44.97 (14.52)	48.37 (14.35)	0.350	45.75 (21.80)	42.28 (16.48)	0.477
PUFA	61.36 (18.24)	65.12 (15.42)	0.377	61.33 (19.64)	63.85 (16.58)	0.582	16.85 (8.65)	15.29 (6.51)	0.420
18:2n-6	30.83 (10.25)	32.29 (8.92)	0.545	48.06 (15.57)	50.03 (13.50)	0.592	12.24 (6.74)	11.06 (5.11)	0.435
18:3n-6	0.10 (0.06)	0.10 (0.05)	0.936	0.58 (0.20)	0.65 (0.22)	0.197	0.14 (0.06)	0.14 (0.06)	0.942
20:3n-6	5.02 (1.84)	5.55 (1.44)	0.207	1.02 (0.40)	1.09 (0.29)	0.424	0.32 (0.17)	0.31 (0.15)	0.811
20:4n-6	14.97 (5.33)	16.17 (4.45)	0.331	8.31 (3.62)	8.62 (2.91)	0.710	0.85 (0.43)	0.81 (0.35)	0.717
22:4n-6	0.42 (0.14)	0.45 (0.12)	0.267	0.00 (0.01)	0.00 (0.02)	0.720	0.11 (0.06)	0.12 (0.07)	0.537
22:5n-6	0.33 (0.18)	0.32 (0.13)	0.796	0.13 (0.02)	0.12 (0.02)	0.866	0.07 (0.05)	0.09 (0.09)	0.183
Sum n-6	51.66 (15.49)	54.89 (13.37)	0.377	58.09 (18.64)	60.50 (15.78)	0.580	13.73 (7.21)	12.54 (5.57)	0.464
18:3n-3	0.31 (0.12)	0.29 (0.10)	0.653	0.87 (0.40)	0.83 (0.39)	0.716	1.64 (1.01)	1.38 (0.71)	0.239
20:5n-3	1.11 (0.63)	1.14 (0.64)	0.854	0.91 (0.60)	0.94 (0.54)	0.864	0.24 (0.17)	0.17 (0.10)	0.064
22:5n-3	1.14 (0.47)	1.21 (0.32)	0.545	0.03 (0.05)	0.03 (0.05)	0.627	0.24 (0.17)	0.22 (0.10)	0.638
22:6n-3	6.43 (2.58)	6.80 (2.20)	0.534	0.41 (0.07)[2]	0.40 (0.07)[2]	0.601	0.70 (0.53)[2]	0.62 (0.37)[2]	0.511
Sum n-3	8.99 (3.60)	9.44 (2.90)	0.582	2.73 (1.30)	2.77 (1.05)	0.905	2.81 (1.67)	2.39 (1.05)	0.238
20:3n-9	0.70 (0.40)	0.79 (0.39)	0.382	0.51 (0.36)	0.58 (0.37)	0.426	0.31 (0.20)	0.36 (0.25)	0.398

[1]Data were analyzed by independent-samples *t*-test. [2]Eluted with 24:1 in the analyses. *P < 0.05.

The correlation of maternal and child fatty acids. Previously, an increase in the sum of n-3 FA, PUFA and DHA was observed in maternal plasma PL at third trimester with similar changes in CE and TAG fractions, but not at 1 month *postpartum* (Hautero *et al.*, 2013). Although there were no differences in child FA status between the counseling and control group at 1 month of age, the majority of the maternal and child serum fatty acids were strongly positively correlated at the same time, **Table 10**.

Table 10. Correlations between maternal and child serum fatty acids at 1 month *postpartum*.

	Phospholipids		**Cholesteryl esters**		**Triacylglycerols**	
	R	*P*	*R*	*P*	*R*	*P*
SFA (%)	0.363	0.003*	0.073	0.567	0.644	<0.001*
SFA (mg)	0.181	0.153	0.312	0.012*	0.098	0.443
MUFA (%)	0.142	0.261	0.173	0.171	0.670	<0.001*
MUFA (mg)	0.134	0.293	0.283	0.023*	0.052	0.685
PUFA (%)	0.090	0.480	0.188	0.138	0.606	<0.001*
PUFA (mg)	0.173	0.172	0.354	0.004*	0.171	0.177
18:2n-6 (%)	0.126	0.322	0.129	0.310	0.536	<0.001*
18:2n-6 (mg)	0.205	0.104	0.361	0.003*	0.268	0.032*
18:3n-6 (%)	0.085	0.504	0.247	0.049*	0.306	0.014*
18:3n-6 (mg)	0.074	0.560	0.337	0.006*	0.234	0.063
20:3n-6 (%)	0.518	<0.001*	0.242	0.054	0.338	0.006*
20:3n-6 (mg)	0.293	0.019*	0.189	0.134	-0.070	0.583
20:4n-6 (%)	0.373	0.002*	0.354	0.004*	0.383	0.002*
20:4n-6 (mg)	0.233	0.064	0.302	0.015*	0.012	0.927
Total n-6 (%)	0.281	0.024*	0.156	0.218	0.573	<0.001*
Total n-6 (mg)	0.218	0.083	0.357	0.004*	0.223	0.076
18:3n-3 (%)	0.469	<0.001*	0.636	<0.001*	0.767	<0.001*
18:3n-3 (mg)	0.270	0.031*	0.464	<0.001*	0.123	0.332
20:5n-3 (%)	0.735	<0.001*	0.837	<0.001*	0.564	<0.001*
20:5n-3 (mg)	0.490	<0.001*	0.574	<0.001*	0.370	0.003*
22:5n-3 (%)	0.531	<0.001*	0.056	0.659	0.613	<0.001*
22:5n-3 (mg)	0.248	0.048*	0.061	0.631	0.106	0.407
22:6n-3 (%)	0.500	<0.001*	0.445	<0.001*	0.583	<0.001*
22:6n-3 (mg)	0.218	0.083	0.294	0.019*	0.366	0.003*
Total n-3 (%)	0.636	<0.001*	0.794	<0.001*	0.730	<0.001*
Total n-3 (mg)	0.255	0.042*	0.448	<0.001*	0.109	0.390
n6/n3	0.552	<0.001*	0.540	<0.001*	0.579	<0.001*

R = Pearson's correlation coefficient. *P < 0.05. n = 64.

5.3.3. Allergic manifestations in the child (III)

The dietary counseling with or without probiotic supplementation had no significant effect on the risk of child cumulative incidence of atopic eczema or asthma by 4 years of age, **Table 11**. Of all the background factors (other than dietary compounds) tested, only a child being male was a significant risk factor for atopic eczema [OR 3.73 (95% CI 1.68, 8.27), P = 0.001]. Also, the risk of asthma tended to be higher among boys than girls [OR 2.29 (95% CI 0.90, 5.80), P = 0.082].

Table 11. Cumulative incidence of atopic eczema, eczema and asthma in the study groups by the age of 4 years.

		Cumulative incidence [1]	**Non-adjusted logistic regression analysis**			**Adjusted[2] logistic regression analysis**		
			OR	*95% CI*	*P*	*OR*	*95% CI*	*P*
Atopic eczema	Control/Placebo	12.9 (9/70)	1.00			1.00		
	Diet/Probiotics	18.7 (14/75)	1.56	0.63, 3.86	0.341	1.68	0.66, 4.27	0.275
	Diet/Placebo	22.7 (17/75)	1.99	0.82, 4.81	0.128	2.29	0.92, 5.69	0.076
Eczema[3]	Control/Placebo	41.4 (29/70)	1.00			1.00		
	Diet/Probiotics	36.5 (27/74)	0.81	0.42, 1.59	0.543	0.85	0.43, 1.69	0.645
	Diet/Placebo	40.0 (30/75)	0.94	0.49, 1.83	0.861	1.01	0.51, 1.99	0.979
Asthma	Control/Placebo	7.2 (5/69)	1.00			1.00		
	Diet/Probiotics	7.6 (6/79)	1.05	0.31, 3.61	0.936	1.08	0.31, 3.72	0.907
	Diet/Placebo	16.2 (12/74)	2.48	0.83, 7.44	0.106	2.59	0.85, 7.87	0.092

[1]Values are % (number of subjects with diagnosis / total number of subjects). [2]Adjusted for sex. [3]Both non-atopic and atopic eczema.

5.4. Dietary exposures defining child allergy

5.4.1. Maternal diet during pregnancy (III)

In univariate analyses, non-linear associations to child risk of atopic eczema were found with dietary intakes of ALA (18:3n-3) (P = 0.071), vitamin C (P = 0.087), folate (P = 0.053), vegetables (P = 0.066), cheese (P = 0.072), milk (P = 0.013) and margarine (P = 0.098), and association to child risk of asthma with protein (P = 0.046). A positive linear association with atopic eczema was seen in the intake of dietary fibre (P = 0.057).

In multivariate analyses, the risk for atopic eczema was lower in children whose mothers had consumed milk between 231-392 g/day [OR 0.27 (95% CI 0.10, 0.73), P = 0.009] with a similar trend for consumption exceeding 392 g/day when compared to children whose mothers had consumed less than 231 g/day (**Table 12**, Model 1B). Conversely, the risk of atopic eczema was higher in children whose mothers had consumed cheese 37.3-55.9 g/day during pregnancy [OR 2.65 (95% CI 1.02, 6.89), P = 0.046] with similar trend for consumption exceeding 55.9 g/day, compared to mothers with intakes less than 37.3 g/day.

Table 12. Multivariate logistic regression analyses of the dietary factors during pregnancy associated with child cumulative incidence of atopic eczema and asthma by the age of 4 years. Nutrients and foods were included in separate models.

Study outcome	Model	Dietary factors	Predictors[1]		Multivariate logistic regression[2]	
					OR (95% CI)	*P*
Atopic eczema	1A.	Nutrients	Folate			0.014
				Medium intake	2.05 (0.75, 5.55)	0.160
				High intake	0.52 (0.15, 1.79)	0.302
			Dietary fibre			0.084
				Medium intake	1.19 (0.41, 3.41)	0.751
				High intake	3.05 (0.99, 9.42)	0.052
	1B.	Foods	Cheese			0.085
				Medium intake	2.65 (1.02, 6.89)	0.046*
				High intake	1.28 (0.46, 3.52)	0.636
			Milk			0.028
				Medium intake	0.27 (0.10, 0.73)	0.009*
				High intake	0.51 (0.21, 1.20)	0.123
Asthma	2A.	Nutrients	Protein (E%)			0.037
				Medium intake	2.76 (0.96, 7.92)	0.060
				High intake	0.70 (0.18, 2.65)	0.597
	2B.	Foods [3]	–			

[1]Low intake is the reference category. [2]Adjusted for study group and gender. Intakes of nutrients and foods were introduced to the forward stepwise logistic model if $P < 0.10$ in univariate analysis. [3]The multivariate model was not conducted as $P > 0.10$ for intakes of nutrients and foods in univariate analyses. *$P < 0.05$.

5.4.2. Maternal diet during breastfeeding

In univariate analyses, positive linear associations with asthma were observed with vitamin C ($P = 0.012$), vitamin E ($P = 0.012$), folate ($P = 0.070$), fat (MJ) ($P = 0.067$) and fruits and berries ($P = 0.059$). A negative linear association with atopic eczema existed with the intake of egg ($P = 0.041$).

In multivariate analyses, the children, whose mothers had received C-vitamin over 140 mg/day [OR 5.85 (95% CI 1.19, 28.7), $P = 0.029$] had a higher risk for atopic eczema when compared to children, whose mothers had intakes less than 80 mg/day. The risk of atopic eczema was lower in children, whose mothers had consumed hen's egg over 20 g/day during pregnancy [OR 0.23 (95% CI 0.07, 0.73), $P = 0.012$], compared to mothers with intakes less than 8 g/day, **Table 13.**

Table 13. Multivariate logistic regression analyses of the dietary factors during breastfeeding associated with child cumulative incidence of atopic eczema and asthma by the age of 4 years. Nutrients and foods were included in separate models.

Study outcome	Model	Dietary factors	Predictors[1]		Multivariate logistic regression[2]	
					OR (95% CI)	*P*
Atopic eczema	1A.	Nutrients[3]	–			
	1B.	Foods	Hen's egg			0.034
				Medium intake	0.44 (0.17, 1.19)	0.108
				High intake	0.23 (0.07, 0.73)	0.012*
Asthma	2A.	Nutrients	C-vitamin			0.031
				Medium intake	1.50 (0.24, 9.54)	0.665
				High intake	5.85 (1.19, 28.7)	0.029*
	2B.	Foods	Fruits & berries			0.113
				Medium intake	0.86 (0.16, 4.60)	0.856
				High intake	3.01 (0.74, 12.2)	0.123

[1]Low intake is the reference category. [2]Adjusted for study group and gender. Intakes of nutrients and foods were introduced to the forward stepwise logistic model if $P < 0.10$ in univariate analysis. [3]The multivariate model was not conducted as $P > 0.10$ for intakes of nutrients and foods in univariate analyses. *$P < 0.05$.

5.4.3. Timing of complementary feeding (IV)

The children, who had been introduced to cereals and fish after 7 months of age, had significantly more atopic eczema than those children with introduction before 7 months ($P = 0.005$ and $P = 0.013$, respectively) (**Figures 6** and **7**). When controlling for the effects of the parents' perception that their child may show symptoms of allergic disease at six months of age, the sex of the child and the study group, the associations between the introduction of cereals or fish and atopic eczema were no longer significant (**Table 14**). The only factors that were significant were parental perception of adverse reactions in both models and male gender in the model for the introduction of cereals.

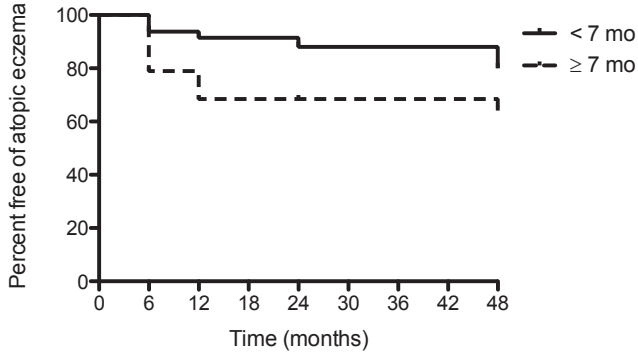

Figure 6. Kaplan-Meier analysis of the association between the age at introduction of cereals (<7 months vs. ≥ 7 months) and atopic eczema by 4 years of age. The figure has been published previously in the original publication IV.

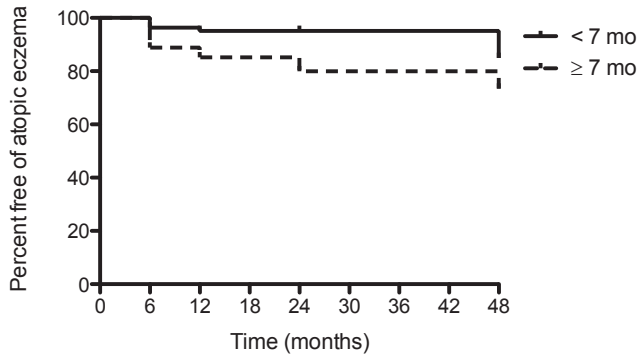

Figure 7. Kaplan-Meier analysis of the association between the age at introduction of fish (< 7 months vs. ≥ 7 months) and atopic eczema by 4 years of age. The figure has been published previously in the original publication IV.

Table 14. The effect of introduction of a) cereals and b) fish after 7 months of age on cumulative incidence of atopic eczema by 4 years of age.

a)

	HR	95% CI	P
Group[a]			
Diet/probiotics	1.14	0.49, 2.66	0.764
Diet/placebo	1.29	0.57, 2.94	0.538
Male gender[b]	2.73	1.28, 5.80	0.009
Cereals ≥ 7 mo[c]	1.43	0.73, 2.80	0.303
Parental perception*	10.0	4.51, 22.2	<0.001

b)

	HR	95% CI	P
Group[a]			
Diet/probiotics	0.90	0.37, 2.24	0.828
Diet/placebo	1.11	0.44, 2.79	0.822
Male gender[b]	2.67	1.16, 6.11	0.021
Fish ≥ 7 mo[d]	1.73	0.75, 3.99	0.196
Parental perception*	9.79	4.33, 22.1	< 0.001

Multivariate analysis performed with Cox's regression analysis. HR = hazard ratio. Reference groups: [a] control group, female gender, [c] cereals < 7 mo and [d] fish < 7 mo. *Parental perception that the child's reactions at 6 months of age could be allergic. The table has been modified from original publication IV.

6. DISCUSSION

6.1. Modification of infant fatty acid status by dietary counseling (I and II)

Maternal dietary counseling was reflected in infant cord serum FA as a lower PL eicosatrienoic acid (20:3n-9, marker for essential fatty acid deficiency) status and more favorable relation of functional indices, suggesting a better essential fatty acid status in infants, whose mothers received counseling. Further, an increased proportion of n-3 FA and reduced proportion of SFA in TAG fraction was observed. Although TAG fraction reflects changes from very recent intake, these findings together demonstrate the delivery of maternal dietary changes due to dietary counseling during pregnancy to the infant. In previous intervention studies during pregnancy, erythrocyte and plasma PL FA were used as biomarkers of child FA supply (Helland *et al.*, 2001; Montgomery *et al.*, 2003; de Groot *et al.*, 2004; Dunstan *et al.*, 2004; Sanjurjo *et al.*, 2004; Helland *et al.*, 2006; Krauss-Etschmann *et al.*, 2007), but this is the first study to investigate the impact of maternal dietary modification also on infant serum TAG and CE FA. Both TAG and CE are markers for dietary intake in adults (Baylin & Campos, 2006) and children (Moilanen *et al.*, 1983; Nikkari *et al.*, 1983; Lagström *et al.*, 1998). However, no effect of dietary counseling on cord CE FA fraction was observed in this study.

A possible explanation for the lack of effect in CE fraction and the relatively modest changes seen in PL and TAG fractions is that the magnitude of changes in maternal dietary FA intakes achieved through counseling were not sufficient to induce more pronounced modification of cord FA. The changes in maternal FA intakes during pregnancy in the subgroup analysis were significant (intakes of total PUFA were 2.03 g/day more in the intervention than in the control group) (Hautero *et al.*, 2013), but on the basis of approximate n-3 to n-6 ratio of 1:4 in Finland (Helldán *et al.*, 2013) it may be presumed that the increase in total n-3 FA and EPA + DHA remained smaller when compared to a recent dietary intervention study providing salmon to expecting mothers (intakes of total n-3 FA were 1.0 g/day and EPA + DHA 370 mg/day more in the salmon versus the control group) (Miles *et al.*, 2011). There seems to be a dose-related response between administered n-3 FA amounts and the change in respective FA in blood (Harris *et al.*, 1983; Brown *et al.*, 1990) and intervention studies using n-3 LC-PUFA supplements to the mother during pregnancy and lactation have verified the delivery to the infant (Jensen *et al.*, 2000; Helland *et al.*, 2006; Krauss-Etschmann *et al.*, 2007). Moreover, as FA are transferred to the fetus via placenta, biomagnification of especially n-3 LC-PUFA (selective transportation of placenta) (Larqué *et al.*, 2003) might equalize differences between different maternal dietary intakes and thus between study groups.

At 1 month of age, there were no differences in FA status between those children whose breastfeeding mothers received and those who did not receive dietary counseling. These

results are in line with previous findings that there were no differences in maternal plasma FA at 1 month *postpartum* (Hautero *et al.*, 2013). Simultaneously, maternal and infant serum FA were demonstrated to be highly correlated. Therefore, these results support the idea that the magnitude of changes in maternal diet and thus in the breast milk are insufficient to induce FA status modification of the infant at group level during the postnatal period.

Generally, the capacity of adults to convert ALA to longer-chain derivatives is limited. Increased consumption of ALA results in increases in blood EPA concentrations while the conversion to DHA seems to be restricted (Burdge & Calder, 2005; Brenna *et al.*, 2009). However, there is some data indicating significant gender differences in conversion capacity, and the rationale for this study was based on the increased capacity of pregnant women to convert ALA to EPA and DHA (Burdge & Wootton, 2002; Pawlosky *et al.*, 2003). Therefore a strategy of ensuring a continuous supply of PUFA from the diet, through provision of rapeseed oil-based food products and taking notice of the diet as a whole was considered sensible. In pregnant and breastfeeding women there are physiological changes in metabolism, which have to be taken in consideration whilst interpreting the results. Firstly, the metabolic demand of FA is increased and FA is utilized differently (Sala-Vila *et al.*, 2008). Secondly, nearly all the women participating in this study were highly educated and all were well nourished. Therefore, achieving significant changes in FA composition by regular diet might be challenging, as it may be presumed that the women already present with relatively high proportions of unsaturated fatty acids in the diet or in the body. FA metabolism is hypothesized to differ between atopic and non-atopic individuals, but no consistent differences has been reported in cord blood fatty acids between infants of allergic compared to non-allergic mothers (Sala-Vila *et al.*, 2008). The quality of breast milk that allergic mothers provide their infants might be different from non-atopic mothers, although evidence is very inconsistent (Sala-Vila *et al.*, 2008). However, eighty percent of the mothers in both groups had an allergic disease, thus any difference or lack of it between the groups due to maternal allergic disease is unlikely.

6.2. Nutrition to prevent allergic diseases

Historically, nutrition was considered a source of energy and growth, and its importance for health was appreciated centuries ago: "Let thy food be thy medicine and thy medicine be thy food" was the famous quote by Hippocrates. Hints about the significance of a balanced diet were obtained from the first scientific experiment with sailors in the 18th century, when scurvy could be prevented by adding limes to their diet (Carpenter, 2003). Later on, physicians learned how to adapt diet in association of specific conditions like diabetes mellitus and to use specific dietary preparations in treatment of diseases: e.g., hydrolyzed formulae in cow's milk allergy. The next step is to use and modify our daily diet to promote health and prevent diseases, **Figure 8**. Thus, the dietary prevention of

allergic diseases is an area of intense research and debate and data about specific nutrients with benefit in allergic diseases is accumulating.

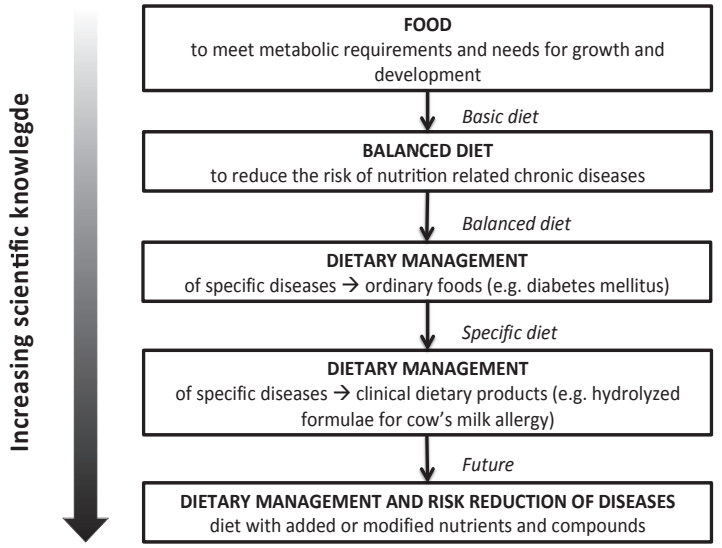

Figure 8. The use of diet to promote health (modified from Laiho & Isolauri, 2004).

6.2.1. Probiotic supplementation (III)

Dietary counseling with probiotics or with placebo did not affect a child's risk for atopic diseases. The result of the probiotic supplementation remained disappointing compared to previous studies. No effect on allergic outcomes was observed at group level. A previous subgroup analysis of the same study found that probiotic supplementation reduced the risk of atopic eczema in those infants, whose mothers were sensitized in prick testing (Huurre *et al.*, 2008). Similarly, a recent study by Rautava et al. (Rautava *et al.*, 2012b) reported a reduced risk of eczema in infants of allergic prick-positive mothers with combined pre- and postnatal maternal use of probiotics, albeit with different strains (similar effects of both *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 or *L. paracasei* ST11 and *B. longum* BL999). Countervailing evidence was reported from a recent study from Taiwan (Ou *et al.*, 2012), where supplementation with LGG in IgE-sensitized mothers had no effect, but this study was hampered by methodological issues, as outcomes were assessed with a parental questionnaire alone without any clinical judgment of eczema. Therefore, a specific protective effect on children of IgE-sensitized mothers may be hypothesized, but remains to be confirmed.

On the basis of previously published trials (**Table 3**, page 27), *Lactobacillus rhamnosus* strains may confer the best protection of eczema when intervention is started prenatally and continued postnatally. Of those trials using combined pre-and postnatal supplementation, eight reported interventions were effective in reducing the risk of eczema and/or atopic eczema (Kalliomäki *et al.*, 2001; Abrahamsson *et al.*, 2007; Kukkonen *et al.*, 2007;

Wickens *et al.*, 2008; Niers *et al.*, 2009; Dotterud *et al.*, 2010; Kim *et al.*, 2010; Rautava *et al.*, 2012b), albeit two studies reported no effect (Kopp *et al.*, 2008; Ou *et al.*, 2012). Those studies with prenatal maternal supplementation alone (Boyle *et al.*, 2011) or only postnatal intervention directly to infants reported no effects (Rautava *et al.*, 2006; Taylor *et al.*, 2007; Soh *et al.*, 2009) with one exception (West *et al.*, 2009). As follow-up beyond two years of age is reported in only two studies thus far (Kalliomäki *et al.*, 2007; Kuitunen *et al.*, 2009), it remains to be seen whether the preventive effect is long-term or just shifts the age when atopic eczema commences forward. Nonetheless, it is premature to give recommendations for routine use of probiotics, as the published trials vary in probiotic strains, mode, quantity and timing of administration, time of follow-up and target populations.

6.2.2. Diet in pregnancy (III)

Significant dietary differences during pregnancy at group level were seen as lower intakes of SFA and butter and higher intakes of PUFA, fiber, folate, vitamins C and E, vegetables, fruits and berries and margarine in the counseling groups *versus* the control group. During breastfeeding, lower intakes of SFA and butter and higher intakes of PUFA, MUFA, vitamin E, vegetables, fruits and berries, margarine and vegetable oil were evident in the counseling groups *versus* the control group. Nearly all of the above-mentioned nutrients or food are associated with the risk of atopic diseases in previous epidemiological studies. It may be, that these subtle changes in the daily diet require a larger population to show an effect on clinical outcomes at group level, or respectively the changes would have to be far greater in a study population of this size.

Numerous observational studies report the associations of maternal diet during pregnancy to the development of allergic diseases in the next generation, but the results generally are inconsistent and conflicting. The most consistent finding is a decreased risk of allergies with increasing maternal consumption of (oily) fish (Fitzsimon *et al.*, 2007; Romieu *et al.*, 2007b; Sausenthaler *et al.*, 2007; Willers *et al.*, 2007; Kremmyda *et al.*, 2011), which is in contrast with the results of this study, as the consumption of oily fish did not have any associations with allergic outcomes. Oily fish is known to provide plenty of n-3 PUFA (e.g., salmon: 2.3-2.8 g/100g) (THL, 2014b), which is hypothesized to lie behind the observed effects, but fatty acid intakes in this study had no effect on allergy risk. However, due to methodological limitations, the intakes of n-3 and n-6 PUFA could not be accurately separated and thus their individual effects could not be parceled out. Moreover, between the study groups, the intakes of fish and oily fish were comparable.

Maternal consumption of vegetables was associated with atopic eczema in univariate analyses, but not in the final multivariate model. However, a fairly constant protective effect of total and individual fruits and vegetables is demonstrated in previous prospective birth cohort studies (Nurmatov *et al.*, 2011). Conversely, a greater risk of allergies exists with an increased maternal consumption of fat spreads (Fitzsimon *et al.*, 2007), vegetable

oils, citrus fruits (Sausenthaler *et al.*, 2007), meat (Saito *et al.*, 2010) and nuts (Willers *et al.*, 2008), none of which came up significant in this study.

A novel finding of opposing effects of milk and cheese on atopic eczema was discovered, although previously maternal intakes of total dairy products, milk and cheese relate with protective effects on wheeze, asthma and allergic rhinitis during pregnancy (Miyake *et al.*, 2010b; Bunyavanich *et al.*, 2014), on sensitization during lactation (Nwaru *et al.*, 2011) and with other clinical endpoints (Larsson *et al.*, 2012). Further, the consumption of farm milk associates with a reduced risk of asthma, atopy and hay fever in many previous studies (Loss *et al.*, 2011; von Mutius, 2012), but this association is hypothesized to be related with increased bacterial counts in unpasteurized milk. Milk contains immunomodulatory factors, e.g., bioactive peptides, which are gaining interest in many inflammatory conditions (Agyei & Danquah, 2012). There is increasing literature that bovine whey proteins might act against excessive inflammation, but the mechanisms behind these actions are poorly understood (Puddu *et al.*, 2009; Rusu *et al.*, 2009; 2010). Milk processing influences the immunomodulatory properties and bioavailability of bioactive peptides, as heating affects more whey proteins than heat stable caseins (Loss *et al.*, 2011). Interestingly, cheese contains mainly casein whereas other milk products contain all milk proteins, as whey is separated in cheese production. Most store bought milk is pasteurized, but in Finland, it is not customary to high heat-treat other than low-lactose milk, unlike in several European countries (Elliott, 2007; Lacroix *et al.*, 2008). Therefore, it may be hypothesized that whey proteins in milk might still be bioactive, but this remains elucidated and requires further studies to justify the present observation of reduced risk of atopic eczema with increased milk consumption.

A further puzzle is the observation that the medium category of cheese and milk consumption was significantly related to the end-points, but the highest category was not, although the trend was parallel to that of the medium category. Usually, it is common to expect that biological effects are dose-dependent, and therefore one would anticipate also a significant association of the highest category. However, animal studies demonstrate that biological associations are not always simple and linear, as selenium diets in mice led to most florid allergic inflammatory responses at medium intakes (Hoffmann *et al.*, 2007). This finding might be one explanatory factor for inconsistencies in human dietary studies.

None of the studied nutrients remained significant in final models, but a recent systematic review and meta-analysis investigating the associations of maternal diet during pregnancy and child allergic manifestations (Nurmatov *et al.*, 2011) summarized that most uniform evidence amongst specific nutrients across observational studies is reported for vitamins A, D, E and zinc. Inconsistent findings exist for vitamin C and selenium (Nurmatov *et al.*, 2011). An increased risk of allergies with folic acid supplementation is reported in two studies (Håberg *et al.*, 2009; Whitrow *et al.*, 2009). Animal studies with antioxidant supplementation before allergic sensitization, suggest that antioxidants may have potential to reduce the risk of developing allergic disease (Zheng *et al.*, 1999; Okamoto

et al., 2006). To date, randomized controlled trials studying the effects of foods or specific nutrients other than fatty acids during pregnancy on child allergic manifestations have not been published. Only one study regarding vitamin C and E supplementation during pregnancy with allergic diseases as a secondary outcome exists with no effect (Greenough *et al.*, 2010). Vitamin supplementation studies are hampered by possible risks, as a meta-analysis of randomized controlled trials in adults observed an increased mortality, especially in studies of β-carotene and vitamin E, but again not for vitamin C or selenium (Bjelakovic *et al.*, 2007). Therefore, modification of diet rather than high-dose vitamin supplementation would seem to be a safer approach, especially when it comes to a vulnerable population like pregnant women.

Diet, however, is a complex mixture where the increase of one nutrient results in relative decrease of another. These interactions are beyond the present knowledge and might have unpredictable outcomes. One approach to the problem of these complex interactions resides in the evaluation of different dietary patterns – covering the diet as whole instead of dealing with individual nutrients and foods. Some interesting results exist: maternal Mediterranean dietary pattern associates with reduced risk of wheeze and sensitization in the offspring (Chatzi *et al.*, 2008) and a "Western pattern" was inversely associated with risk of wheeze (Miyake *et al.*, 2011). This notwithstanding, several studies have found no associations with Mediterranean diet, the Alternate Healthy Eating Index, "Western", "Prudent", "Health conscious", "Traditional", "Processed", "Vegetarian" and "Confectionery" patterns during pregnancy and offspring allergic diseases (de Batlle *et al.*, 2008; Shaheen *et al.*, 2009; Lange *et al.*, 2010).

To conclude, this contradictory evidence emphasizes the complexity of dietary research and constitutes a reminder that we are far from understanding nutrient interactions with the immune system. The findings of this study require repetition in future studies to justify any further conclusions.

6.2.3. Diet during breastfeeding

Studies investigating maternal dietary intakes during lactation are scarce, as most studies have concentrated on examining the duration of breastfeeding and dietary avoidance of potentially allergenic foods during lactation with limited and conflicting data (Friedman & Zeiger, 2005; Oddy, 2009). Only few observational prospective studies on dietary intakes during lactation exist. Observational studies identifying the effects of postnatal dietary exposures are frequently "set back" by several conflicting factors – the effects of the length of breastfeeding, the quality of breast milk and the timing of introduction of different complementary feeding are very difficult, if not impossible, to separate from each other. Further, it is not ethically acceptable to perform randomized studies with a non-breastfeeding arm. Therefore, data is largely inconsistent and difficult to compare.

The observed association of higher vitamin C intake (over 140 mg/day) during breastfeeding and increased risk of asthma in this study is in agreement with a previous

study, where maternal intake of vitamin C [mean intake 171 (SD 142) mg/day] during lactation increased the risk of sensitization to cat allergen (Nwaru *et al.*, 2011). These intakes exceed the recommended intake of 100 mg/day during breastfeeding, although maximum intake still considered safe is set as high as 1000 mg/day (THL, 2014c). Further, here the association of increased total intakes of fruits and berries was associated positively with asthma in univariate analyses, but not in the final model, however this may suggest that the association of vitamin C is plausible. On the other hand, vitamin C concentration from breast milk is reported to be inversely associated with atopic sensitization (Hoppu *et al.*, 2005b). Studies concentrating on antioxidant intakes during lactation and child allergic outcomes are lacking, thus these observations need confirmation from larger studies. Reports investigating vitamin C intakes during pregnancy have yielded conflicting results, with a systematic review concluding unsupportive evidence for preventive effects (Nurmatov *et al.*, 2011).

Maternal intake of egg during lactation was associated with a reduced risk of atopic eczema. This result contrasts that of Nwaru et al. (Nwaru *et al.*, 2011), who reported that maternal intake of egg during breastfeeding increased the risk of sensitization to cat allergen, but not to other allergens studied. Again, studies evaluating dietary intakes during lactation are lacking, as previous literature has concentrated primarily on investigating elimination diets targeting allergenic foods, such as eggs, seafood and cow's milk. Most studies show that these diets are not useful in preventing allergic diseases (Kramer & Kakuma, 2003), although two studies reported protective effects on eczema (Businco *et al.*, 1983; Lovegrove *et al.*, 1994). Interestingly, a proportion of infants already manifest with clinical reactivity before first exposure, thus sensitization has occurred much earlier (Palmer & Prescott, 2012). Food allergens are secreted in breast milk and represent an important potential source of early exposure (Palmer *et al.*, 2005; Vance *et al.*, 2005) and thus might promote tolerance in newborn (Mosconi *et al.*, 2010).

Maternal fat consumption did not come up significant in this study, although associations are reported in previous studies. Nwaru et al. reported that higher maternal consumption of butter and saturated fatty acids were associated with an increased risk, while margarine was associated with a decreased risk of sensitization to wheat allergen, but not to other tested food or inhalant allergens (Nwaru *et al.*, 2011). These results are in line with another study, where higher maternal intakes of saturated fat during lactation were associated with increased risk of atopic sensitization (Hoppu *et al.*, 2000). In the same study by Nwaru et al., consumption of potatoes, milk products, margarine and low-fat spreads were associated with decreased risk of birch allergic sensitization. Two additional Finnish studies examined dietary fat consumption during breastfeeding. Hoppu et al. reported an inverse association of maternal n-3 PUFA intakes with risk of atopic dermatitis (Hoppu *et al.*, 2005a), while Lumia et al. found no association of any fatty acid consumption with asthma, reporting instead an increase in asthma with higher maternal consumption of margarines (Lumia *et al.*, 2012). The association of fatty acid supplementation during breastfeeding to atopic diseases is of interest, but a recent

review on the matter concluded no effect on asthma, food allergy, or atopy (Klemens *et al.*, 2011).

In summary, data to date is scarce and thus limits any definite conclusions that can be made about the effects of maternal diet during breastfeeding on child risk of allergy. The findings of this thesis are intriguing and find some support from previous studies, thus further studies with focus on diet during breastfeeding could give an answer to these questions.

6.2.4. Introduction of complementary feeding (IV)

In the present study, no association of timing of complementary feeding introduction was found with atopic eczema, when adjusted for parental perception of child symptoms. This result is in concordance with the current perspective that early complementary feeding does not necessarily promote allergy, but controversy and debate on the subject still remains active among experts (Cattaneo *et al.*, 2011; Palmer & Prescott, 2012).

Early cohort studies, in the 80's and 90's, reported an increased risk of eczema and asthma in children whom were introduced to complementary feeding very early (under three months of age) (Fergusson *et al.*, 1982; Kajosaari & Saarinen, 1983; Zeiger *et al.*, 1989; Fergusson *et al.*, 1990). Therefore, expert opinions recommended postponing weaning especially in high-risk infants until 6 months and delaying certain foods even later (Høst *et al.*, 1999; American Academy of Pediatrics, 2000; Zeiger, 2003). In 2001, the WHO revised their recommendation for exclusive breastfeeding from 4 months of age to 6 months of age for all infants, although this recommendation was not aimed at allergy prevention (WHO, 2001). Thus, as a consequence delayed introduction of complementary feeding beyond 6 months of age was recommended globally. A further debate stems around the introduction of special "allergenic foods", such as cow's milk, eggs, nuts, and seafood. Most studies investigating elimination diets have done so in combination with other allergy prevention strategies (Zeiger *et al.*, 1989; Arshad *et al.*, 1992; Bardare *et al.*, 1993; Marini *et al.*, 1996; Exl *et al.*, 2000; Chan-Yeung *et al.*, 2005), thus the sole effect of elimination is not clear. However, in the 21st century, a series of publications reporting potential increases in allergy after delaying complementary feeding and avoiding specific food allergens emerged and raised concerns of increasing immune dysregulation (Høst *et al.*, 2008; Prescott *et al.*, 2008).

Early introduction is hypothesized to result in allergic sensitization, as the mucosal barrier of the infant is still immature with increased permeability and therefore early exposures could promote allergy (Halken & Høst, 1996; Bailey *et al.*, 2005; Grimshaw *et al.*, 2013) thus justificating delayed introduction. However, evidence to support this beyond 4 months of age is very limited. Evidence from animal studies demonstrate that oral tolerance induction is actually an active process, driven by early regular exposure to antigens (food proteins) (Lack, 2007). Moreover, a study investigating children with an immature gastrointestinal tract found no increased risk of food allergies (Liem *et al.*,

2007). In accordance, several cohort studies report either no benefit (Zutavern *et al.*, 2006; Chuang *et al.*, 2011; Joseph *et al.*, 2011) or increased incidences of eczema (Zutavern *et al.*, 2004; Kull *et al.*, 2006; Filipiak *et al.*, 2007; Snijders *et al.*, 2008; Alm *et al.*, 2009; Hesselmar *et al.*, 2010), asthma (Kull *et al.*, 2006; Virtanen *et al.*, 2010), allergic rhinitis (Kull *et al.*, 2006; Virtanen *et al.*, 2010) and sensitization (Kull *et al.*, 2006; Snijders *et al.*, 2008; Nwaru *et al.*, 2010) after delayed introduction of complementary feeding. Based on these new observations most expert panels have revised their recommendations (**Table 1**, page 20), with most suggesting that complementary feeding should be introduced at 4-6 months of age, including allergenic foods, also in high risk infants.

Whilst our results support that no increased risk of allergy is observed with early introduction of complementary feeding, we neither found any association with delayed introduction and allergy after adjusting with parental opinion of child allergy. An important limitation of observational studies is "reverse causality", that is, when families with a history of allergy postpone complementary feeding deliberately. Many early studies have not considered this issue, but most recent studies have considered reverse causality into account by adjusting for family history of allergy (Kull *et al.*, 2006; Zutavern *et al.*, 2006; Hesselmar *et al.*, 2010; Koplin *et al.*, 2010; Nwaru *et al.*, 2010; Virtanen *et al.*, 2010; Joseph *et al.*, 2011) or infants with early allergic symptoms (Kull *et al.*, 2006; Zutavern *et al.*, 2006; Snijders *et al.*, 2008; Hesselmar *et al.*, 2010; Koplin *et al.*, 2010; Virtanen *et al.*, 2010; Chuang *et al.*, 2011). Our study population consisted merely of families with a history of allergy, and in addition of physician diagnosed allergy, we recorded parental opinion, as parents are known to suspect allergies more than can be diagnosed objectively (Eggesbø *et al.*, 2001). Transient adverse reactions to foods are observed in 20 to 35% of infants, manifesting as excessive crying, skin and gastrointestinal symptoms, which however, are not reproducible in double-blind food challenges (Eggesbø *et al.*, 1999; Eggesbø *et al.*, 2001). These reactions cannot be thus considered as true allergic symptoms, but they may lead parents to unnecessary delaying of complementary feeding or self-prescribed elimination diets and cause reverse causality. Moreover, unnecessary elimination diets are a risk for PUFA deficiency, especially for n-3 PUFAs (Aldámiz-Echevarría *et al.*, 2008). To conclude, as parental opinion proved to be a significant factor outweighing the feeding pattern, this must be acknowledged in future observational studies. However, prospective randomized controlled trials are needed to truly eliminate reverse causation. So far, not a single study exists exploring this issue, but many are on the way assessing both normal and high-risk populations (Palmer & Prescott, 2012) and the results are anticipated.

6.3. Methodological aspects

This prospective randomized controlled trial was aimed to investigate whether maternal dietary counseling during pregnancy and breastfeeding is able to modify child risk of atopic diseases. Further, we were able to evaluate if combining probiotic supplementation with dietary counseling has any additional advantage. Moreover, due to prospective

continuous recording of maternal diet from early pregnancy onwards, we could dissect the impacts of specific dietary components on risk of childhood atopic diseases.

The dietary intakes were assessed repeatedly with prospective 3-day food diaries, using household measures from early pregnancy onwards, therefore covering the time-window considered important in the development of allergies. In previous studies, the food diaries were collected at random time points during pregnancy, usually in the third trimester, or studies have used retrospective reminiscence with food-frequency questionnaires. The 3-day recording method is regarded as the most accurate compared to other methods and is considered long enough to allow evaluation of individual intakes of energy and macronutrients on a group level (Willett, 1998). Some under-reporting exists (Scagliusi *et al.*, 2003), especially in overweight or obese individuals (Pietiläinen *et al.*, 2010) and pregnant women (McGowan & McAuliffe, 2012), but the latter applies to the whole study population. The nutrient intakes were calculated with the Micro-Nutrica computer program and therefore for example n-3 and n-6 FA intakes could not be reliably calculated as this information was often missing from the used database. The dietary recommendations have recently been updated, the new Nordic Nutrition Recommendations were published in 2012 (NNR, 2012) and Finnish recommendations in 2014 (Fogelholm *et al.*, 2014). The recommended upper limit for the intake of total fat is now higher (range 25-40 E%, compared to earlier 25-35 E%), the lower limit for the intake of carbohydrates lower (45-60 E% compared to 50-60 E%) and the upper limit for intake of MUFA higher (10-20 E% compared to 10-15 E%). The recommendations for PUFA quality and intakes have remained the same and the ranges for recommended intakes are in line with previous recommendations, thus it is likely that the results of this study would not change even if this study had been conducted with these new recommendations.

The participants in the fatty acid analysis (studies I and II) were included in consecutive order of recruitment, thus selection bias in this subpopulation is unlikely. The number of participants was determined on the basis of earlier studies and therefore considered sufficient (Velzing-Aarts *et al.*, 2001; Dunstan *et al.*, 2004). As a limitation, some blood samples were missing because repeated blood sampling of small infants was considered unethical (attempts restricted to two times) and thus the sample size remained smaller than anticipated. As a further constraint, the analysis of few specific fatty acids was particularly challenging due to very low concentrations, thus especially ETA and DHA were eluted with impurities and had contaminated separation in gas chromatography, however this represents a systematic error and does not affect group differences. We have reported fatty acid composition as both percentages of total fatty acids and concentrations, both of which are rarely reported in literature and this lack of data is considered a problem in many fatty acid studies (Baylin & Campos, 2006). The use of serum FA as biomarkers for FA status is still considered an up-to-date method, although the use of erythrocyte membrane lipids might offer an indication for a longer time-period and thus be a useful tool to consider in future studies (Serra-Majem *et al.*, 2012).

Our study population was homogenous and consisted exclusively of children with an increased risk for allergy, therefore representing a population who would most benefit from novel ways of prevention. We used traditional definitions in diagnostics, separating non-atopic eczema from atopic eczema, thus following the recommendations of Nomenclature Review Committee of the World Allergy Organization (Johansson *et al.*, 2004). In view of the hypothesis that non-atopic and atopic eczema are a continuum, it would have been justified to also combine these forms for analyses. However, the effect of the intervention remained non-significant when atopic and non-atopic eczema were considered together (**Table 11**, page 47). Hence it remains to be seen whether different prevention strategies should be applied for atopic and non-atopic disease. In many previous studies, these definitions are used inaccurately, making the comparison of results across studies difficult.

In this study, all allergic manifestations were diagnosed by a doctor, unlike in many epidemiological approaches in which a questionnaire is used to diagnose atopic diseases. Although 80 percent of all the mothers in this study self-reported an allergic disease, only 58 percent were skin prick positive. As maternal atopic status might work as an effect modifier, this may have an impact on the results. The number of study participants perhaps was too small for the secondary analyses and the number of end-points was lower than expected based on our previous studies in high-risk families. Together with a relatively high dropout rate during the 4 year follow–up, these factors represent the main weaknesses of this study and limit the interpretation and generalizability of the results. However, the numbers of discontinuing women and children and the reasons for discontinuation in the study groups were comparable.

6.4. Considerations regarding the present results and future studies

One of the most important considerations for future studies is to retain clear definitions of allergic outcomes to allow comparison between studies. Eczema, atopic eczema and atopic dermatitis are being used haphazardly in current literature, but it should be clearly indicated whether these definitions denote IgE-positive or negative forms of disease, or both. Secondly, many studies have used vague terminology and diagnostic methods in collecting data about allergic diseases. The use of plain parental questionnaires without clinical data for diagnosis is not recommended due to parental bias. Further, the associations with mere sensitization in some studies (either positive skin prick test or elevated serum IgE) is questionable; sensitization to an allergen does not denote clinical allergic disease (Sicherer *et al.*, 2012). In summary, allergic diseases should always be diagnosed by a competent physician with up-to-date methods and clear terminology.

Studies investigating perinatal maternal dietary intakes are fraught with several methodological and practical difficulties, which should be carefully addressed in the future. Our approach was a prospective repeated assessment of the maternal diet at each trimester of pregnancy, as the exact time window when the maternal dietary intake would

have the most influence on the development of allergies is not known. Previous studies have used random time-points and the results are therefore difficult to compare. Further, retrospective collection of dietary data used in many studies is subject to recall bias. However, as we combined these three time points for a mean estimate of dietary intakes during pregnancy in statistical analyses, we might have on the other hand overlooked short periods when a specific nutrient would exert effect on the infants' developing immune system. Therefore, in the future it would be interesting if well-powered studies would consider collecting and analyzing dietary data at separate stages of pregnancy.

Although maternal diet during pregnancy is a popular subject on observational studies, those investigating maternal diet during lactation are clearly under-represented in current literature. It may be well presumed that the effect of maternal diet on child risk of allergy differs during pregnancy and lactation, as the physiology of nutritional influx to the child is completely different through placenta or breast milk. Therefore, a more intense effort should be addressed trying to separate these periods. In this context, however, it is important to acknowledge periods of exclusive and total breastfeeding, which are not clearly stated in all studies. There is some evidence showing that continued breastfeeding during introduction of complementary feeding could promote tolerance induction, however this evidence is limited (Agostoni *et al.*, 2008; Grimshaw *et al.*, 2013). In the present study, there was no effect of total breastfeeding under or over six months of age (III and IV), but a significant constraint was met, as exact data on whether the infant was breastfed at the time of complementary feeding introduction was not available in all cases. In addition, the comparatively high proportion of breastfed infants and duration total breastfeeding could mask the possible protective effects. Thus, future studies should aim to collect precise data on breastfeeding while introducing new foods to the infant.

Until recently, there has not been any recommendation on which background factors should be considered when adjusting the results in statistical analyses for maternal diet. Nurmatov et al. published a very thorough examination on the subject in 2012, suggesting primary and secondary confounders (maternal characteristics, birth measurements, socioeconomic characteristics and environmental exposures) that should be considered in future observational studies (Nurmatov *et al.*, 2012). As the present study was commenced already over decade ago, all suggested confounding information was not available, and due to the relatively low number of subjects and end-points, the number of confounding variables had to be restricted to allow sufficient power in the statistical analyses. However, the study population was homogenous due to a stringent inclusion and exclusion criteria of this prospective randomized controlled study. Nevertheless, the emphasis in the future should be on randomized and well-powered controlled trials, which are the ultimate way to eliminate possible confounders.

The changing doctrine of introduction of complementary feeding requires attention in subsequent observational study settings. In the present study (IV), the families' adherence to continuously making notes of introduction of new foods was clearly poorer

after 6 months of age. Prior to that age, introductions were mostly recorded meticulously by exact dates, but thereafter, the accuracy was often by a month's precision, thereby restricting the potential to evaluate whether the child was still breastfed at the same time. Therefore, it should be stressed to the parents how important precision is and to encourage the families to maintain a diary, as keeping the diary is tedious in the long run and lapses are understandable. Moreover, clear terminology is important: the term complementary feeding is not used similarly in all studies. The WHO defines complementary feeding as any non-breast milk foods or liquids (WHO, 1991), whereas for example ESPGHAN allows formula feeding (Agostoni *et al.*, 2008). We used the definition by the WHO, because infant formulae contain cow's milk protein, one of the most important allergens.

Finally, parental opinion on whether their child might have symptoms of allergic origin or not turned out to be a significant factor outweighing the feeding pattern. Therefore, it may be suggested that this should be taken into account in future studies in addition of family history of allergy in order to minimize reverse causation.

7. SUMMARY AND CONCLUSIONS

This study aimed to evaluate the prospects of dietary counseling, which complied with current dietary recommendations in tandem with probiotic intervention to affect child risk of atopic diseases. Dietary counseling achieved significant changes in maternal diet during pregnancy and lactation, and was reflected to some extent in infant cord blood fatty acids. These changes confirm the delivery of dietary changes to the child, indicating a better essential fatty acid status in infants in the counseling group. Data from previous literature suggest that modification of infant fatty acids might have clinical relevance to future allergy risk. However, as no differences in allergic outcomes were observed at group level and differences in infant fatty acid status did not exist at 1 month of age, these results suggest that more potent interventions might be necessitated to induce reduction in allergy risk.

Furthermore, the results of the present study demonstrate interesting associations between maternal diet and child risk of allergic diseases. A novel finding of opposing effects of milk and cheese on atopic eczema during pregnancy was revealed. In addition, higher intakes of vitamin C during lactation increased the risk of asthma, but higher intakes of egg decreased the risk of atopic eczema. These observations together with earlier data suggest that maternal diet during pregnancy and lactation is an intriguing way to target child risk of allergy, but the mechanisms and the specific active factors in the diet are far from understood. It is likely that any association of the diet with allergic diseases is related to a number of other dietary and environmental factors, microbiota, and genetic susceptibility. Interactions between nutrients and the developing immune system are likely to be complex, thus a single answer is unlikely to be discovered. Future studies combining individual nutrients, foods and dietary patterns might be useful. Moreover, the present findings support the notion that maternal diet might exert differential effects during pregnancy and lactation, but the confirmation of this hypothesis calls for well-designed intervention studies.

Finally, the timing of introduction of complementary feeding was not found to be associated with child risk of atopic eczema when adjusted to parental opinion of child allergy. This finding indicates that parental opinion is a significant contributing factor, which should be taken into account in further studies as it might cause reverse causation and unnecessary elimination diets. The optimal window of introducing complementary feeding is not clear, but it seems that a safe period for introduction is between 4 and 7 months of life and that there is no advantage of delaying complementary feeding beyond this to prevent allergy in high-risk infants (Prescott *et al.*, 2008).

In conclusion, while any dietary recommendations cannot be given based on this study, high-risk families can safely adhere to current dietary recommendations for pregnant and lactating women. Public health care systems should continue their work to promote awareness in preventing unnecessary elimination diets. Advice on healthy diet during pregnancy and lactation should be offered actively in prenatal clinics. While the benefit of

probiotic supplementation could not be confirmed in the present study, current literature indicates that they may provide protection against eczema and atopic eczema in high-risk infants when supplementation is started prenatally and continued during lactation. In addition, complementary feeding may be introduced between 4 and 7 months of age and no additional benefit is gained with delaying introduction beyond this period.

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APPENDIX

Intake of nutrients and foods during lactation. The lower tertile (33rd percentile) and the upper tertile (67th percentile) divide the subjects into three categories, low intake (<33rd percentile), medium intake (33rd percentile - 67th percentile) and high intake (>67th percentile), each containing a third of the population of 227 lactating women.

a) Nutrients

	Median	Range	Lower tertile	Upper tertile
Carbohydrate, E%	48.6	31.9-61.4	46.6	50.9
Carbohydrate, g	235	103-412	211	263
Protein, E%	16.7	10.3-28.8	15.9	18.1
Protein, g	81.5	30.8-148	74.3	89.8
Total fat, E%	33.0	20.7-46.9	30.8	35.0
Total fat, g	70.4	18.2-133	63.3	79.9
SFA, E%	12.5	5.9-20.9	11.3	13.5
SFA, g	27.3	6.1-56.6	23.5	31.1
MUFA, E%	11.2	5.6-19.5	10.4	12.2
MUFA, g	24.5	6.8-44.3	21.2	27.7
PUFA, E%	5.8	2.2-10.2	5.2	6.4
PUFA, g	12.6	3.3-25.3	10.6	14.4
18:2n-6, LA	9.7	1.5-21.7	8.4	10.8
18:3n-3, ALA	2.0	0.4-5.4	1.7	2.3
Fiber, g	19.0	6.3-50.9	16.7	21.4
C vitamin, mg	104	9.0-327	78.2	138
D vitamin, µg	5.4	0.7-23.8	4.5	6.5
E vitamin, mg	9.9	3.1-19.3	8.5	11.3
Folate, µg	273	109-639	246	306
Zinc, mg	11.8	5.2-19.5	10.7	12.8

b) Foods (g)

	Median	Range	Lower tertile	Upper tertile
Fruits and berries	209	0.0-814	152	275
Vegetables	259	8.0-635	214	304
Meat	102	0.0-250	80.3	128
Fish	19.5	0.0-167	11.0	30.4
Fish g/week	136	0.0-1170	76.7	212
All milk products	489	3.9-1420	367	589
Milk	271	3.9-1100	189	407
Cheese	43.3	0.0-223	32.5	56.7
Margarine	21.37	0.0-60.3	16.0	25.4
Butter	1.45	0.0-29.3	0.5	2.8
Oil	9.73	0.0-54.2	6.6	13.3
Egg	12.32	0.0-75.0	7.7	19.7