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BLACKCURRANT SEED OIL FOR PREVENTION OF ATOPIC DERMATITIS

by

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To my boys

ABSTRACT

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Blackcurrant seed oil for prevention of atopic dermatitis

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One hypothesis for the increased incidence of atopic diseases has been that it is associated with changing dietary habits, especially the changed intake of essential fatty acids (EFAs). The metabolism of EFAs produces eiconasoids, prostaglandins and leukotrienes, which are essential to organs and play a major role in regulation of inflammation and immune response. In some studies persons with atopic dermatitis have been found to have reduced levels of EFAs. The first year of infancy as well as the foetal period are crucial for the development of atopic immune response. The composition of blackcurrant seed oil (BCSO) corresponds to the recommended ratio of EFAs n-3 and n-6 in the diet (1/3-1/4) and as a dietary supplement could, even in small doses, modify the unbalance of EFAs in an efficient way. The purpose of this study was to find out whether atopic allergies can be prevented by supplementing the diet of pregnant mothers with blackcurrant seed oil and whether it could affect the immunological balance of a child. We also sought to find out whether a blackcurrant seed oil supplementation can affect the composition of breast milk to suppress the T helper 2 lymphocyte (Th2) responses in infants.

313 pregnant mothers were randomly assigned to receive BCSO (n=151) or olive oil as placebo (n=162). Supplementation was started at the 8th to 16th weeks of pregnancy, 6 capsules per day (dose of 3 g), and continued until the cessation of breastfeeding. It was thereafter followed by direct supplementation to infants of 1 ml (1 g) of oil per day until the age of two years. Atopic dermatitis and its severity (SCORAD index) were evaluated, serum total IgE was measured and skin prick tests were performed at the age of 3, 12 and 24 months. Peripheral blood mononuclear cell (PBMC) samples were taken at the age of 3 and 12 months and breast milk samples were collected during the first 3 months of breastfeeding. Parental atopy was common (81.7%) in the studied infants, making them infants with increased atopy risk. There was a significantly lower prevalence of atopic dermatitis in the BCSO group (33%) than in the olive oil group (47%) at the age of 12 months. Also, SCORAD was lower in the BCSO group than in the olive oil group. Dietary intervention with BCSO had immunomodulatory effects on breast milk, inducing cytokine production from Th2 to Th1 immunodeviation. It decreased the level of IL-4 and elevated the level of IFN-γ. BCSO intervention did not affect cytokines in the children's PBMC. However, children of smoking parents in the combined BCSO and olive oil group had significantly elevated levels of Th2 type cytokines IL-4, IL-5 and the proinflammatory cytokine TNF. Dietary supplementation with BCSO is safe. It is well tolerated and transiently reduces the prevalence of atopic dermatitis at the age of 12 months. It can possibly become a potential tool in prevention of atopic symptoms when used at the early stages of life.

Keywords: Atopic dermatitis, black currant seed oil, EFA, PUFA, parental smoking, breast milk, cytokines

TIIVISTELMÄ

pia Linnamaa

Atooppisen ihottuman ehkäisy mustaherukansiemenöljyvalmisteella

Iho- ja sukupuolitautioppi, Turun yliopisto
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Yhdeksi syyksi atooppisten allergioiden lisääntymiseen on esitetty muutoksia ruokailutottumuksissamme, erityisesti välttämättömien rasvahappojen osuuteen on kiinnitetty huomiota. Välttämättömien rasvahappojen metabolian tuloksena syntyy elimistölle välttämättömiä eikosanoideja, prostaglandiineja ja leukotrieenejä, joilla on tärkeä merkitys tulehdusreaktion ja immuunivasteen säätelyssä. Aikaisemmissa tutkimuksissa atoopikoilla on todettu alhaisia välttämättömien rasvahappojen pitoisuuksia. Ensimmäinen vuosi ja jo sikiöaika ovat tärkeitä allergisen puolustusmekanismin kehittymisen kannalta. Mustaherukan siemenöljyn rasvahappokoostumus vastaa suositeltua n-3- ja n-6- rasvahappojen suhdetta ravinnossa (1/3-1/4) ja voi jo pieninä annoksina auttaa tasapainottamaan välttämättömien rasvahappojen osuutta elimistössä. Tutkimuksen tavoitteena oli selvittää, pystytäänkö raskauden aikana ravintolisänä aloitetulla mustaherukan siemenöljyllä ehkäisemään atooppisia allergioita, vaikuttamaan lapsen immunologiseen tasapainoon ja vaikuttamaan rintamaidon koostumukseen siten, että se vaimentaa lapsen Th2-vastetta.

313 raskaana olevaa äitiä satunnaistettiin saamaan joko mustaherukan siemenöljyä (n=151) tai lumeena toimivaa oliiviöljyä (n=162). Äidin öljykorvaus annettiin kapselien muodossa 6 kapselia/pv (3 g/kerta-annos). Se aloitettiin 8.-16. raskausviikolla ja sitä jatkettiin imetyksen loppuun asti. Lapsi alkoi saada samaa öljykorvausta 1 ml/pv (1 g), kun yksinomainen rintamaitoruokinta päättyi, ja öljykorvausta jatkettiin, kunnes lapsi täytti kaksi vuotta. Atooppinen ihottuma ja sen vaikeusaste (SCORAD) arvioitiin, seerumin kokonais-IgE määritettiin ja ihopistotestit (prick) tehtiin 3, 12 ja 24 kuukauden iässä. Perifeerisen veren mononukleaaristen solujen näytteet kerättiin 3 kuukauden ja 12 kuukauden iässä ja rintamaitonäytteet kerättiin 3 kuukauden sisällä. Vanhempien atopia oli yleistä (81,7 %), mikä lisäsi lasten atopian riskiä. Herukkaöljyryhmässä todettiin vähemmän atooppista ihottumaa (33 %) verrattuna plaseboryhmään (47 %) 12 kuukauden iässä. Myös ihottuman vaikeusaste oli lievempi SCORAD-asteikolla mitattuna. Raskauden aikaisella herukkaöljyinterventiolla todettiin immunomodulatorisia vaikutuksia rintamaidon sytokiinituotantoon kohti Th1-vastetta. Interleukiini-4:n taso laski ja IFN-γ:n taso nousi. Mustaherukan siemenöljyllä ei ollut vaikutusta sytokiineihin lasten perifeerisen veren mononukleaarisissa soluissa. Sen sijaan todettiin, että tupakoivien vanhempien lapsilla yhdistetyssä ryhmässä oli merkitsevästi enemmän Th-2-tyypin sytokiineja, IL-4 ja IL-5 sekä proinflammatorista TNF:ää. Mustaherukan siemenöljy on turvallinen ja hyvin siedetty. Raskauden aikana käytettynä se vähentää lapsen atooppisen ihottuman esiintyvyyttä 12 kuukauden iässä. Varhaisessa lapsuudessa käytettynä se voisi olla mahdollinen vaihtoehto atooppisen ihottuman ehkäisyssä.

Avainsanat: Atooppinen ihottuma, mustaherukan siemenöljy, EFA, PUFA, vanhempien tupakointi, rintamaito, sytokiinit

CONTENTS

ABBREVIATIONS

AA	arachidonic acid
AD	atopic dermatitis
ALA	α-linolenic acid
ANOVA	analysis of variance
BCSO	blackcurrant seed oil
CD	cluster of differentiation
CE	cholesteryl esters
ConA	concanavalin A
COX	cyclo-oxygenase
DBPC	double-blind placebo-controlled
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
EFA	essential fatty acid
Eos	eosinophil
EPA	eicosapentaenoic acid
ETA	eicosatetraenoic acid
FA	fatty acid
FoxP3	forkhead/winged helix transcription factor
GLA	γ-linolenic acid
HBSS	Hanks' balanced salt solution
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LA	linolic acid
LOX	lipo-oxygenase
LT	leukotriene
mRNA	messenger RNA
n-3	omega-3
n-6	omega-6
NK	natural killer cell
PBMC	peripheral blood mononuclear cell
PDAR	Pre-Developed Assay Reagents
PG	prostaglandin
PUFA	polyunsaturated fatty acid
RT-PCR	reverse transcription polymerase chain reaction
SCORAD	severity scoring of atopic dermatitis
SD	standard deviation

SDA	stearidonic acid
SPT	skin prick test
RPMI	Rosswell Park Memorial Institute
TAG	triacylglyserols
TLR	toll-like receptor
Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
TGF	transforming growth factor
TNF	tumor necrosis factor
Tk	cytotoxic T cell
Treg	regulatory T lymphocyte cell
TXA	thromboxane

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-III).

I Linnamaa P, Savolainen J, Koulu L, Tuomasjukka S, Kallio H, Yang B, Vahlberg T, Tahvonen R. Blackcurrant seed oil for prevention of atopic dermatitis in newborns: a randomized, double-blind, placebo-controlled trial. Clin Exp Allergy 2010;40:1247-55.

II Linnamaa P, Nieminen K, Koulu L, Tuomasjukka S, Kallio H, Yang B, Tahvonen R, Savolainen J. Black currant seed oil supplementation of mothers enhances IFN-γ and suppresses IL-4 production in breast milk. Pediatr Allergy Immunol 2013;24:562-6.

III Linnamaa P, Nieminen K, Koulu L, Tuomasjukka S, Kallio H, Yang B, Tahvonen R, Savolainen J. Pro-inflammatory and Th2- type cytokine responses in PBMC in infants are associated with parental smoking. Clin Exp Allergy 2012;42:1472-8.

1. INTRODUCTION

Atopic diseases, such as atopic dermatitis, allergic rhinitis and asthma, have become a prevalent public health problem in the industrialized world. The rapid increase of atopic diseases has been associated with environmental factors; urban lifestyle, reduced infections (hygiene theory), "Western" diet and reduced diversity in the bacterial gut flora, antibiotic exposure, traffic-related air pollution and tobacco smoke. Suggested protective factors are rural living, maternal contact with farm animals during pregnancy, endotoxin exposure in early life and probiotics.

One hypothesis for the increase in atopic diseases is that it is, in part, associated with changing dietary habits such as changes in fat consumption and thus the intake of essential fatty acids (EFAs). EFAs consist of two different fatty acid groups, n-3 fatty acids (FAs) and n-6 FAs. N-3 FAs are derived from α-linolenic acid (ALA) and n-6 FAs from linolic acid (LA). EFAs must be included in the diet, since they can not be synthesized in the body.

Atopics have reduced levels of EFAs and it has also been hypothesized that atopic persons may have a lower activity of Δ^6-desaturase enzyme. This is the regulatory enzyme starting the conversion of EFAs from phospholipids into other FAs which are then further converted into a series of eiconasoids, such as tromboxanes, prostaglandins and leucotrienes. These have an important role in the regulation of epidermal inflammation and immune function. The fetal period and the first year of infancy are crucial for the development of immune function and the deviation toward atopic immune response.

Blackcurrant seed oil (BCSO) contains both n-3 FAs and n-6 FAs in recommended optimal dietary intake, proportion 1/3 to 1/4. Significant parts of the FAs of BCSO are metabolites beyond the Δ6-desaturase step. It is possible that the low activity of Δ^6-desaturase in atopics can be compensated with BCSO supplementation. This study investigates whether early BCSO compensation could prevent the atopic immune response.

2. REVIEW OF THE LITERATURE

2.1 Atopic allergy in infancy

Atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or dermatitis. The term atopy is recommended for describing this clinical trait and predisposition (Johansson et al 2004).

Today, in the Western societies, approximately one in three children suffers from an atopic disease. According to the International Study of Asthma and Allergies in Childhood, globally the prevalence of allergic rhinoconjunctivitis, dermatitis and asthma in children aged from 13 to 14 years was 14.6%, 7.3% and 14.1%, respectively. In the 6-7-year-old group the prevalence of rhinoconjunctivitis, dermatitis and asthma was 8.5%, 7.9%, and 11.7%, respectively (Mallol et al 2013). In Finland more than 40% of school children appear to be sensitized to one or more allergens (Haahtela et al 2008). The lifetime occurrence of atopic dermatitis was 20% in children aged 3 to 11 years. Atopic dermatitis develops in the first 6 months of life in 45% of the children and 12 months of life in 60% of who had the condition (Kay et al 1994). In one study the cumulative incidence of atopic dermatitis over the first two years of life amounted to 20.1% and there was a significant association with AD history of the parents (Bergmann et al 1997). In another study, cumulative incidence of AD in 0-4-year-old children was 27.1% without atopic parents and in those with single or double parental history 37.9% and 50%, respectively (Böhme et al 2003).

The development of atopic disease depends on a complex interaction between genetic and several environmental factors such as environmental exposure to food and inhalant allergens and non-specific adjuvant factors, e.g. tobacco smoke, air pollution and infections (Magnusson et al 1986, Kulig et al 1998, Herzen et al 2006, Laan et al 2000, Boyce et al 2012). It is estimated that genetic factors account for around 50% of asthma and allergy (Halken et al 2000, Leung et al 2003, Halken et al 2004). Around 30% of newborns have at least one parent or older sibling with atopic disease. 20-30% of these children develop allergic disease. In children without atopic heredity, approximately 10% develop an allergic disease (Bergman et al 1997, Hansen et al 1993, Kjellman et al 1977, Böhme et al 2003, Halken et al 2004, Nissen et al 2013).

The atopic march is the natural history of atopic manifestations, characterised by a typical sequence of progression of clinical signs of atopic disease. Atopic dermatitis (AD) and food allergies are usually the first clinical manifestations in infancy, whereas asthma and allergic rhinoconjunctivitis are the main problems later in childhood (Dharmage et al

2014). The infantile phase of AD typically begins on the cheeks, forehead or scalp and is intensely itchy. Lesions usually remain localized to the face or might extend to the trunk or particularly to the extensor aspects of the extremities. Exacerbation of facial dermatitis on the medial cheeks and chin is often seen concomitant with teething and initiating solid foods, probably because of the exposure to irritating saliva and foods (Spergel et al 2003). Characteristic of this infantile phase is the tendency to show significant oedema of affected areas, leading to oozing and crusting (Spergel et al 2003). In a study conducted amongst 2-year-old children with AD, 45% presented mild, 53% moderate and 2% severe AD. Twenty-seven percent of them had a positive prick for food allergens and 15% had positive Phadiatop (Böhme et al 2001). During the first 2 years of life there is a significant association between atopic dermatitis and other atopic disease manifestations, and also respiratory infections, increased rate of acute otitis media, pneumonia and use of antibiotics (Böhme et al 2002). Infants with atopic dermatitis have an increased risk for development of asthma (Bergmann et al 1998, Kurukulaaratchy et al 2002, Carlsten et al 2013).

Sensitization to cow's milk and hen's egg are common in the first year of life. This is predictive for sensitization to respiratory allergens and also for clinical allergic disease, atopic dermatitis and allergic rhinitis as well as asthma, later in childhood especially in high-risk infants or infants with early atopic dermatitis or wheezing (Kulig et al 1998, Laan et al 2000, Hill et al 2004). IgE-mediated allergic disease early in childhood is associated with a high risk of allergic disease later on. This natural course of the allergic diseases - the so-called "allergic march" - is important to understand when evaluating factors that may influence the development of allergic disease or elaborating strategies in the management of AD (Halken 2004, Laan et al 2000, Bieber et al 2012).

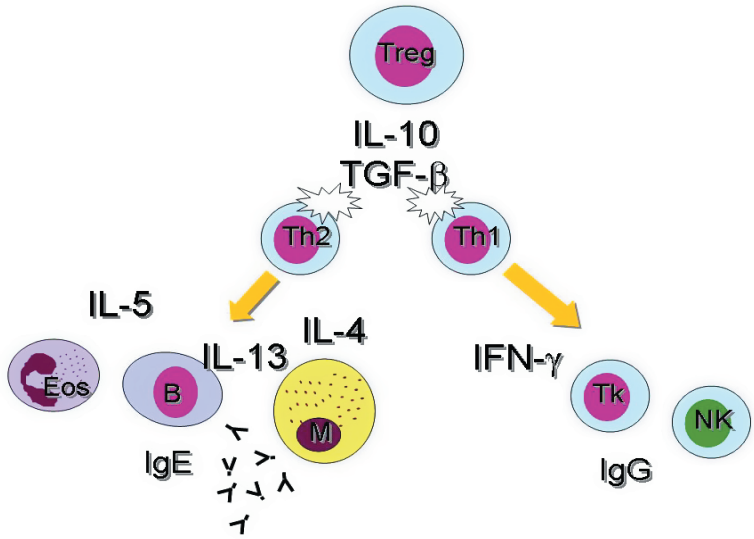
2.2 Development of the allergic immune response

The immune system of a newborn infant is not fully developed. It is influenced by maternal immunity, both transplacentally and by breast milk (Billington 1992, Böttcher et al 2000). There is close immunological interaction between the mother and her baby during gestation and the period of breastfeeding, and the mother may provide her offspring with protective factors and immunomodifying components as well as antigenic stimulation (Böttcher et al 2000, Goldman 2007). It seems that the initial sensitization to allergens starts as early as in utero (Holt 1996).

There are many studies showing that the balance between specific regulatory and Th2 cells is disturbed in atopic persons (Cottrez et al 2000, Akdis et al 2004, Ling et al 2004). Atopic allergy is associated with the production of type -2 cytokines, e.g.

interleukin-4 (IL-4), IL-5 and IL-13 (Böttcher et al 2000). Cytokines are a large family of polypeptide regulators that are produced throughout the body by nearly every cell. Cytokines are primarily involved in host responses to disease or infection (Dinarello 2000).

Th2 cells differentiate under the effect of IL-4 and boost with their cytokine production, IL-4, IL-5 and IL-13, the IgE antibody synthesis of B cells and maturation, differentiation and activation of eosinophile cells and mast cells (de Vries et al 1999). Th1 cells differentiate under the effect of IL-12 and boost with the production of cytokines interferon-γ (INF-γ) and IL-18 the maturation of cytotoxic T cells (Tk) and natural killer cells (NK) involved in microbe-specific immune defence (de Vries et al 1999). There is counteraction between T helper 1 lymphocyte (Th1) and T helper 2 lymphocyte (Th2). INF–γ secreted by Th1 cells suppresses the Th 2 response and IL-4 secreted by Th2 cells (de Vries et al 1999). Interleukin-10 secreted by regulatory T lymphocytes (Treg) suppresses the Th2 response (Cottrez et al 2000) as shown in figure 1. Table 1 presents the major cytokines and their functions.



Treg, regulatory T lymphocyte; Th2, T helper 2 lymphocyte; Th1, T helper 1 lymphocyte; Eos, eosinophil; B, B-lymphocyte; M, Mast cell; Tk, cytotoxic T cell; NK, natural killer cell

Figure 1. The regulation of allergic immune response.

Table 1. Major cytokines and their functions.

TH1 cytokines	
IL-2	Growth factor for B cells, proliferation of effector T and B cells
IL-12	Induce Th1 cells differentiation and cytotoxicity
IL-15	T-cell activation, proliferation and activation of NK cells
IL-18	Induction of Th1 cells
IL-21	Regulation of proliferation, differentiation, apoptosis, cytotoxic activity
IL-23	Stimulate production of IL-17 and promote memory T-cell proliferation
IL-27	Induction of Tbet promoting Th1 differentiation
IFN-γ	Antiviral properties, Th1 differentiation, cytotoxic activity, growth inhibition of TH2 cells
TH2 cytokines	
IL-4	Induction of Th2 differentiation, IgE class switch
IL-5	Growth and stimulation of eosinophiles
IL-13	Growth and specialisation of B cells, production of IgG4 and IgE
Treg cytokines	
IL-10	Immune suppression
TGF-β	Cell growth inhibition, controls proliferation and differentiation, induces apoptosis
Proinflammatory cytokines	
IL-1α, IL-1β	Induction of proinflammatory proteins, differentiation of Th17 cells
IL-6	T cell activation and differentiation; B cell differentiation and production of IgG, IgG, IgA
TNF	Cell activation, proliferation, differentiation, apoptosis, antimicrobial defence

Modified from Akdis et al 2011, Borish et al 2003

Cytokines IL-10, along with transforming growth factor-β (TGF-β) and regulatory T-cells (Treg) are essential factors in the regulation of the balance between tolerance and allergy. A healthy immune system implies allergen-specific Treg responses against common allergens. In addition, healthy and allergic subjects have different ratios of allergen-specific Th1, Th2 and Treg cells (Prescott et al 1999, Akdis et al 2004, Ling et al 2004, Verhagen et al 2006). Cord blood Treg responses are predictive of later outcome of atopic allergy (Cottrez et al 2000, von Hertzen et al 2009). The functionally impaired Treg cells, and thus imbalance between tolerance and allergy, exist at birth and most likely already during the fetal period (Schaub et al 2008, Smith et al 2008).

Enhanced production of Th2 cytokine and reduced IFN-γ in the newborn high-atopy-risk infant is behind the evolved prominent Th2 cytokine profile in children who later become atopic (van der Velden et al 2001, Herberth et al 2010). Enhanced IL-4 and low IFN-γ production has been found already in cord blood CD4+ T cells of children who developed atopic dermatitis during the first 2 years of life (Herbert et al 2010). During the first year of life a weak Th2 response of the newborn subsides and a Th1 response gradually matures in healthy children (Prescott et al 1998, 1999). It has also been shown that in atopic children the allergen-induced IL-10 production at 12 months of age is lower than in non-atopic children, resulting in poor regulatory control of the inflammatory process (Van der Velden et al 2001).

2.3 Breast milk and the allergic immune response

Breast milk contains numerous components protecting the infant against infections, including such factors that provide specific immunity, e.g. antibodies and viable lymphocytes (Böttcher et al 2000, Goldman 2007). Several immune cells and immunomodulatory chemokines and cytokines such as TGF-β and IL-10 are present in human milk (Oddy et al 2010, Iyengar et al 2012, Ismail et al 2013). It also contains toll-like receptor (TLR) agonists that induce innate immune responses (Newburg et al 2007). Human milk is also rich in non-specific protective factors, such as the iron-chelating protein, lactoferrin, lysozyme and oligosaccharides (Böttcher et al 2000). The composition of breast milk shows individual variation (Rodriquez et al 1999). These factors influence the maturation of the infant`s immune system. Chemokines are characterised as a family of small cytokines, or proteins secreted by cells. Their major role is to act as a guide to the migration of cells (Ono et al 2003). Differences in cytokine production have been observed between breast milk and peripheral blood mononuclear cells (PBMC) from the same mothers. Breast milk cells collected at 5 weeks postpartum spontaneously produced significantly less IL-1β, IL-6, and tumor necrosis factor-α (TNF) than PBMCs. In vitro stimulation of human milk cells with lipopolysaccharide increased cytokine production by 40-50%, whereas PBMC responded to lipopolysaccharide with increased cytokine production of up to 350%. These observations suggest that mature milk cells maintain their capacity to participate in the production of cytokines well after the colostral phase of lactation is over (Hawkes et al 2002a).

There are differences in the leukocyte, cytokine and chemokine composition of milk from atopic and non-atopic mothers (Böttcher et al 2000, Järvinen et al 2002, Laiho et al 2003, Oddy et al 2010, Ochiai et al 2013). Allergic mothers have higher IL-4 concentrations in colostrums than non-allergic mothers and similar trends are seen for IL-5 and IL-13 (Böttcher et al 2000). On the other hand, despite the differences in the composition of breast milk, the effect of breast milk on cytokine production does not appear to influence the risk of infant sensitization or allergic disease. TNF and IFN-γ production of PBMC was significantly lower in infants with cow‘s milk allergy than in healthy children (Österlund et al 1999). In a comparison between Swedish and Estonian mothers, the breast milk of Estonian mothers was found to have lower breast milk levels of TGF-β2 but higher levels of secretory IgA, IL-10 and IFN-γ, possibly because of differences in microbial load (Tomicić at al 2010).

Maternal diet has a strong influence on breast milk composition. Breast milk provides all the dietary essential fatty acids, linoleic acid, and α-linolenic acid and their metabolites, including arachidonic acid (AA) and docosahexaenoic acid (DHA) to support the growth and development of the breast-fed infant. Linolic acid (LA) levels of milk have increased in the Western world from the mean levels over the last century (Black et al 1997,

Simopoulos 1999, Innis 2007). This is explained by the increase in the dietary intake of LA-rich vegetable oils. DHA levels are lowest in countries in which the intake of fish and other animal tissue lipids is low (Innis 2007). Reduced γ-linolenic acid (GLA) levels have been found in the breast milk of allergic mothers (Duchén 2000, Kankaanpää et al 2001, Laitinen et al 2006). The breast milk inflammatory factors and fatty acid composition were shown to be related. A positive association was observed between TGF-β2 and the proportion of polyunsaturated fatty acids, suggesting that it may be possible to influence the immunologic properties of breast milk by dietary intervention of mothers (Laiho et al 2003). Supplementation with fish oil during pregnancy significantly increased early postpartum breast milk fatty acid composition and IL-10 and IL-6 levels correlate with n-3 fatty acid levels (Dunstan et al 2004). Supplementation with DHA-rich tuna oil also increased the n-3 fatty acid concentration in breast milk, but no relation to cytokine concentrations was seen (Hawkes et al 2002b). It has been shown that administering probiotics during pregnancy and to lactating mothers increased the TGF-β2 in breast milk (Rautava et al 2002). A recent study showed that probiotic supplementation increased IL-10 and decreased IgA antibodies in breast milk (Kuitunen et al 2012). Dietary intervention which included dietary counselling and provision of rapeseed oil based food products, or dietary intervention with probiotics, increased n-3 acid fatty acid and the concentrations of TNF, IL-10, IL-4 and IL-2 compared with controls (Hoppu et al 2012).

Breast milk fatty acids possess immunomodulatory properties, and a new strategy is to modify maternal diet with dietary intervention (Sala-Vila et al 2008, West et al 2010, Iyengar et al 2012).

2.4 Smoking and the allergic immune response

Earlier studies show that parental smoking is associated with higher cord blood immunoglobulin E (IgE) levels (Magnusson 1986). Because IgE does not cross the placenta, this provided preliminary evidence of a direct effect of maternal smoking on fetal immune function (Prescott et al 2008).

Many earlier studies have linked parental smoking with markers of atopy in children, including serum IgE levels, eosinophilia and positive skin prick tests (Noakes et al 2003). Maternal smoking is associated with increased exhaled nitric oxide value and poorer lung function in steroid-naive preschool children with multiple trigger wheeze (Kalliola et al 2013). Passive smoking increases the incidence of wheeze and asthma in children and young people by at least 20% (Burke et al 2012). There is still very limited information about the effects on the developing neonatal immune responses.

More recently, parental smoking has also been associated with increased serum IL-4 and nasopharyngeal IL-13, lower peripheral blood IFN-γ and more frequent respiratory

symptoms in children (el-Nawawy et al 1996, Kulig et al 1999, Feleszko et al 2006, Prescott et al 2008, Tebow et al 2008). It has also been found that mitogen-induced cord blood IL-13 was higher and IFN-γ was lower if mothers smoked during pregnancy (Noakes et al 2003).

These findings indicate that maternal and parental smoking during pregnancy can modify the immune function of children in favour of Th2 response over Th1 responses.

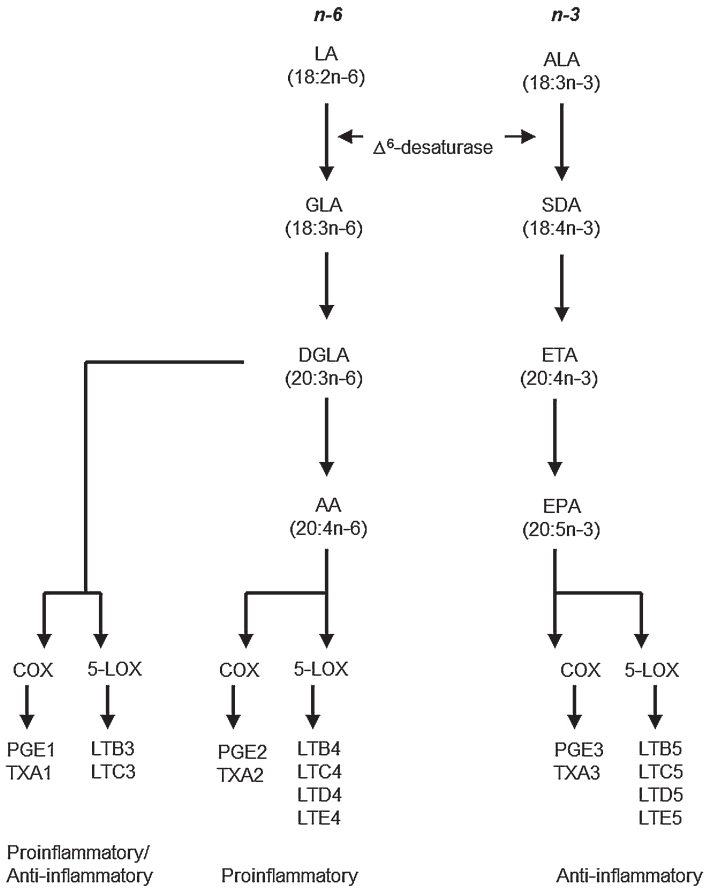
2.5 Essential fatty acid (EFA)

There are two classic essential fatty acids. These are linoleic acid (LA) and α-linolenic acid (ALA), which cannot be synthesized in animal cells and therefore must be obtained from the diet. LA is an n-6 polyunsaturated fatty acid (PUFA), described by 18:2n-6, which refers to the 18-carbon fatty acid with two double bonds, the first of which is on carbon atom 6 from the metyl end. ALA is an n-3 PUFA with a shorthand notation of 18:3n-3, describing an 18-carbon fatty acid with three double bonds, the first being positioned at carbon atom 3 from the methyl end (Yaqoob et al 2007)**.** Human cells cannot form a double bond so close to a metyl group, which is why these fatty acids have to be obtained from nutrition to prevent deficiencies (Simopoulos 1999, Das 2006a). Dietary sources of LA and ALA include plant seeds and nuts, plant oils and margarines. Over the past 100-150 years there has been an enormous increase in the consumption of LA (5 to 20-fold) compared to ALA, due to the increased intake of vegetable oils from corn, sunflower seeds, safflower seeds, cottonseeds and soybeans. Relative deficiencies of essential fatty acids in humans are thought to be closely linked to many common diseases in Western countries, such as atherosclerosis, hypertension, diabetes and multiple sclerosis (Simopoulos 1999, Sanders 2000). The role of fatty acids in these diseases has been intensively studied because the basic chemistry and biology of fatty acids are better understood than those of many other substances, such as oligosaccharides.

Both essential FAs can be further elongated and saturated forming longer chains and more unsaturated members of the n-6 and n-3 families of PUFAs. The metabolism of the n-6 and n-3 fatty acids is competitive, since both pathways employ the same enzymes (Yaqoob et al 2007). Δ^6 -desaturase is the regulatory enzyme starting the conversion of EFAs from phospholipids into other FAs to be further converted into a series of eicosanoids, such as tromboxanes, prostaglandins and leucotrienes (Das 2006a, Lee et al 2014). These have an important role in the regulation of epidermal inflammation and immune function (Ziboh et al 2000, Calder et al 2002). Arachidonic acid (AA, 20:4n-6) derived eicosanoids are mostly proinflammatory, whereas eicosapentaenoic acid (EPA, 20:5n-3) derived eicosanoids are inhibitory. Eicosanoids derived from dihomo-γ-linolenic acid (DGLA, 20:3n-6), the intermediate metabolite of n-6 series,

are either anti-inflammatory or inactive (Nakamura et al 2004). In addition, a novel AA-derived eicosanoid, lipoxin A4, has been found to possess anti-inflammatory properties (Serham et al 2008). Both n-6 and n-3 FAs and their metabolites regulate and modulate inflammation and chronic diseases via several pathways (Das 2006a, Calder 2002). Figure 2 presents the metabolism and synthesis of EFAs.

Recently it has been suggested that the fetal period is important for EFAs and their metabolites in programming or imprinting the development of the immune system (Prescott et al 2007, Enke et al 2008). Perinatal supplementation of long-chain PUFAs can modulate TH1 and TH2 cell generation and their cytokine production (Das 2006b, Steer et al 2011, Shek et al 2012).



LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; SDA, stearidonic acid; DGLA, dihomo-γ-linolenic acid; ETA, eicosatetraenoic acid; COX, cyclo-oxygenase pathway; 5-LOX, 5-lipo-oxygenase pathway; PGE; prostaglandin E; TXA, tromboxane; LTB, leukotriene B; LTC, leukotriene C; LTD, leukotriene D and LTE, leukotriene E.
Modified from Calder et al 2002.

Figure 2. The metabolism of n-6 and n-3 fatty acids (**I**)

2.6 Essential fatty acid and atopic dermatitis

Although atopic diseases are genetically determined, it alone cannot explain their rapid increase. This rapid increase is more likely a result of a combination of genetic risk, changing lifestyle and dietary changes. It has been suggested that increased n-6 PUFA (margarine, vegetable oil) and decreased n-3 PUFA (oily fish) intakes may explain the increase in atopic diseases during the last decades (Black et al 1997, Sanders 2000). The similarities between the manifestation of skin symptoms encountered in the deficiencies of essential fatty acids and skin symptoms of atopic dermatitis inspired the researchers to find out more about the effects of supplemental fatty acids on the skin.

Subjects with atopic dermatitis have in some studies been shown to have reduced levels of EFAs, with considerable variation in the plasma levels of n-3 and n-6 FA (Devereux et al 2005, Laitinen et al 2006, Yen et al 2008). γ-linolenic acid levels are lower in atopics compared with healthy infants (Kankaanpää et al 2001). Atopic dermatitis (AD) was suggested to be associated with abnormal EFA intake or metabolism as early as in the 1930s (Hansen et al 1933). Children with AD had lower blood levels of PUFA and it was assumed that the addition of LA to the diet would improve the dermatitis. Later, a controlled study showed that a small dose of LA produced no positive effects in children with AD (Pettit 1954). An elevated level of LA in plasma phospholipids was reported in adults with atopic dermatitis as well as lower levels of its metabolites; GLA, DGLA and AA (Manku et al 1984). It was speculated that these patients had a defect in the enzyme delta-6-desaturase, which converts LA into GLA (Horrobin et al 1993, Lattka et al 2009**).** Lower levels of n-6 PUFAs (DGLA and AA) and their correlation with Δ^6-desaturase (FADS2) mRNA expression have since been demonstrated in children with atopic dermatitis (Chisaguano et al 2013). This would imply that the direct administration of GLA, to bypass the apparent metabolic block, would have a beneficial effect on AD. Δ^6-desaturase also converts α-linolenic acid to steridonic acid (SDA), and thus adding SDA would enhance the production of longer chain n-3 fatty acid. (Chisaguano et al 2013).

Sources of GLA are, amongst others, evening primrose oil, blackcurrant seed oil and borace oil. Many positive results have been published on primrose oil, but no beneficial effects have been established in independent studies (Williams 2003). Supplementing a normal diet with borage oil has resulted in beneficial effects on subjective symptoms (Foster et al 2010). Moreover, one study showed that infants given borage oil for the first 6 months of life had reduced severity of AD (van Gool et al 2003). GLA supplementation did not prevent AD development in infants (Van Gool et al 2003, Kitz et al 2006, Foolad et al 2013). Oral supplementation of sea buckthorn seed oil increased the plasma levels of ALA and EPA in patients with AD but showed no effects on AD (Yang 2000).

Large amounts of saturated fats in the mother's diet have been linked to atopic sensitization in the child (Hoppu et al 2000). Also, decreased levels of cholesterol have been found in children and adolescents with allergic symptoms, in positive skin prick tests and elevated IgE levels as compared with nonatopic subjects. As the difference in cholesterol concentrations is detectable as early as from the age of 2 months onward, it is unlikely to be caused by dietary alterations related to atopy (Pesonen et al 2007). Examination of the dietary data in 1980 showed that atopic children had used less butter before the expression of atopy and that children with atopic disease consumed more margarine (Dunder et al 2001). A low proportion of n-3 FAs in breast milk and a large ratio of saturated to PUFAs has also been found to be connected with AD in children (Hoppu et al 2005, Thijs et al 2011)). Controversial results have been observed in earlier post-natal supplementation studies (van Gool et al 2004). It has also been shown that if fish oil substitution was given during pregnancy, the children had less severe AD at the age of 1 year (Dunstan et al 2003). The results of both prenatal and antenatal studies suggest that for successful modulation of the immune system, a prenatal supplementation of diet is required. Several types of FA composition abnormalities have been found in atopic disease; however, any really clear pattern of altered status of particular FA or a particular FA family seems not to exist (Sala-Vila et al 2008).

2.7 Blackcurrant seed oil (BCSO)

Blackcurrant (Ribes nigrum) is one of the most common cultivated berries in the western countries. Blackcurrant seed oil (BCSO) contains both n-6 FAs [LA> 40% and γ-linolenic acid (GLA, 18:3n-6) >10%] and n-3 FAs [ALA >15% and stearidonic acid (SDA, 18:4n-3) ~3%]. The content of oleic acid (OA, 18:1n-9) is around 10% (Johansson et al 1997). BCSO constitutes as little as 1% of the total weight of the berry (Johansson et al 1997). It is, however, produced as a by-product from the seeds of the berries by the food processing industry. The composition of BCSO corresponds to the recommended optimal dietary intake of n-3 and n-6 FAs, the oil has a ratio of n-3 FAs to n-6 FAs from 1/3 to 1/4 (The British Nutrition Foundations Task Forse 1992, Recommendations for intake of polyunsaturated fatty acids in healthy adults 2004). Due to its favourable fatty acid composition it has been used in different kinds of foods, such as yoghurts, juices and salad dressings, and dietary supplements in Europe and in the USA for the last 30 years. It has been tested in various long-term clinical trials without side effects (Spielmann et al 1989, Deferne et al 1996, Wu et al 1999, Sori et al 1993, Watson et al 1993, Tahvonen et al 2005). In the metabolic chain, a significant part of FAs (15%) of BCSO are metabolites beyond the Δ^6 - desaturase step, whereas in sea buckthorn seed oil, no FAs are Δ^6-desaturase-derived (Manku et al 1982).

Dietary supplementation of BCSO increases the proportion of GLA in triacylglyserols (TAG) and cholesteryl esters (CE), and dihomo-γ-linolenic acid (DGLA) in addition in glycerophospholipids in young healthy volunteers (Tahvonen et al 2005). In another study, serum levels of GLA and DGLA increased significantly with BCSO supplementation and an encouraging, yet not significant, healing process of canine atopic dermatitis was observed (Noli et al 2007). BCSO supplementation leads to increased levels of GLA and DGLA but not AA (Gauvreau et al 1999, Tahvonen et al 2005). In agreement with this, BCSO reduces the production of prostaglandin E_2 (PGE_2) (Watson et al 1993). This may explain why supplementation with n-6 FAs can be beneficial in atopic dermatitis. On the other hand, in murine models of asthma PGE2 has been shown to reduce allergic inflammation and expression of Th2 type cytokines IL-4 and IL-5, thus showing anti-inflammatory properties (Gauvreau et al 1999, Martin et al 2002).

A previous study on the effects of alpine currant seed oil produced by supercritical carbon dioxide extraction has shown a reduction of dermatitis symptoms in a group (n=40) of atopic children aged 5 months to 4.5 years. In the DBPC randomized parallel study with rapeseed oil as placebo, the intensity of the dermatitis symptoms, including itching, decreased significantly more in the alpine currant group than in the placebo group (Johansson et al 1999). The composition of alpine currant seed oil is close to that of blackcurrant. It grows wild in Scandinavian countries (Johansson et al 1997).

Dietary supplementation of BCSO increased the content of DGLA in plasma phospholipids, which is an indication of the increased capacity of precursors of anti-inflammatory eicosanoids (Tahvonen et al 2005). It should be possible to modify the unbalance of the PUFAs and eicosanoids in an efficient way with small doses of supplementation of BCSO.

3. AIMS OF THE STUDY

I To find out whether atopic allergies can be prevented by supplementing the diet of pregnant mothers with blackcurrant seed oil already during the pregnancy.

II To find out whether a blackcurrant seed oil supplement can affect the composition of breast milk so that it enhances the suppression of Th2 response in a child.

III To assess whether a blackcurrant seed oil supplement administered as early as during the pregnancy can affect the Th1/Th2 balance of the infant

4. MATERIALS AND METHODS

4.1 Subjects

A randomized double-blind placebo-controlled intervention trial was carried out during the years 2004-2008 at the public health care centres in the Salo and Kaarina-Piikkiö regions in Southwest Finland. All pregnant mothers willing to participate were included in the study; the only inclusion criterion was less than 16 weeks of gestation. Public health nurses informed all pregnant mothers about the study when the mothers first visited the maternity care units and asked for a permission to be contacted by the study nurse. The study nurse sent an information leaflet about the study to the mothers who had given their consent and contacted them by telephone. Around 500 mothers were interested in the study and 322 of them wanted to participate in it. Altogether 322 mothers were randomly assigned by a random number list to receive either BCSO or olive oil as placebo. The random allocation sequence was concealed until interventions were assigned. The randomization and allocation of interventions occurred at a different location from the recruitment of participants. Nine of the mothers cancelled their participation, leaving 313 mothers participating in the study. Of the 313 mothers, 151 were assigned to receive BCSO and 162 placebo. 31 mothers and infants from the BCSO group and 30 from the placebo group were randomly chosen into the breast milk study (**II**). Thirty-four infants from the BCSO group and 34 infants from the placebo group were randomly selected for the third immunological study (**III**). The treatment codes were not opened until the study material had been analysed in 3/2008.

4.2 BCSO preparations

BCSO was manufactured by supercritical CO_2 extraction technology 125 (Aromtech Ltd, Tornio, Finland). BCSO and olive oil (Santagata Luigi s.r.l., Genova, Italia) as reference oil (placebo) were raffinated in an identical way. Olive oil was manufactured from the pulp of the olive. The peroxide values of the raffinated oils were less than 1.0 (meq. O_2/g) and the acid values less than 0.5 (mg KOH/g). The fatty acid compositions (expressed as weight percentage of total) of the oils are presented in Table 2. Both oils were encapsulated in soft gelatine capsules for supplementation to the mothers and bottled as loose oil for delivering to the infants. They were packed in plastic bottles with a volume of 15 ml. The bottles were stored in the freezer and only bottles in use were stored in the refrigerator. The sensory properties of the oils and capsules were close to each other. They looked, smelled and tasted identical.

Table 2. Major fatty acids in blackcurrant seed oil and olive oil (weight percentage of total) (**I**).

Fatty acids	Blackcurrant seed oil	Olive oil
Palmitic acid (16:0)	6	11
Palmitoleic acid (16:1n-7)	0	1
Stearic acid (18:0)	2	3
Oleic acid (18:1n-9)	14	73
cis-Vaccenic acid (18:1n-7)	1	0
Linoleic acid (LA, 18:2n-6)	48	9
g-Linolenic acid (GLA, 18:3n-6)	12	0
a-Linolenic acid (ALA, 18:3n-3)	14	0
Stearidonic acid (SDA, 18:4n-3)	3	0

4.3 Study design

The first doses were given to the mothers during the 8th to 16th weeks of pregnancy and the supplementation continued until the end of the exclusive breastfeeding period. The BCSO dose was 3 grams per day given in capsules. The infants started to receive the same oil in the form of drops, dose 1 ml per day (1 gram), when the exclusive breastfeeding was ended, and they continued receiving the oil until the age of two years. The treatment codes were not opened until the study material had been analysed in 3/2008. The study physician or the study nurse met the mothers every 3 months and the mothers received a 3-month batch of capsules at a time. During the mothers' first visit the study physician or the nurse filled out a preliminary information form (appendices) which contained evaluations of, among other things, the mothers' residential environment, pets, smoking, usage of vitamins and additional nutrients and tendency for atopic diseases in the family. The children were examined at the ages of 3 months, 1 year and 2 years, at which points any occurrences of atopic dermatitis were evaluated, skin prick testing was carried out and blood was drawn for a serum sample (serum stored at -20^{o} C). The breast milk samples were collected mainly after delivery, but because of early discharge and/or delayed start of lactation, this was not possible with all mothers. Those mothers gave their samples at the 3-month control visit. Also, a data form (appendices) was filled in at each evaluation point to document any occurrences of atopic dermatitis and signs of allergies, as well as the child's general health, use of supplements and any changes in the living environment since the last visit.

Of the babies of the 322 randomized mothers, 241 were examined at the age of three months. Altogether 112 of these belonged to the blackcurrant group and 129 to the placebo group. The blackcurrant group also included a pair of twins. At 12 months of age 210 babies were examined, 100 of which belonged to the blackcurrant group and 110 to the placebo group. Altogether 177 babies took part in the study from the beginning till the end and were examined at the age of 24 months. Of those children, 85 belonged to the BCSO group and 92 to the placebo group. Sick children and those born prematurely (altogether 8), who required more intensive care, naturally dropped out of the study. Out of the group receiving BCSO, 29 mothers withdrew from the study during pregnancy due to miscarriage, problems with swallowing the capsules, pregnancy-related nausea, stomach problems, abortion or poor compliance. Post-birth reasons for dropping out included premature birth, colic and poor compliance. Out of the placebo group, 28 mothers withdrew from the study during pregnancy due to pregnancy-related nausea, problems with swallowing the capsules, miscarriage and poor compliance. Post-birth reasons for interruption included premature birth, colic and poor compliance. After the 3-month follow-up visit, 12 children withdrew from the blackcurrant group and 18 from the placebo group. The most common reasons for withdrawal were postpartum tiredness of the mother, other worries in the family and poor compliance. After the one-year-examination, 15 withdrew from the blackcurrant group and 19 from the placebo group. The most common reasons were again other worries in the family and poor compliance. The flow chart of the participants is presented in Figure 3. There were no significant differences in the baseline characteristics between the BCSO and placebo groups (Table 3). Baseline and clinical characteristics of studies **II** and **III** are presented in Table 4 and Table 5.

The primary endpoint of the study was the prevalence of atopic dermatitis by the age of 12 months, as the prevalence was predicted to be highest at this point of time. Secondary endpoint was the prevalence of atopic dermatitis by the age of 24 months. The prevalence of atopic dermatitis by the age of 3 months was predicted to be too low to detect a significant difference.

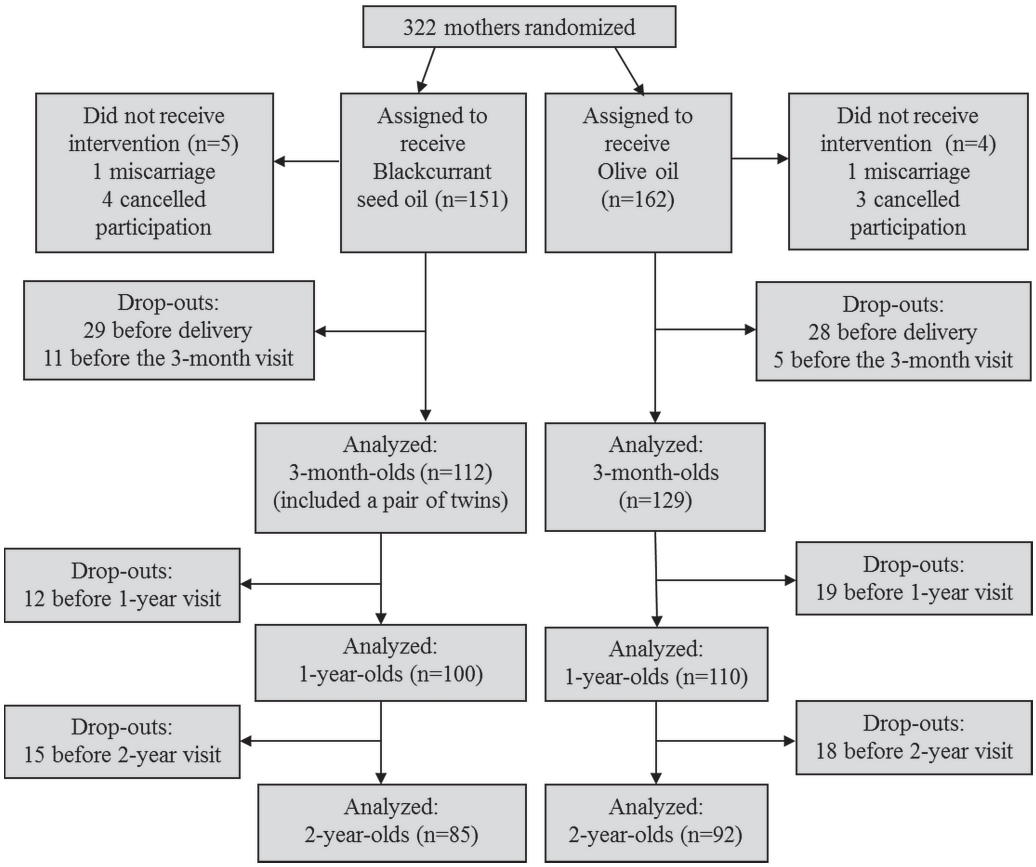


Figure 3. The flow chart of the participants enrolled in the study (**I**).

Table 3. Baseline Demographic and Clinical Characteristics for study **I**.

	BCSO	**Olive oil**
Number	112	129
Gender; n (%)		
Male	54 (48.2)	59 (45.7)
Female	58 (51.8)	70 (54.3)
Parental atopy; n (%)		
Both	29 (25.9)	34 (26.4)
One	61 (54.5)	73 (56.5)
None	22 (19.6)	22 (17.1)
Birth weight (g); mean (SD)	3595 (461)	3599 (468)
Parental smoking; n (%)	28 (25.0)	40 (31.0)
Breastfeeding (weeks); mean (SD)	33.8 (22.7)	30.8 (18.0)
Any furred pets at home; n (%)	48 (42.9)	52 (40.3)
Older siblings (n); mean (SD)	0.78 (0.87)	0.71 (0.90)

Table 4. Baseline Demographic and Clinical Characteristics for study **II**.

	BCSO	**Olive oil**
Number	31	30
Parental atopy; n (%)		
Both	6 (19.4)	8 (26.7)
One	16 (51.6)	16 (53.3)
None	9 (29.0)	6 (20.0)
Birth weight (g); mean (SEM)	3557 (86)	3653 (93)
Parental smoking; n (%)	10 (32.3)	7 (23.3)
Excl. breastfeeding (months); mean (SEM)	3.9 (0.3)	4.2 (0.3)
Breastfeeding (months); mean (SEM)	9.0 (1.0)	9.5 (0.9)
Any furred pets at home; n (%)	14 (45.2)	12 (40.0)
Older siblings; n (%)		
None	11 (35.5)	14 (46.7)
One	13 (41.9)	10 (33.3)
Two or more	7 (22.6)	6 (20.0)
Atopic dermatitis at; n (%)		
3 months	3 (9.7)	4 (12.9)
12 months	11 (35.5)	15 (50.0)
24 months	14 (45.2)	17 (56.7)
SCORAD at; mean (SEM)		
3 months	2.5 (1.2)	2.6 (0.8)
12 months	3.5 (0.7)	4.9 (1.1)
24 months	3.6 (0.9)	4.4 (1.2)

Table 5. Baseline Demographic and Clinical Characteristics for study **III**.

	BCSO	**Olive oil**
Number	34	34
Gender; n (%)		
Male	14 (41.2)	16 (47.1)
Female	20 (59.8)	18 (52.9)
Parental atopy; n (%)		
Both	5 (14.7)	5 (14.7)
One	22 (64.7)	23 (67.6)
None	7 (20.6)	6 (17.6)
Birth weight (g); mean (SD)	3636 (483)	3726 (541)
Parental smoking; n (%)	12 (35.3)	13 (38.2)
Breastfeeding over 4 months; n (%)	25 (73.5)	25 (73.5)
Any furred pets at home; n (%)	15 (44.1)	14 (41.2)
Older siblings; n (%)		
None	13 (38.2)	17 (50.0)
One	12 (35.3)	12 (35.3)
Two or more	9 (26.5)	5 (14.7)
Atopic dermatitis at; n (%)		
3 months	4 (11.8)	2 (5.9)
12 months	7 (20.6)	12 (35.3)
24 months	9 (26.5)	13 (38.2)
Sensitisation to egg at; n (%)		
3 months	0 (0.0)	1 (2.9)
12 months	3 (8.8)	3 (8.8)
24 months	0 (0.0)	2 (5.9)
Sensitisation to milk at; n (%)		
3 months	1 (2.9)	0 (0.0)
12 months	1 (2.9)	0 (0.0)
24 months	0 (0.0)	0 (0.0)

4.4 Clinical evaluation

A specialist in dermatology evaluated the skin of the child at each visit. Atopic dermatitis was defined as a chronic or relapsing itchy dermatitis with characteristic morphology and distribution (Hanifin et al 1980). The SCORAD index, an internationally used batch of criteria for AD, was used to assess eczema severity (Severity scoring of Atopic Dermatitis 1993, Pucci et al 2005). AD was also recorded if there was a history of chronic or relapsing itchy dermatitis with a typical localisation (Hanifin et al 1980). At each follow-up visit a data form was filled out, which charted the skin symptoms observed in the children, suspected food allergies, allergic nose or eye symptoms, asthma symptoms, any other diseases and the use of vitamins and additional nutrients by the children and their mothers. Any changes in the residential environment, household animals and smoking were also registered in the forms.

4.5 Skin prick testing (SPT) and serum total IgE

The skin tests were carried out by an experienced study nurse at the 3-month, 12-month and 24-month follow-up visits of the children according to the EAACI guidelines (European Academy of Allergology and Clinical Immunology 1993). The tested allergens were standardized allergen extracts of birch, cat, dog, cow's milk, egg, wheat, gliadin and house dust mite (SoluPrick, ALK187 Abellò, Horsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as a positive control and the ALK-Abello NSA-diluent as a negative control. In addition, 12-month and 24-month testing included *Candida albicans* (Soluprick, ALK-Abellò). Half or more of the histamine reaction size was regarded as a positive reaction (Meinert et al 1994). Serum total IgE was analyzed by Pharmacia CAP System (Phadia, Uppsala, Sweden).

4.6 Peripheral blood mononuclear cell (PBMC) cultures

The PBMC were isolated from heparinized blood samples by Ficoll-Paque density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and washed twice with Hanks' balanced salt solution (HBSS) buffered with $NaHCO_3$ (pH 7.4). After washings, the cells were resuspended in RPMI-1640 culture medium (Invitrogen Co., Carlsbad, CA, USA) supplemented with 5% autologous serum, 2.5 mM L-glutamine (Sigma-Aldrich Co.) and 100 μg/ml gentamycin sulfate (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) and applied on 48-well flat-bottomed cell culture plates (Costar, Corning Inc.) at a density of 10^6/ml. The cells were stimulated in the presence of ConcanavalinA mitogen (Pharmacia, Uppsala, Sweden) (10μ g/ml).

Medium alone served as unstimulated control. All incubations were performed at +37 ºC in humidified atmosphere with 5% CO_2. After 72 hours the cells and the culture supernatants were collected, and the cells were resuspended in TRIzol reagent (Gibco Life Technologies, Paisley,UK) for total RNA extraction.

4.7 Breast milk samples (II)

The breast milk samples were centrifuged and the supernatants were stored in aliquots at -70º C. The cytokine levels in the breast milk samples were detected with high-sensitivity human cytokine Lincoplex kits (LINCO Research, St. Charles, MO, USA). The assays were performed in accordance with the manufacturer's protocol by employing Luminex technology. The breast milk samples were collected mainly after delivery, but because of early discharge and/or delayed start of lactation, this was not possible with all mothers. Those mothers gave their samples at the 3-month control visit. There were no significant differences in the cytokine or in the demographics of the subjects with regard to the sampling time (Table 6).

Table 6. Demographic data and cytokine levels in subjects grouped according to timepoint of the breast milk sample for study **II**.

	Colostrum	Three-month	P *
Number	39	22	
BCSO (%)	20 (51.3)	11 (50.0)	0.92
Olive oil (%)	19 (48.7)	11 (50.0)	
Maternal atopy; n (%)	26 (66.7)	15 (68.2)	0.90
Maternal atopic dermatitis; n (%)	13 (50.0)	10 (45.5)	0.35
Birth weight (g); mean (SEM)	3540 (68)	3718 (124)	0.22
Breastfeeding (months); mean (SEM)	9.2 (0.9)	9.4 (1.0)	0.88
Any furred pets at home; n (%)	21 (53.8)	10 (45.5)	0.84
Older siblings; n (%)			0.26
None	19 (48.7)	6 (27.3)	
One	13 (33.3)	10 (45.4)	
Two or more	7 (18.0)	6 (27.3)	
Atopic dermatitis at; n (%)			
12 months	17 (43.6)	9 (40.9)	0.92
24 months	21 (53.8)	10 (45.5)	0.70
Cytokines (pg/ml); mean (SEM)			
IL-4	251 (55)	258 (72)	0.88
IL-5	1.5 (0.4)	1.5 (0.7)	0.90
IL-10	51 (28)	39 (22)	0.25
IL-12	3.5 (1.7)	6.4 (3.9)	0.14
IFN-γ	8.7 (5.1)	12.8 (6.5)	0.64
TNF	6.7 (0.7)	4.3 (0.7)	0.15

* Chi-square test, Mann-Whitney U-test or multivariate logistic regression analysis (cytokines)

4.8 Total RNA extraction and RT-PCR (III)

The RNA isolation and the TaqMan® RT-PCR were performed as described (**III**, Savolainen et al 2007). RNA was isolated according to Trizol instructions and the RT reaction was performed with First-strand cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden) using oligo (dT) primers. The amplification of IL-4, IL10 and IFN-γ was performed in MicroAmp® optical 96-well reaction plate (PE Applied Biosystems, Foster City, CA, USA). The reaction was performed in ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). For analysis of IL-10, Predeveloped Assay Reagents (PDAR) kits were purchased from PE Applied Biosystems. The primer and probe sequences for detection of β-actin, IFN-γ and IL-4 mRNA expression were as described (II). For the analysis of IL-10, a cDNA-specific PDAR (Pre-Developed Assay Reagents) kit was purchased from Applied Biosystems. The data analysis was performed according to the manufacturer's instructions (User Bulletin #2, P/N 4303849, Applied Biosystems) using comparative Ct $^{(2-\Delta\Delta Ct)}$ method, where β-actin served as an endogenous reference gene and unstimulated cell culture as a calibrator.

4.9 Cytokine assay (II, III)

The cytokine responses from PBMC and breast milk samples were detected 72 hours after the beginning of the stimulation by measuring cytokine secretion with high-sensitivity human cytokine Lincoplex kits (LINCO Research, St. Charles, MO, USA). The assays were performed in accordance with the manufacturer's protocol by employing Luminex technology.

4.10 Statistical methods

In the first study **(I)** we predicted the prevalence of AD at the age of 12 months to be 34% in the olive oil group and 17% in the BCSO group. Based on 80% power to detect a significant difference (p=0.05, two-sided χ^2 –test) and 30% early discontinuation rate a sample size of 147 subjects was required for each study group. In the per-protocol analysis the difference in proportions between the groups was tested using the χ^2 –test. Graphical methods were used for testing the normality of the continuous variables. In the case of normally distributed variables a two-sample t-test was applied to test the mean differences between the groups and otherwise the non-parametric Mann-Whitney U-test was used. P-values less than 0.05 were considered as statistically significant. The statistical analyses were carried out using SAS/STAT(r) software, Version 9.2 of the SAS System for Windows (SAS Institute Inc., Cary, NC).

In the second and third study, statistical analyses were performed with multivariate logistic regression analysis and ANOVA using StatView 5.0 statistical software. Variables included in the analysis were (**II**) cytokines, study intervention, atopy, number of the newborns' siblings, having pets at home, atopic dermatitis of the mother and the child; and (**III**) cytokines, study intervention, atopy, birthweight, breastfeeding, parental atopy, parental smoking, number of the newborns' siblings, having pets at home and atopic dermatitis. P-values less than 0.05 were considered to be statistically significant. In a two-group analysis, chi-square test and Mann-Whitney U-test were also used. P-values less than 0.05 were considered to be statistically significant.

4.11 Ethical considerations

The trial was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Ethical Committee of the Hospital District of South West Finland. All subjects participating signed a written informed consent.

5. RESULTS

5.1 Atopic dermatitis and sensitization (I)

The flow chart of the participants and the baseline and clinical characteristics are presented in figure 3 and in table 3 in section Study design. In study **I** there was a significantly lower prevalence of atopic dermatitis in the BCSO (33%) group than in the olive group (47.3%) at the age of 12 months (p=0.035, χ^{-test}). The difference did not remain significant (p=0.18) at the age of 24 months, with AD in 38.8% of the children in the BCSO group as compared to olive oil group (48.9%). At the age of three months the prevalence of atopic dermatitis was low, no significant differences were found between the treatment groups (BCSO group: 13.4%; placebo group 12.4%; p=0.82) in the prevalence of AD (Figure 4).

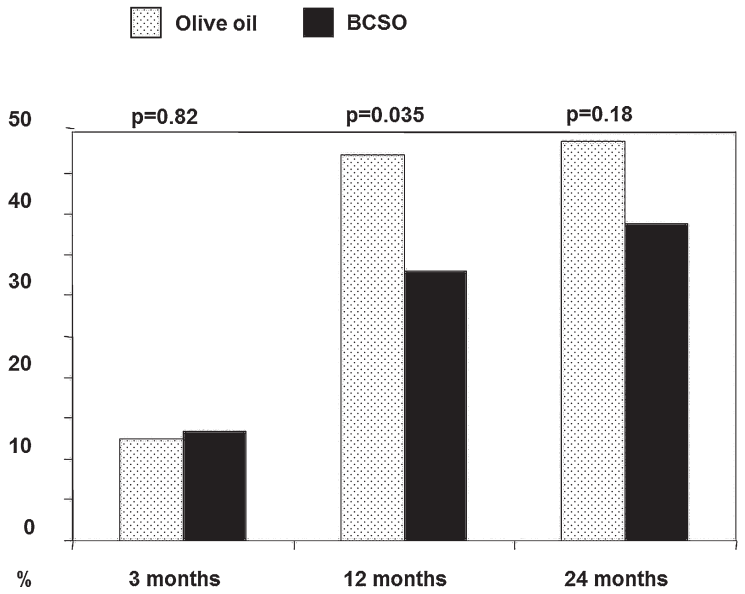


Figure 4. Prevalence of atopic dermatitis in the BCSO group (grey bar) and the placebo group (white bar) at the age of 3, 12 and 12 months. Statistical comparison with χ^2–test. (**I**)

According to SCORAD (table 7), AD was less severe in atopic children in the BCSO group as compared to those in the placebo group at the age of 12 months (p=0.035, Mann- Whitney U-test).

There were no significant differences in serum IgE levels or skin prick test with egg at the age of 3, 12 and 24 months between the BCSO and placebo groups (**I** table 4). Over 90% of all sensitization in SPT was to egg.

Table 7. SCORAD classes (**I**).

	Class	Blackcurrant seed oil	Olive oil	P b *
3 months				0.65
	1	91 (81.3%)	107 (83.0%)	
	2	7 (6.3%)	11 (8.5%)	
	3	11 (9.8%)	9 (7.0%)	
	4	3 (2.7%)	2 (1.6%)	
12 months				0.035
	1	80 (80.0%)	74 (67.3%)	
	2	9 (9.0%)	13 (11.8%)	
	3	8 (8.0%)	18 (16.4%)	
	4	3 (3.0%)	5 (4.6%)	
24 months				0.24
	1	70 (83.3%)	68 (76.4%)	
	2	6 (7.1%)	8 (9.0%)	
	3	8 (9.5%)	12 (13.5%)	
	4	0 (0.0%)	1 (1.1%)	

*1 = 0-5 pts, 2 = 6-10 pts, 3 = 11-20 pts, 4 = >20 pts
b Mann-Whitney`s U-test

5.2 BCSO and breast milk cytokines (II)

In study **II** the baseline and clinical characteristics are presented in Table 4 in section Study design. Mothers and infants from an intervention study by BCSO (n=31) or placebo (n=30) were included in the study. Breast milk samples were collected during the first 3 months of breastfeeding. BCSO intervention group had decreased level of IL-4 (p=0.044) and elevated level of IFN-γ (p=0.014) in breast milk as compared to the olive oil group (Figure 5).

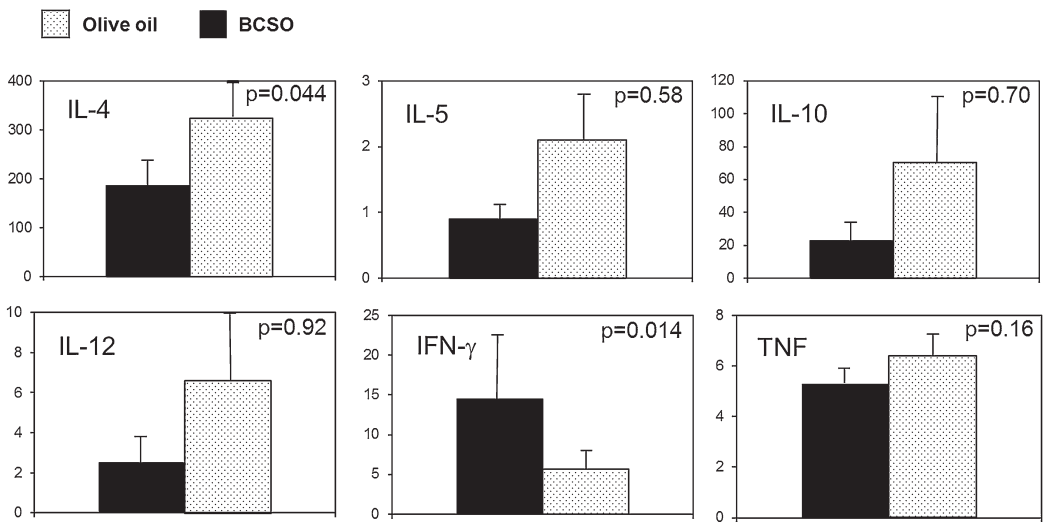


Figure 5. Breast milk IL-4, IL-5, IL-10, IL-12, IFN-γ and TNF concentrations (pg/ml, mean and SEM) of mothers undergoing BCSO (n=31, grey column) or olive oil (n=30, white column) intervention analyzed by high-sensity human cytokine Lincoplex[R] kits using Luminex [R] technology. (**II**)

Table 8 shows the cytokine levels of mothers with and without atopic dermatitis. Mothers who had atopic dermatitis had significantly decreased level of IL-10 (p=0.044) in breast milk at 3 months. No significant differences were observed in other cytokines.

Table 8. Breast milk cytokine concentration (pg/ml) of mothers with and without atopic dermatitis; mean (SEM) (**II**).

	Mothers with atopic dermatitis		
	Yes (n=23)	**No (n=38)**	**P-value***
IL-4	228 (71)	269 (56)	0.091
IL-5	1.6 (0.7)	1.5 (0.4)	0.37
IL-10	36.7 (21.5)	52.5 (28.9)	0.044
IL-12	6.5 (3.9)	3.3 (1.6)	0.064
IFN-γ	9.2 (5.2)	10.8 (5.6)	0.50
TNF	5.1 (0.8)	6.3 (0.7)	0.43

* Multivariate logistic regression analysis

Breast milk of the mothers of the children who developed atopic dermatitis had lower levels of IFN-γ (p=0,039) as compared to the breast milk of the mothers of the children without dermatitis (Table 9).

Table 9. Breast milk cytokine concentration (pg/ml) of mothers of children with and without atopic dermatitis at 12 months; mean (SEM) (**II**).

	Children with atopic dermatitis at 12 months of age		
	Yes (n=26)	No (n=33)	P-value*
IL-4	307 (74)	225 (54)	0.65
IL-5	2.3 (0.8)	0.9 (0.2)	0.56
IL-10	76.5 (44.3)	25.6 (9.4)	0.58
IL-12	7.1 (3.8)	2.7 (1.3)	0.64
IFN-γ	4.8(1.9)	14.9 (7.1)	0.039
TNF	6.4 (0.9)	5.5 (0.6)	0.38

* Multivariate logistic regression analysis

5.3 Smoking and cytokines (III)

In study **III** the baseline and clinical characteristics are presented in table 5 in section Study design.

In study **III** infants from an intervention study by using BCSO (n=34) or placebo (n=34) were included in the study. BCSO intervention did not have any effect on cytokine production or mRNA expression. No significant differences were observed between the BCSO and olive oil groups in cytokine production, IL-4, IL-5, Il-10, Il-12, TNF and IFN-γ of PBMC at 3 months of age. Children of smoking parents had elevated levels of IL-4 (p=0.0004), IL-5 (p=0.0002), IFN-γ (p=0.039) and TNF (p=0.0003) at 12 months of age (Figure 6).

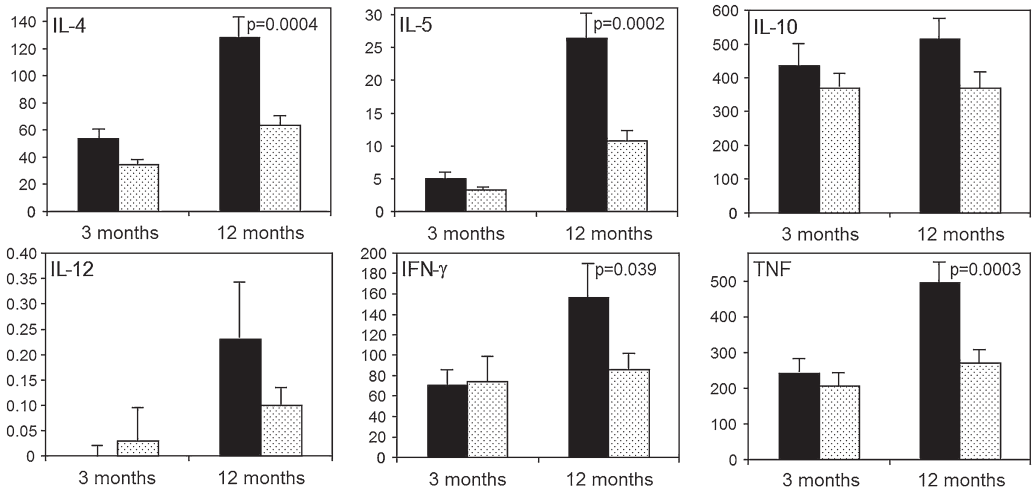


Figure 6. Mitogen (Concanavalin A) induced IL-4, IL-5, IL-10, IL-12, IFN-γ and TNF productions (pg/ml, mean and SEM) in peripheral blood mononuclear cell (PBMC) of infants with smoking (n=25, grey column) or non-smoking (n=43, white column) parents analysed at 3 and 12 months of age by high-sensitivity human cytokine Lincoplex^{R} kits using Luminex^{R} technology. (**III**)

Children who had atopic dermatitis by the age of 3 months showed elevated levels of IL-5 at 3 months (p = 0.0027) and 12 months of age (p = 0.022) as shown in Table 10**.** No significant differences were observed in the cytokine production when children were grouped according to the cumulative development of atopic dermatitis by the age of 12 or 24 months.

Table 10. Atopic dermatitis at 3 months and mitogen (ConA)-induced net cytokine production (pg/ml); mean (SEM) (**III**).

	Cytokine production at 3 months Atopic dermatitis at 3 months			Cytokine production at 12 months Atopic dermatitis at 3 months		
	Yes (n=6)	No (n=62)	P-value	Yes (n=6)	No (n=62)	P-value
IL-4	59.6 (12.6)	41.7 (3.3)	0.41	128.5 (41.7)	90.5 (9.5)	0.14
IL-5	9.2 (3.2)	3.5 (3.9)	0.0027	30.1 (10.9)	16.9 (2.1)	0.022
IL-10	565 (270)	376 (28)	0.17	391 (139)	431 (36)	0.78
IL-12	0.03 (0.03)	0.01 (0.05)	0.97	-0.10 (0.13)	0.19 (0.05)	0.11
IFN-γ	39.7 (15.0)	74.7 (15.5)	0.39	63.0 (16.8)	116.6 (16.3)	0.18
TNF	232 (51)	219 (23)	0.25	323 (65)	365 (31)	0.11

The production of cytokines in the infants grouped according to parental atopy is shown in Table 11. The production of TNF at the age of 3 months was higher in infants whose parents were atopic (p=0.010). On the contrary, the production of IL-12 at the age of 12 months was lower in infants whose parents were atopic (p=0.025).

Table 11. Parental atopy and mitogen (ConA) induced net cytokine production (pg/ml); mean (SEM) (**III**).

	Cytokine production at 3 months Parental atopy				Cytokine production at 12 months Parental atopy			
	No (n=13)	One (n=45)	Both (n=10)	P-value	No (n=13)	One (n=45)	Both (n=10)	P-value
IL-4	41.9 (7.3)	43.2 (4.0)	45.3 (9.3)	0.60	71.2 (13.6)	104.2 (13.0)	77.1 (16.3)	0.52
IL-5	3.5 (0.9)	4.3 (0.7)	3.3 (0.9)	0.83	14.3 (3.4)	20.8 (2.9)	10.7 (4.6)	0.98
IL-10	377 (47)	391 (45)	419 (105)	0.82	425 (59)	437 (47)	385 (88)	0.44
IL-12	-0.05 (0.03)	0.04 (0.06)	-0.03 (0.03)	0.53	0.34 (0.16)	0.12 (0.05)	0.00 (0.02)	0.025
IFN-γ	58.1 (18.7)	72.6 (20.1)	84.5 (26.1)	0.34	150 (519)	104 (15.9)	97.5 (31.0)	0.33
TNF	180 (49)	207 (194)	331 (100)	0.010	314 (66)	379 (37)	346 (71)	0.71

6. DISCUSSION

6.1 BCSO and atopic dermatitis (I)

Diet is an important factor in the prenatal and the postnatal periods affecting the early immune system. Modern diets include more synthetic and processed foods, with less fruits, vegetables and fresh fish than before. Modern dietary changes are implicated in the rise of many inflammatory diseases (West et al 2010). Decrease in the intake of n-3 PUFA in favour of n-6 PUFA has been implicated in the allergy epidemic. This has led to the interest of n-3 PUFA supplementation in the prevention of allergic diseases.

Human intervention studies concerning FA and fetal immune development are so far rare and the results are inconclusive. However, the results of both prenatal and antenatal studies suggest that for successful modulation of the immune system, a prenatal supplementation of diet is needed (Kukkonen et al 2007, Kalliomäki al 2001). In our study (**I**) we show that the use of BCSO in pregnant and breastfeeding mothers and their infants after breastfeeding results in a reduced outcome of atopic dermatitis at the age of 12 months. The prevalence of AD in the placebo group was 49%, which is considerably higher than in randomly chosen healthy infants in Finland. It is explained by the fact that the study attracted families with an atopic background. Over 80% of the children had at least one atopic parent and in one quarter of the families both parents were atopic. The symptoms of AD (SCORAD) were less severe in BCSO group than in olive oil group. In addition to this, previous study on the effects of alpine currant seed oil produced by supercritical carbon dioxide extraction revealed significant effects on dermatitis of atopic children aged 5 months to 4.5 years. In the DBPC randomized parallel study with rapeseed oil as placebo the intensity of the dermatitis symptoms, including itching, decreased significantly more in the alpine currant group than in the placebo group (Johansson et al 1999).

Polyunsaturated FAs are precursors for several lipid mediators, important in the regulation of inflammation (Serhan et al 2008). BCSO supplementation leads to increased levels of GLA and DGLA but not AA (Nakamura et al 2003, Tahvonen et al 2005). The composition of BCSO corresponds to the recommended optimal dietary intake of n-3 and n-6 FAs; the oil has a ratio of n-3 FAs to n-6 FAs from 1/3 to 1/4. In the metabolic chain, 15% of the FAs of BCSO are Δ^6-desaturase derived metabolites, whereas in sea buckthorn seed oil no FAs are Δ^6 -desaturase derived and they do not have an effect on AD (Yang et al 2000). Therefore it is possible that the low activity of Δ^6 -desaturase in atopics can be compensated with BCSO supplementation (Manku et al 1982, Chisaguano et al 2013).

BCSO reduces the production of prostaglandin E2 (Wu et al 1999). In murine models of asthma, PGE_2 reduces allergic inflammation and expression of Th2 type cytokines

IL-4 and IL-5 showing anti-inflammatory properties (Gauvreau et al 1999, Martin et al 2002). This exlains why supplementation with n-6 FAs may be beneficial also in AD. In addition a novel AA-derivated eicosanoid, lipoxin A4, has anti-inflammatory properties (Serham et al 2008). The presence of stearidonic acid (SDA) in BCSO may play a role in controlling inflammatory response by regulating the release of AA from phospholipids by the activity of phospholipase A2 and by inhibiting the 5-lipo-oxygenase pathway (Finnen et al 1991, Guichardant et al 1993, Chilton et al 2008). There are thus several reasons why the n-6 FAs traditionally considered proinflammatory may not always be so.

No distinction could be made between the groups at three months of age because the prevalence of AD was low as predicted. The diet of mothers during pregnancy and breastfeeding naturally also affected the fat supply of the foetus and the infant. The variation of starting time of the supplementation as well as the length of sole breastfeeding and low prevalence of atopic allergy at the age of 3 months in general may all have contributed to the missing difference between groups at the age of 3 months. The difference was no more significant at the age of two years. The reason for this transient significant difference was that the prevalence of atopic eczema increased during the second year of life in the BCSO group. It is possible that the effect of BCSO on the EFA balance is disturbed by the more diversified diet usually established at that age. According to a recent Finnish report (Kyttälä et al 2008), the diet of one-year-old children is good, but then worsens rapidly as the children begin to eat conventional family food instead of industrial baby foods or self-made small children's food. Already at the age of two years the intake of saturated fat is high and the intake of polyunsaturated fat is low. It remains to be investigated in further studies what kind of additional interventions are required to maintain a better long-lasting effect. The result of our study is nevertheless significant. The decreased and less severe atopic dermatitis at the age of one year increases the well-being of the child and lessens the burden on the family and makes its everyday life easier.

Maternal diet has great potential to influence immune development. There is clear evidence of the anti-inflammatory properties of n-3 PUFA. Modifications to the diet should remain a priority for researchers in the prevention of allergic disease. Further comprehensive studies are still needed before recommendations for the use of n-3 PUFA or BCSO in allergy prevention can be given. Future research should take genetic and environmental interactions into consideration.

6.2 BCSO and breast milk cytokines (II)

Breast milk contains several different cytokines, growth factors, nucleotides and hormones which regulate the immunological responses of the gastrointestinal tract of the newborn

(Böttcher et al 2000, Goldman et al 2007, Walker 2010, Iyengar et al 2012). Their concentrations in breast milk are typically quite small and their significance for infants has not been fully investigated. Breast milk contains all the dietary essential fatty acids, linoleic acid, α-linolenic acid and their metabolites including arachidonic acid (AA) and docosahexaenoic acid (DHA) to support the growth and development of the breast-fed infant. It is well established that the FA composition of breast milk is highly influenced by diet (Black et al 1997, Simopoulos 1999, Innis 2007). Due to various dietary habits and life styles worldwide, fatty acid composition of breast milk is different not only between countries but also within regions of a country (Chen 1995, Samur et al 2009).

It has been found earlier that concentrations of Th2-type cytokines are higher in breast milk from allergic than non-allergic mothers (Böttcher et al 2000). Breastfeeding has been shown to affect the outcome of AD and cytokine responses of infants (Chuang et al 2011, Belderbos et al 2012). Increased levels of several cytokines in breast milk, including IL-4, predict the development of AD by the age of 6 months (Ochiai et al 2013). Significant reduction in toll-like reseptor 7-mediated IL-10 production in peripheral blood was seen in breastfed infants at the age of 1 month (Belderbos et al 2012). In one study breastfeeding reduced the risk of asthma, but was a risk factor for sensitization to milk, nuts and egg (Brew et al 2012). In another study, long duration of breastfeeding increased the risk of atopic dermatitis at the age of 18 months (Chuang et al 2011).

Mothers with atopic dermatitis have a lower concentration of TGF-β2 in breast milk compared with those without atopic dermatitis (Kalliomäki et al 1999, Laiho et al 2003, Savilahti et al 2005). A positive association was also observed between TGF-β2 and a proportion of PUFA and a negative association between TGF-β2 and the proportion of saturated FA in breast milk, suggesting that it might be possible to influence the immunological properties of breast milk by dietary intervention of the mother (Laiho et al 2003).

In our present study (II) we found that dietary intervention with BCSO had immunomodulatory effects on breast milk cytokine production towards Th2 to Th1 immunodeviation. BCSO intervention group had decreased level of IL-4 and elevated level of IFN-γ. This is in line with our earlier trial **I**, in which we demonstrated that BCSO supplementation to pregnant and breastfeeding mothers and their infants after breastfeeding resulted in a reduced outcome of atopic dermatitis at the age of 12 months. In our study there were no differences in breastfeeding or exclusive breastfeeding between the intervention groups, and breastfeeding was not associated with the cytokine responses or outcome of atopic dermatitis.

Earlier studies on fatty acid intervention of pregnant and breastfeeding mothers have produced contradictory results on breast milk cytokine composition. Supplementation with fish oil during pregnancy altered breast milk fatty acid composition but had no effect

on IL-6, IL-10 and IL-13 levels in breast milk (Dunstan et al 2004). Also supplementation with tuna oil rich in docosahexaenoic acid increased n-3 fatty acid concentration in breast milk but did not affect the concentrations of IL-1β, IL-6 and TNF (Hawkes et al 2002b). Dietary intervention with rapeseed oil and intervention with probiotics during pregnancy increased n-3 fatty acid concentration in breast milk and raised the levels of IL-2, IL-4, IL-10 and TNF (Hoppu et al 2012).

Comparison of Swedish and Estonian mothers' breast milk showed that Estonian mothers have lower breast milk levels of TGF-β but higher levels of secretory IgA, IL-10 and IFN-γ. This might be due to differences in dietary habits and microbial exposure (Tomicić et al 2010). Children with higher risk of developing atopic dermatitis during the first 2 years of life have also been shown to have reduced IFN-γ and enhanced IL-4 producing CD4+ T cells in the cord blood (Herberth et al 2010). All these findings are in line with our findings on fatty acid intervention and outcome of atopic dermatitis and the association of these with breast milk IL-4 and IFN-γ.

In our **II** study we also show that the mothers of children who developed atopic dermatitis by the age of 12 months had considerably lower levels of IFN-γ in their breast milk. Earlier studies have been conducted, according to which the concentrations of IL-4 are higher in breast milk from allergic than non-allergic mothers and similar trends were seen for IL-5 and IL-13 (Böttcher et al 2010). Comparison of Swedish and Estonian mothers is in line with our findings on fatty acid intervention and outcome of atopic dermatitis and the association of these with breast milk IL-4 and IFN-γ. However, in a recent study maternal atopy was associated with increased IgA antibodies and decreased TGF-β, whereas probiotic supplementation was associated with decreased IgA antibodies and increased IL-10 (Kuitunen et al 2012).

AA-derivated PGE 2 has been proven to inhibit the production of Th1 cytokine IFN-γ, but not that of Th2 cytokines IL-4 and IL-5 in human T cells (Betz et al 1991), and in line with this AA-derivated leukotriene B4 induces both IL-4 and IL-5 in human T cells (Yamaoka et al 1993, Yamaoka et al 1994). BCSO contains LA and GLA, precursors of DGLA, as well as ALA and SDA, precursors of EPA. Dietary supplementation of BCSO increases the content of DGLA in plasma phospholipids. During BCSO intervention the increased production of DGLA and EPA instead of AA could be the way to influence the Th cell balance from Th2 to Th1.

6.3 Smoking and cytokines (III)

The adverse effects on the child of smoking during pregnancy are well known. Exposure to passive smoking can also lead to disturbance of growth in the foetus and disturbances

in the development of its organ systems. Children are more sensitive to the adverse health effects of smoking on, for example, the development of their respiratory system as well as their immunological and physiological development after birth. One possible mechanism by which environmental tobacco smoke may function is the impairment of the immune system. There is still very limited information about the effects on the developing neonatal immune responses.

In study **III** we found that children of smoking parents had significantly elevated levels of IL-4, IL-5, IFN-γ and TNF at the age of 12 months. Blackcurrant seed oil supplementation did not affect the studied cytokine secretion or cytokine mRNA expression of white blood cells in the children's peripheral blood. Earlier studies indicate that maternal cigarette smoking during pregnancy can modify the immune function of children in favour of Th2 response over Th1 responses. Neonatal IL-13 was higher and IFN-γ lower if mothers smoked in pregnancy (Noakes et al 2003). In another study it was shown that children exposed to parental smoking, from pregnancy to 11 years of age, had less mitogen ConA (Concanavalin A) induced IFN-γ production of PBMC than children of non-smoking parents. As opposed to this, no significant effects of parental smoking were found on IL-4 production (Tebow et al 2008). However, one study did observe that there was a significant increase in IL-4 in children of smoking parents (El-Nawawy et al 1996). These findings are in line with our findings. In addition to this, we demonstrated an increase of another pro-inflammatory cytokine, TNF, in children of smoking parents. Another association of TNF with allergic responses was seen as we demonstrated that increased TNF was associated with parental atopy. Parental atopy was also associated with low IL-12 levels reflecting Th2 dominance over Th1 in infant with atopic parents.

Our study also demonstrated the effect of parental smoking on cytokines post-natally at three and 12 months of age, suggesting a more prolonged effect on the immune function of the newborn child. Out of the 68 mothers in our study, only four reported having smoked during pregnancy, whereas 14 reported having stopped smoking upon noticing the pregnancy. Of the fathers, 25 smoked. These results confirm that even passive smoking affects the developing immune system of the newborn infant. This is a surprising result, especially when taking into account that according to the mothers, most fathers smoked out-of-doors and it is actually quite rare for anyone in Finland to smoke indoors nowadays.

Our intervention study could not show any significant effect of BCSO on the cytokine production or mRNA expression in PBMC. There was no association with cytokine production and outcome of atopic dermatitis either. We did, however, discover a trend of increased IFN- γ and IL-12 in the BCSO group. The multivariate analysis failed to show any statistical significance, suggesting that the cofounding factors, such as parental

smoking and parental atopy, had a stronger effect on the cytokine production than the BCSO intervention itself.

Children who developed atopic dermatitis by the age of 3 months had considerably elevated levels of Th2 cytokine IL-5 at the age of 3 and 12 months. An earlier observation has been made that children with reduced IFN-γ enhanced IL-4 producing CD4+ T cell in the cord blood had a higher risk of developing atopic dermatitis during the first 2 years of life (Herberth et al 2010). It has been shown that the selective development of Th2 cytokine profile in children with high risk of atopy is due to increased production of Th2 cytokines, possibly caused by impaired allergen-induced IFN-γ production in the neonatal period (van der Velden et al 2001, Herberth et al 2010).

7. SUMMARY AND CONCLUSIONS

Dietary supplementation with BCSO significantly reduced the prevalence of atopic dermatitis in infants at the age of one year. Atopic dermatitis was also less severe in the BCSO group than in the placebo group. BCSO is safe and well tolerated by mothers and their infants.

BCSO had immunomodulatory effects on breast milk cytokine production. The intervention group had decreased level of IL-4 and elevated level of IFN-γ. In addition, mothers who had atopic dermatitis had significantly decreased level of IL-10 in breast milk, and breast milk of the mothers of the children who developed atopic dermatitis had lower levels of IFN- γ.

Children exposed to parental smoking had elevated levels of IL-4, IL-5, IFN-γ and TNF at 12 months of age. Our study shows the detrimental effects of parental smoking on the child's immune function. The father's smoking also poses a risk for the newborn immune system by enhancing pro-inflammatory and Th2 cytokines. This may be connected to increased risk of asthma later in life.

It is evident that even more attention should be paid by the maternal health centres to supporting both mothers and fathers in their efforts to stop smoking.

BCSO offers an interesting alternative for re-balancing the profile of EFAs in atopic patients. BCSO can become a potential tool among others in prevention of atopic dermatitis at the early stages of life.

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APPENDICES

Appendix 1. Preliminary information form

TYKS Ihotautien klinikka/ TY, Biokemian ja elintarvikekemian laitos ___/___200

HERUKKAÖLJYTUTKIMUS

ESITIETOKAAVAKE

Nimi: _____

Sotu: _____

Osoite: _____

_____

Puh: _____

Koodi: _____

Pituus: _____

Paino: _____

1. PERHEEN ALLERGIAT (rastita)

	Lapsen äiti	Lapsen isä	Perheen lapset 1	2	3	4
Atooppinen ihottuma						
• Extrinsic-tyyppi (IgE)						
• Intrinsic-tyyppi						
Allergiset nuha-/silmäoireet						
Astma						
Nokkosihottuma						
Ei tiedossa						

2. ASUINYMPÄRISTÖ

Asuinpaikka: Maaseudulla ☐ Kaupungissa ☐

Kerrostalo ☐ Omakotitalo ☐ Rivitalo ☐ Maatalo ☐

Kotona asuvien aikuisten lukumäärä:_____

Kotona asuvien lasten lukumäärä: _____

Asunnon huoneiden lukumäärä: _____

Asunnon pinta-ala: _____ m^2

TUPAKOINTI	Lapsen äiti	Samassa taloudessa asuva kumppani	Muu perheenjäsen
Ei koskaan			
Aiemmin, ei enää			
Kyllä			

ELÄIMET

Onko Teillä kotieläimiä?

Ei ole	☐	Marsu	☐
Koira	☐	Hiiri/rotta	☐
Kissa	☐	Muu, mikä? _____	
Hamsteri	☐		

Onko Teillä hyötyeläimiä?

Ei ole	☐	Kanoja	☐
Hevosia	☐	Sikoja	☐
Lehmiä	☐	Muu, mikä? _____	
Lampaita	☐		

3. TUTKITTAVAN TERVEYDENTILA

Terve, ei ole todettu kroonisia sairauksia ☐

Minulla on todettu krooninen sairaus ☐

joka on: _____

LÄÄKKEET

Minulla ei ole säännöllistä lääkitystä ☐

Minulla on säännöllinen lääkitys ☐

Lääkkeen nimi ja annostus: _____

VITAMIINIT JA LUONTAISTUOTTEET

Onko käytössäsi raskauden aikana vitamiinivalmisteita tai luontaistuotteita?

Ei ☐

Kyllä ☐

Rautavalmiste ☐

Monivitamiinivalmiste ☐

Muu vitamiini tai luontaistuote ☐

Valmisteen nimi: _____

Tämän kyselylomakkeen täytti _____/_____200

_____ _____

Allekirjoitus Nimen selvennys

Appendix 2. Data form

TYKS, Ihotautien klinikka/TY, Biokemian ja elintarvikekemian laitos

HERUKKAÖLJYTUTKIMUS

Lääkärin suorittama haastattelu ja lapsen kliininen tutkimus ___/___ 200

Lapsen ikä _____ pituus_____ cm paino_____ g

Lapsen syntymäpituus _____ cm

Lapsen syntymäpaino _____ g

LAPSEN RAVINTO

Imetys: Saako lapsi vielä äidinmaitoa

EI ☐ Imetys päättyi _____ iässä

KYLLÄ ☐

Maidonvastike aloitettiin _____ iässä

Kiinteät lisäruoat aloitettiin _____ iässä

ALLERGIAT

Onko lapsella epäilty allergioita?

EI ☐

KYLLÄ ☐

Maito ☐

Muna ☐

Ruis ☐

Vehnä ☐

Kaura ☐

Ohra ☐

Peruna ☐

Muu ruoka-aine, mikä ☐_____

Kissa ☐

Koira ☐ Muu eläin, mikä ☐_____

Heinä ☐

Koivu ☐ Muu siitepöly, mikä ☐_____

Miten mahdollinen allergia oireilee?

Ihottuma ☐

Hengitystieoireet ☐

Nuha-tai silmäoireet ☐

Vatsaoireet ☐

Yleisreaktio ☐

IHOTTUMAT

Onko lapsella esiintynyt ihottumaa ?

EI ☐

KYLLÄ ☐

Atooppinen ihottuma ☐

Muu ihottuma ☐ mikä _____

Täytetään **SCORAD**-kaavio

Minkä ikäisenä atooppinen ihottuma on alkanut?

0-1 kk ☐ 7-12 kk ☐

2-3 kk ☐ 1-2 v ☐

4-6 kk ☐

Onko atooppinen ihottuma kestänyt yli kuukauden?

EI ☐

KYLLÄ ☐ Kuinka kauan?_____

Onko se toistunut?

EI ☐
KYLLÄ ☐

Paheneeko atooppinen ihottuma ruoka-aineista?

EI ☐
KYLLÄ ☐

Mistä _____

Onko atooppista ihottumaa hoidettu?

EI ☐
KYLLÄ ☐

Perusvoide ☐
Kortisonivoide ☐
Takrolimuusivoide ☐
Pimekrolimuusivoide ☐
Antihistamiini ☐

NUHA- TAI SILMÄOIREET

Onko lapsella esiintynyt allergisia nuha- tai silmäoireita?

EI ☐
KYLLÄ ☐

Juokseva nuha allergeenikontaktin yhteydessä ☐
Nenän tukkoisuus ☐
Silmien kutina/punoitus/turvotus ☐

Ovatko nuha- ja silmäoireet vaatineet lääkitystä viimeisen 6 kk aikana?

EI ☐
KYLLÄ ☐ Lääkkeen nimi:_____

HENGITYSTIEOIREET

Onko lapsella todettu astma?

EI ☐

KYLLÄ ☐ Lääkitys aloitettu ☐

Lääkkeen nimi:_____

Onko lapsella esiintynyt:

Hengityksen vinkumista ☐

Yli 2 viikkoa kestänyt yskä ☐

Hengityksen rohina ☐

Toistuvia keuhkoputkentulehduksia ☐

Toistuvia korvatulehduksia ☐

Onko lapsella putket korvissa?

EI ☐

KYLLÄ ☐

VATSAOIREET

Onko lapsella esiintynyt vatsaoireita mahdolliseen allergiaan liittyen?

EI ☐

KYLLÄ ☐

Ripuli ☐

Oksentelu ☐

Vatsakipu ☐

MUUT

Onko lapsen suussa ollut sammasta?

EI ☐
KYLLÄ ☐

Onko lapsella todettu jokin muu sairaus?

EI ☐
KYLLÄ ☐

Mikä_____

Lääkitys_____

VITAMIINIT JA LUONTAISTUOTTEET

Onko lapsi saanut D-vitamiinitippoja?

EI ☐
KYLLÄ ☐

Onko lapsi saanut muita vitamiineja tai luontaistuotteita?

EI ☐
KYLLÄ ☐ Valmisteen nimi_____

Onko herukkaöljykorvaukseen liittynyt ongelmia?

EI ☐
KYLLÄ ☐ Minkälaisia?_____