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DIET AND PROBIOTICS DURING PREGNANCY –

Endorsing developmental programming

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ABSTRACT

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Maternal obesity has been shown to increase the risk for adverse reproductive health outcomes such as gestational diabetes, hypertension, and preeclampsia. Moreover, several studies have indicated that overnutrition and maternal obesity adversely program the development of offspring by predisposing them to obesity and other chronic diseases later in life. The exact molecular mechanisms leading to developmental programming are not known, but it has recently been suggested that obesity-related low-grade inflammation, gut microbiota and epigenetic gene regulation (in particularly DNA methylation) participate in the developmental programming phenomenon. The aim of this thesis was to evaluate the effect of diet, dietary counseling and probiotic intervention during pregnancy in endorsing favorable developmental programming.

The study population consisted of 256 mother-child pairs participating in a prospective, double-blinded dietary counselling and probiotic intervention (*Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb12*) NAMI (Nutrition, Allergy, Mucosal immunology and Intestinal microbiota) study. Further overweight women were recruited from maternal welfare clinics in the area of Southwest Finland and from the prenatal outpatient clinic at Turku University Hospital. Dietary counseling was aimed to modify women's dietary intake to comply with the recommended intake for pregnant women. Specifically, counseling aimed to affect the type of fat consumed and to increase the amount of fiber in the women's diets. Leptin concentration was used as a marker for obesity-related low-grade inflammation, antioxidant vitamin status as an efficiency marker for dietary counselling and epigenetic DNA methylation of obesity related genes as a marker for probiotics influence.

Results revealed that dietary intake may modify obesity-associated low-grade inflammation as measured by serum leptin concentration. Specifically, dietary fiber intake may lower leptin concentration in women, whereas the intakes of saturated fatty acids and sucrose have an opposite effect. Neither dietary counselling nor probiotic intervention modified leptin concentration in women, but probiotics tended to increase children's leptin concentration. Dietary counseling was an efficient tool for improving antioxidant vitamin intake in women, which was reflected in the breast milk vitamin concentration. Probiotic intervention affected DNA methylation of dozens of obesity and weight gain related genes both in women and their children. Altogether these results indicate that dietary components, dietary counseling and probiotic supplementation during pregnancy may modify the intrauterine environment towards favorable developmental programming.

Keywords: Obesity, overweight, dietary counseling, probiotics, leptin, epigenetics, DNA methylation

TIIVISTELMÄ

Sanna Vähämiko

RAVITSEMUS JA PROBIOOTIT RASKAUDEN AIKANA – tukemassa sikiökautista ohjelmoitumista

Turun yliopisto, Lääketieteellinen tiedekunta, Lastentautioppi, Turun yliopiston kliininen tohtoriohjelma ja Funktionaalisten elintarvikkeiden kehittämiskeskus, Turku, Suomi

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Raskaudenaikainen ylipaino ja lihavuus lisäävät muun muassa raskausdiabeteksen ja kohonneen verenpaineen riskiä. Lisäksi useat tutkimukset ovat osoittaneet, että äidin raskaudenaikainen lihavuus lisää syntymässä olevan lapsen riskiä ylipainoon ja muihin kroonisiin sairauksiin hänen omassa aikuisuudessaan. Tähän niin sanottuun sikiökautiseen ohjelmoitumiseen johtavat molekyylitason yksityiskohtaiset mekanismit eivät ole selvillä, mutta viime aikoina useissa tutkimuksissa on esitetty, että lihavuuteen liittyvä matala-asteinen tulehdus, suoliston mikrobisto sekä epigeneettinen geenien säätely (erityisesti DNA-metylaatio) osallistuisivat sikiökautiseen ohjelmoitumiseen molekyylitasolla. Tämän väitöskirjatyön päätarkoituksena oli arvioida raskaudenaikaisen ruokavalion, ravitsemusohjauksen ja probiootti-intervention kykyä tukea terveellistä sikiökautista ohjelmoitumista.

Väitöskirjan aineisto koostui 256 äiti-lapsiparista, jotka osallistuivat NAMI-tutkimusryhmän prospektiiviseen, kaksoissokkoutettuun ravitsemusohjaus-probiootti-interventiotutkimukseen (*Lactobacillus rhamnosus GG* ja *Bifidobacterium lactis Bb12*), ja lisäksi ylipainoisista äideistä, jotka oli rekrytoitu mukaan tutkimukseen suoraan Lounais-Suomen alueen äitiysneuvoloista tai Turun yliopistollisen keskussairaalan poliklinikalta. Ravitsemusohjauksella tavoiteltiin suositusten mukaista ruokavaliota. Erityisesti äitejä neuvottiin kiinnittämään huomiota käytetyn rasvan laatuun sekä kuidun määrän kasvattamiseen. Työssä äitien ja lasten seerumin leptiinipitoisuutta analysoitiin lihavuuteen liittyvän tulehduksen mittarina. Äitien ja lasten antioksidanttivitamiinitilaa puolestaan seurattiin ravitsemusohjauksen tehokkuuden mittarina. Lihavuuteen tai painonnousuun liittyvien geenien DNA:n metylaation muutosta seurattiin probioottien vaikutuksen mittarina.

Tutkimuksen tulokset osoittivat, että raskaudenaikaisella ruokavaliolla voidaan vaikuttaa lihavuuteen liittyvään matala-asteiseen tulehdukseen, kun tulehduksen mittarina käytetään leptiinipitoisuutta. Erityisesti runsas kuidunsaanti voi laskea leptiinin pitoisuutta kun taas runsas tyydyttyneiden rasvojen ja sokerin saanti voivat kasvattaa äidin leptiinin pitoisuutta. Ravitsemusohjaus ja probiootti-interventio eivät vaikuttaneet äitien leptiinipitoisuuteen mutta probiooteilla oli taipumusta kasvattaa lasten leptiinipitoisuutta. Ravitsemusohjaus puolestaan osoittautui tehokkaaksi tavaksi parantaa antioksidanttivitamiinien saantia naisilla, ja tämä heijastui edelleen rintamaidon antioksidanttipitoisuuksiin. Probiootti-interventio vaikutti DNA-metylaatioon kymmenissä lihavuuteen tai painonnousuun liittyvissä geeneissä sekä naisilla että lapsilla. Yhteenvetona nämä tulokset osoittavat, että raskaudenaikaisella ruokavalion laadulla, ravitsemusohjauksella sekä probioottien käytöllä voidaan tukea lapsen myöhemmän terveyden ohjelmoitumista.

Avainsanat: lihavuus, ylipaino, ravitsemusohjaus, probiootti, leptiini, epigenetiikka, DNA-metylaatio

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ABBREVIATIONS

AdoMet	S-adenosyl-L-methione
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BMI	Body mass index
CpG	Cytocine-guanine islands
CD	cluster of differentiation
CI	Confidence interval
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease -hypothesis
HOMA	Homeostasis model assessment
IL	Interleukin
JAK/STAT	Janus kinase/Signal transducers and activators of transcription pathway
LEP	Leptin
LGG	Lactobacillus rhamnosus GG
NAMI	Nutrition, Allergy, Mucosal immunology and Intestinal microbiota project
Ob	Obese-gene
PUFA	Polyunsaturated fatty acids
QUICKI	Quantitative insulin check index
RNA	Ribonucleic acid
SAFA	Saturated fatty acids
Th	T helper cell
TNFα	Tumor necrosis factor α
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

I. Vähämiko S, Isolauri E, Pesonen U, Koskinen P, Ekblad U, Laitinen K. Dietary sucrose intake is related to serum leptin concentration in overweight pregnant women Eur J Nutr. (2010) 49, 83-90.

II. Vähämiko S, Isolauri E, Laitinen K. Weight status and dietary intake determine serum leptin concentrations in pregnant and lactating women and their infants. Br J Nutr. (2013) 110, 1098-106.

III. Vähämiko S, Isolauri E, Poussa T, Laitinen K. The impact of dietary counselling during pregnancy on vitamin intake and status of women and their children. Int J Food Sci Nutr. (2013) 64, 551-60.

IV. Vähämiko, Isolauri, Laiho, Lund, Salminen, Laitinen. The impact of probiotic supplementation during pregnancy on DNA methylation of obesity-related genes in mothers and their children. Submitted.

1. INTRODUCTION

Overweight is a global epidemic affecting about 39 % of the world's adult population, which means 1.9 billion individuals are overweight or obese. The prevalence of obesity has doubled during the last three decades, and World Health Organization (WHO) has declared obesity as one of the top adverse health risk conditions in the world (World Health Organization, 2015; World Health Organization, 2016) (http://www.who.int/mediacentre/factsheets/fs311/en/, 19.2.2016). The increasing prevalence of obesity causes a particular concern because obesity is associated with a large set of adverse health outcomes such as hypertension, lipid disorders, hyperglycemia, insulin resistance, type 2 diabetes and inflammation, which again are associated with increased morbidity and mortality (Correa and Marcinkevage, 2013). Moreover, an American study found that obesity correlates with poorer health-related quality of life compared to normal weight individuals (Kroes et al., 2016). Altogether these factors contribute to increased economic costs in health care, reduced productivity and increased disability rates (McKinnon et al., 2015).

Childhood obesity is one the most serious public health problems in the world. Globally, it was estimated that in 2014 there were 41 million overweight children under five years old in the world (World Health Organization, 2016). Overweight and obese children likely stay obese into adulthood and are more likely to develop obesity-related co-morbidities at a younger age (Hoey, 2014; World Health Organization, 2016). This is why childhood obesity creates a significant economic burden on the society. To stop the increase in both childhood and general obesity, WHO has developed a package of recommendations to address childhood obesity. These include promotions of healthy food, physical activity and weight management during three important life periods: preconception/ pregnancy, early childhood and older childhood into adolescence (World Health Organization, 2016). Indeed, numerous studies have revealed the importance of early life in obesity risk formation. The general population-based Northern Finland Birth Cohort has shown that a long-term overweight parent carries a major risk for having overweight children (Jaaskelainen et al., 2011). In particular, maternal lifestyle factors such as being overweight, obese or gaining excessive weight during pregnancy increase the risk for obesity in offspring's later life (Brenseke et al., 2013; Nathanielsz et al., 2013). This developmental programming phenomenon is widely known as the "Developmental origins of heath and disease"- hypothesis (DOHaD), and it states that chronic non-communicable diseases originate through unbalanced nutrition early in life (Heindel et al., 2015; Portha et al., 2014). However, there are no simple practical methods to halt the rise of obesity and therefore intervention studies are needed to evaluate and endorse favorable developmental programming to ultimately stop the increase of obesity.

2. REVIEW OF THE LITERATURE

2.1 Maternal obesity

Along with the rise in general obesity, also young women have become overweight and obese, which has also increased the rate of maternal obesity. In the USA, over 50% of pregnant women are overweight or obese, and 8% of women in the reproductive age group are morbidly obese (BMI >40). Worldwide, over 30% of women in childbearing age are obese (Zambrano and Nathanielsz, 2013). Globally, Oceania, Europe and the Americas have the highest proportion of overweight and obese women, but countries in Africa and Asia are rapidly catching up with the continuous increase in maternal overweight (Ojha et al., 2015). In Finland, the national FINRISK study has shown that the proportion of obese women has increased during the last four decades in all areas of the country (Borodulin et al., 2015). In 2014, 19.6 % of all Finnish women over 18 years 'old were obese (World Health Organization, 2015).

Maternal obesity increases the risk for adverse health outcomes both in women and their children (Zambrano and Nathanielsz, 2013). In particular, the risk for adverse reproductive health outcomes, such as decreased fertility and increased cases of miscarriages, gestational diabetes, hypertension, preeclampsia, macrosomia, preterm birth, structural birth defects and even fetal death is increased (Correa and Marcinkevage, 2013). Moreover, several human and animal studies have indicated that overnutrition and maternal obesity adversely program the development of offspring by predisposing them to chronic diseases later in life (Brenseke et al., 2013; Nathanielsz et al., 2013). In particular, maternal obesity and / or excessive weight gain during pregnancy are associated with higher newborn birth weights and an increased risk of obesity and diabetes in later life (Ross and Desai, 2013; Zambrano and Nathanielsz, 2013). Indeed, a recent study showed that greater maternal gestational weight gain was associated with greater offspring BMI, and the risk of obesity was doubled in children whose mothers were overweight/obese before pregnancy and gained excessive weight during pregnancy (Guo et al., 2015). Interestingly, the effect of maternal obesity on offspring has been evaluated by comparing the siblings born to obese mothers before and after bariatric gastrointestinal bypass surgery. It was observed that siblings born after the surgery were less overweight and exhibited improved cardiometabolic risk profiles in adulthood compared to siblings born before maternal surgery. The improved health correlated with different DNA methylation patterns of nearly 6000 genes between siblings. The affected genes were mainly from glucoregulation, inflammation or vascular disease pathways (Guenard et al., 2013).

2.2 Developmental programming theory

Historically low birth weight was considered to indicate maternal undernutrition or specific nutrient deficiencies: therefore, several studies have focused on investigating the programming effect of scarcity. One well-known example comes from Dutch Hunger Winter (Schulz, 2010). During the World War II, part of the Netherlands suffered strict famine from November 1944 to May 1945. By studying individuals who were born soon after the famine, researchers concluded that prenatal exposure to famine has long-term consequences in individual's later life depending on the stage of pregnancy when the exposure occurred. Those who were exposed only during late pregnancy were born small and were glucose intolerant compared to non-exposed individuals. Individuals that were exposed in mid-pregnancy were also glucose intolerant, but they also showed markers of reduced renal functions. Interestingly, those who were exposed to famine at early gestation were affected the most. In addition to glucose intolerance, they experienced elevated rates of obesity, altered lipid profiles, increased tendency of breast cancer and cardiovascular diseases in later life (Ravelli et al., 1998; Roseboom et al., 2006; Schulz, 2010) .

The Dutch famine was unique in that it was a brief period of intense deprivation in a well-nourished population whose level of nutrition was soon restored after the famine. Children who were exposed to famine *in utero* were well nourished in childhood and probably had accelerated weight gain in childhood. This means that they faced a "mismatch" between *in utero* and childhood environments and showed clinical symptoms due to famine (Hales and Barker, 2001; Portha et al., 2014; Ravelli et al., 1998). On the contrary, the siege of Leningrad occurred over the prolonged period 1941 – 1944. The population was already malnourished already before the famine started, and they also remained badly nourished after the siege was lifted. Children born during the siege faced the environment which they had adopted *in utero,* and they did not experience accelerated weight gain in childhood. No association between famine exposure and altered glucose-insulin metabolism were found (Hales and Barker, 2001).

Barker and colleagues proposed "The thrifty phenotype hypothesis" (Hales and Barker, 2001; Portha et al., 2014) to describe the developmental programming phenomenon. Their hypothesis suggested that the epidemiological association between poor fetal and infant growth and subsequent development of type 2 diabetes and the metabolic syndrome results from poor nutrition in early life which again produces permanent changes in glucose-insulin metabolism (Hales and Barker, 2001). Central to this hypothesis was that during the times of nutritional deprivation, the growing fetus adopts to maximize its chances of postnatal survival in similar conditions of poor nutrition (Hales and Barker, 2001). Such adaptations include the programming of metabolism in a way that would storage nutrients when they were available, thus helping offspring to survive if the fetus is born into poor nutrition condition such as populations where there is chronic malnutrition. However, if the fetus is born into conditions that differ from those experienced *in utero*, the "mismatch" between

early and later life environments predispose offspring to metabolic diseases in adulthood (Hales and Barker, 2001; Portha et al., 2014).

Another well-known study, the Helsinki Birth Cohort (Barker et al., 2009; Eriksson et al., 2001) evaluated the effect of early childhood on developmental programming and particularly if childhood growth would modify the effect of birth size on the later disease susceptibility. Results suggested that two disadvantageous paths of early growth precede the development of hypertension in adult life. In the first one, low birth weight and low weight gain during infancy are followed by rapid gain in BMI during childhood (catch-up growth). In the second path slow linear growth *in utero* and during infancy followed by persisting small body size (Barker et al., 2009). The first path has been associated with the development of coronary heart disease and metabolic syndrome (Eriksson et al., 2007), and the second path has been associated with the later development of an atherogenic lipid profile and stroke (Eriksson et al., 2000).

Dabelea et al. studied the associations between birth weight and increased risk of type 2 diabetes in Pima Indians, who live along the Gila River in central Arizona and have the world's highest recorded prevalence and incidence of type 2 diabetes (Dabelea et al., 1999). The study with Pima Indian children and young adults showed the U-shaped relationships between birth weight and 2-h glucose concentration, which suggests that both low and high birth weight compared to normal birth weight may increase the risk for type 2 diabetes. Several other studies (Chung et al., 2013; Ferraro et al., 2012; Starling et al., 2015) have shown associations between maternal pre-pregnancy obesity and / or excessive weight gain during pregnancy with higher birth weight and consequential metabolic diseases in offspring.

Today the term developmental plasticity is used to describe the individual's ability to change its phenotype in response to changes in the environment (Brenseke et al., 2013). Developmental plasticity includes all the processes that generate alternative phenotypes from a single genotype due to environmental factors acting during development. If the resulted change or adaptation is permanent, it is considered developmental programming and it is associated with a persistent effect on an individual's structure and/ or function (Gluckman and Hanson, 2007). Based on the current knowledge, it is clear that the nutritional, hormonal and metabolic environment provided by the mother permanently alters organ structure, cellular responses, and gene expression and ultimately impact the metabolism and physiology of her offspring (Ross and Desai, 2013). Further, it seems that effects vary depending upon the developmental period. Indeed, both pregnancy and early childhood seem to create a critical time window for developmental programming, which is often called a window for opportunity. The developmental programming phenomenon is widely known as "Developmental origins of health and disease"- hypothesis (DOHaD), which states that chronic non-communicable diseases including coronary heart disease, stroke, hypertension, type 2 diabetes etc. originate through unbalanced nutrition early in life, and risk is highest when there is a big "mismatch" between early- and later-life environments.

However, the DOHaD-hypothesis has not yet been generally accepted as the official term (Heindel et al., 2015) and various terminologies such as developmental origins hypothesis and metabolic imprinting have been proposed to describe biological phenomena relevant to DoHaD (Portha et al., 2014). Here the term developmental programming is used.

2.3 Mechanisms of developmental programming

The exact molecular mechanisms leading to the developmental programming are still unknown, although the first studies in the field were published almost 40 years ago. Numerous studies have been conducted to find the answer for question how memory of early events is stored and later expressed (Desai et al., 2015). It is likely that multiple mechanisms are involved in developmental programming. Here the role of obesity-associated low-grade inflammation, gut microbiota and epigenetic gene regulation is covered.

2.3.1 Obesity associated low-grade Inflammation in developmental programming

Obesity is associated with the inflammation, which results from excess energy intake and storage of excess nutrients in adipose tissue. The growth of adipose tissue either by increase in fat cell number (adipogenesis) or by increase in fat cell size is involved. Adipogenesis involves the proliferation and differentiation of preadipocytes to adipocytes, whereas the increase in fat cell size results from excess triglyceride accumulation in adipose tissue (Ross and Desai, 2013). Storage of excess nutrients in the adipose tissue results in lipid dysfunction, mitochondrial dysfunction and endoplasmic reticulum stress. Further, it has been suggested that both high-fat and high-caloric feeding may act as chemotactic signal, which leads to macrophage recruitment in the adipose tissue (Gregor and Hotamisligil, 2011; Procaccini et al., 2013; Toubal et al., 2013). It has been proposed that CD8+ T-cells as well as Th1 CD4+ T cells dominate in the adipose tissue and are able to promote the differentiation and function of infiltrated macrophages to M1 macrophages. These macrophages produce large amounts of pro-inflammatory cytokines such as TNF-α, IL-1β ad IL-6 (Winer et al., 2013) and additionally high quantities of reactive oxygen species which induces oxidative stress (Harford et al., 2011). In lean individuals, different sets of T-cells may dominate in adipose tissue and favor the maintenance of M2 macrophages that produce mainly anti-inflammatory cytokines such as IL-10 (Wensveen et al., 2015).

Since adipose tissue is a mixture of different cell types (in addition to adipocytes and macrophages, it contains also endothelial cells, fibroblasts and leucocytes) it is able to rapidly respond to alterations in the nutrient status by producing and secreting a variety of humoral factors which are collectively called adipokines (including many cytokines) (Procaccini et al., 2013). Most of the adipokines, such as TNF-α, leptin and resistin, show pro-inflammatory activity and their concentrations are increased in obesity. Anti-inflammatory adipokines, such as adiponectin, have low concentration in obesity (Nakamura et al., 2014).

The production of adipokines contributes to the development of localized inflammation in adipose tissue but may later lead to the propagation of overall systemic inflammation associated with the development of obesity-related co-morbidities (Nakamura et al., 2014; Procaccini et al., 2013). During pregnancy, obesity-related inflammation directly affects the fetal environment by exposing fetus to increased expression of cytokines and adipokines in circulation. Indeed, maternal inflammation may results in pre-term birth and subsequently lead to low birth weight, which again predisposes a child to be overweight and suffer diseases in adult life (Rogers and Velten, 2011).

To date, more than 600 adipokines have been discovered (Crujeiras et al., 2015). The first one, leptin, was identified in 1994 as a product of the obese-gene. The gene itself was found from mutant mice that suffered from obesity and diabetes and further exhibited reduced physical activity, metabolism and body temperatures. Interestingly, injections of recombinant Ob-protein lowered body weight, body fat and serum glucose and insulin concentrations in these mice (Pelleymounter et al., 1995). These findings encouraged numerous other studies to evaluate leptin's function as a regulator of energy metabolism but still leptin's role is ambiguous. Leptin (16kDa protein) is primarily produced in adipose tissue and secreted into the circulation. Its expression is regulated by various hormones, such as insulin and glucocorticoids (Zhou and Rui, 2013). Circulating leptin concentrations reflect the amount of body fat and are typically highly elevated in overweight and obese individuals (Fantuzzi, 2005). The increased concentration of leptin acts as an adiposity signal that conveys information about stored energy to the brain. In the brain, the hypothalamus senses and integrates leptin's anti-obesity signals and maintains the homeostasis and body weight by controlling energy intake (eating) and expenditure (Zhou and Rui, 2013). Leptin exerts its biological effects by binding to a leptin receptor, which is a type-1 cytokine receptor. It stimulates the transcriptional activity of several genes via the JAK/STAT (Janus kinase /Signal transducers and activators of transcription) pathways (Gautron and Elmquist, 2011). In general, these pathways are critical for the maintenance of many homeostatic and developmental processes, but the chronic activation of JAK-STAT3 is related to the onset of numerous diseases. In obesity, the increased concentration of circulation leptin chronically activates JAK-STAT3 in the central nervous system. This has suggested leading to the development of leptin resistance state in which leptin loses its ability to regulate the balance between energy intake and expenditure (Wunderlich et al., 2013). Both short and long forms of leptin receptor have been identified. Short forms of the receptor are widely expressed in multiple tissues whereas longer form is highly expressed in particular sites within central nervous system (Gautron and Elmquist, 2011). Animal experiment have been conducted to identify the hypothalamic neurons able to respond to leptin. Results from these studies suggest that the arcuate nucleus of the hypothalamus is the site of leptin's action since direct administration of leptin into the arcuate nucleus have shown to reduce short-term feeding (Gautron and Elmquist, 2011).

Diet may influence leptin metabolism and triggering of leptin resistance. In particular, high fat content in the diet has induced both central and peripheral leptin resistance in animal experiments. However, obese animals with low plasma leptin levels remained sensitive to exogenous leptin before and after exposure to a high-fat diet, which suggests that fat content is not the only cause of leptin resistance (Liu et al., 2011). Therefore also the role of sugars have also been evaluated. Low fructose and high-fat diets induce leptin resistance, but animals fed high-fructose and high-sugar diet were sensitive to exogenous leptin administration (Crujeiras et al., 2015; Haring and Harris, 2011; Shapiro et al., 2008).

DNA methylation of proximal region of LEP promoter silences the leptin gene whereas demethylation is able to increase leptin expression. Moreover, methylation of the CpG island in the leptin promoter plays an important role in leptin expression during pre-adipocyte differentiation and further that DNA methylation of the leptin promoter varied with obesity (Crujeiras et al., 2015).

Pregnancy increases leptin concentrations. Indeed, maternal hyperleptinemia may lead to leptin resistance which again would act as a mechanism to ensure the needs of the developing fetus (Tessier et al., 2013). Further, leptin appears to modify insulin sensitivity, glucose and lipid metabolism as well as stress and immune responses during pregnancy but it also regulates placental functions such as nutrient transport (Tessier et al., 2013). Leptin's role in the pregnancy complications has been evaluated in many studies; in overweight pregnancy, leptin concentrations are highly elevated when compared to normal-weight women (Briffa et al., 2015). Also, preeclampsia and gestational diabetes have been shown to increase leptin concentration (Bao et al., 2015; Tessier et al., 2013). Moreover, it has been shown that fetuses from obesity-complicated pregnancies had higher percentages of body fat, *in utero* insulin resistance and elevated cord blood leptin concentrations when compared to lean women (Catalano et al., 2009). To clarify the role of hyperleptinemia in developmental programming further experiments were conducted in mice (Makarova et al., 2013). Those experiments proposed that hyperleptinemia during pregnancy has gender-specific long-term effects on energy balance regulation in progeny, but high leptin concentration itself does not predispose offspring to developing obesity.

2.3.2 Gut microbiota in developmental programming

Recently it was estimated that the human body is composed of $3x10^{13}$ eukaryotic cells and 4×10^{13} colonizing bacteria (Sender et al., 2016). Most of these microbial cells are located within the digestive track (Hooper et al., 2012; Karlsson et al., 2013; Sonnenburg et al., 2016). Up to 1000 different species have been identified up to now, with at least 160 such species per individual (Power et al., 2013; Szajewska, 2013). Many of those species are potential pathogens whereas others maintain health (Szajewska, 2013). Such as reviewed by Power the majority of bacteria belong either to the phylum *Firmicutes* (including *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus*) or to the phylum *Bacteroidetes* (*Bacteroides*

and *Prevotella*), which constitute over 90% of the known phylogenetic categories found in the human intestine (Power et al., 2013). A number of analyses have concluded that microbiota plays a key role in metabolic, physiologic, nutritional and immunological processes and further that microbiota composition varies between healthy and diseased individuals (Power et al., 2013). Animal models have revealed that composition of gut microbiota is related to the development of obesity. Germ-free mice are leaner than their conventional counterparts and their colonization with an intestinal microbiota resulted in a significant increase in body fat content despite lower food consumption (Backhed et al., 2004; Power et al., 2013). Moreover, it has been shown that germ-free mice are protected against obesity when following consumption of a Western-style, high-fat, sugar-rich diet (Backhed et al., 2007; Power et al., 2013). Further, colonization of germ free mice with obese microbiota has been shown to lead to greater increases in total body fat compared to the mice colonized with lean microbiota (Power et al., 2013; Turnbaugh et al., 2006). Altogether these findings suggest that obese microbiota has better capacity to harvest energy from the diet than lean microbiota (Power et al., 2013). Indeed, some studies have linked alterations in intestinal microbiota composition to obesity; an increased ratio of *Firmicutes* : *Bacteroidetes* has been found in genetically obese mice and also in obese humans. This finding has led to the hypothesis that individuals who carry a microbial community with more efficient energy extraction from the diet, or with the increased ability to promote adiposity through manipulation of host genes and metabolism, may be predisposed to obesity (Turnbaugh and Gordon, 2009).

Probiotics are living micro-organisms that can confer health benefits to the host when given in adequate amounts. Among other things, probiotics have been shown to efficiently reduce the duration of diarrhea in acute gastroenteritis, reduce the incidence of antibiotic-associated diarrhea, and also to prevent upper respiratory tract infections (Szajewska, 2013). The role of probiotics in the development of obesity and related co-morbidities has been studied intensively but results are ambiguous. Probiotics have induced weight changes both in human and animals, but the effect seems to be entirely dependent on the species or strain of probiotic used. Comparative meta-analysis of the effect of *Lactobacillus* species on weight gain concluded that *Lactobacillus* acidophilus administration results in weight gain both in humans and in animals (Million et al., 2012). *Lactobacillus gasseri*, in turn, was associated with weigh loss in obese humans (Million et al., 2012). Table 1 introduces studies that represent probiotic interventions with main or secondary outcomes related to obesity in humans. A limited number of probiotic interventions have been carried out during pregnancy and these have mainly aimed to decrease the risk of allergic diseases in offspring (Ou et al., 2012; Pelucchi et al., 2012; Rautava et al., 2012). Table 2 introduces pregnancy-related probiotic interventions which have been conducted with primary or secondary outcomes to modify weight, obesity risk or metabolic health in women or their children.

Table 1. Probiotic interventions aiming to impact on weight in humans.

Reference	Aim	Method	Result
Osterberg et al., 2015.	To determine the effects of probiotics on body and fat mass	Randomized study with probiotic VSL3 or placebo with subsequent consuming of a high-fat diet. Participants underwent dual x -ray absorptiometry to determine body composition	Body mass and fat mass increased less following high-fat diet in the probiotic group compared with placebo
Lee et al., 2014b	To assess the effect of probiotics when combined with herbal medicine in treatment of obesity	A randomized, double-blind, placebo controlled study with participant with higher BMI and waist circumference to receive Bofotsushosan (herbal medicine) with either probiotics or placebo	No significant differences in body composition and metabolic markers were observed
Gobel et al., 2012	To investigate the effect of the probiotic strain *Lactobacillus salivarius* on biomarkers related to inflammation and the metabolic syndrome in adolescent with obesity	Double-blind placebo-controlled trial with adolescents randomized to receive *Lactobacillus salivarius* for 12 weeks	No differences in changes from baseline to 12 week intervention with regard to any anthropometric measures were found
Kadooka et al., 2010	To evaluate the effects of the probiotic *Lactobacillus gasseri* on abdominal adiposity, body weight and other body measures in adults with obese tendencies	Double-blind randomized placebo-controlled intervention	Probiotic *Lactobacillus gasseri* showed lowering effects on abdominal adiposity, body weight and other measures
Kitajima et al., 1997	To investigate the effects of colonization with *Bifidobacterium breve* of the bowels of very low birthweight infants	Prospective randomized clinical study with very low birth weight infants with *Bifidobacterium breve*	*Bifidobacterium breve* can colonize the immature bowel very effectively and is associated with better weight gain in infants
Vendt et al., 2006)	To evaluate the influence of *Lactobacillus rhamnosus* GG enriched formula on growth and fecal microflora during the first 6 months of life in normal healthy infants	Double-blind randomized study with infants receiving LGG-supplemented formula or regular formula	Infants fed with LGG-enriched formula grew better than those fed with regular formula
Chouraqui et al., 2008	To evaluate infant formulas containing probiotics and synbiotics for safety and tolerance	Prospective, controlled, double-blinded, randomized trial with full-term infants with formula containing *Bifidobacterium longum* + *Lactobacillus rhamnosus* or *Bifidobactrerium longum* + *Lactobacillus paracasei*	Infants fed formula containing probiotics or synbiotics showed a similar rate in weight gain as those fed a control formula

Table 2. Pregnancy-related probiotic interventions with primary or secondary outcome to modify women's or their children's weight, obesity risk and /or metabolic health

Reference	Outcome	Method	Result
Obesity and weight			
Ilmonen et al., 2011	To test whether probiotic supplemented dietary counseling influences maternal anthropometric measures during and after pregnancy	Dietary counseling with supplementation of *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis* during preganancy	Waist circumference was decreased by probiotic supplemented dietary counselling, whilst dietary counseling alone did not affected waist cirmumference
Luoto et al., 2010	To evaluate the impact of perinatal probiotic intervention on childhood growth and the development of obesity during a 10 years follow-up period	Double-blinded supplementation of *Lactobacillus rhamnosus GG* or placebo 4 weeks before expected delivery	Probiotics modified children's growth patterns of the child by restraining the weight gain during the first years of life
Glucose metabolism			
Laitinen et al., 2009a	To determine whether probiotics with dietary counseling affects glucose metabolism in normoglycaemic pregnant women	Randomized, prospective dietary counselling with supplementation of *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb12*	Blood glucose concentrations were lowered through dietary counseling with probiotics in normoglycaemic population
Lindsay et al., 2014	To evaluate the effect of probiotic capsules on maternal fasting glucose in obese pregnant women	Placebo-controlled, double-blind randomized trial in obese pregnant women with *Lactobacillus salivarius* capsules	Probiotic capsules did not influence maternal fasting glucose, the metabolic profile, or pregnancy outcomes in obese women
Lindsay et al., 2015)	To investigate the effect of probiotics capsule intervention on maternal parameters and pregnancy outcomes among women with gestational diabetes	Double-blind placebo-controlled randomized trial of daily *Lactobacillus salivarius* or placebo capsules in women with newly diagnosed with gestational diabetes or impaired glucose tolerance	Probiotic capsules among women with abnormal glucose tolerance had no impact on glycemic control

Reference	Outcome	Method	Result
Asemi et al., 2013	To determine the effects of daily consumption of probiotic yogurt on insulin resistance and serum insulin levels on pregnant women	Randomized controlled trial of daily consumption of commercially available yogurt (prepared with starter cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and enriched with probiotic culture of *Lactobacillus acidophilus* and *Bifidobacterium animalis*) for nine weeks during the third trimester of pregnancy	In contrast to conventional yogurt, daily consumption of probiotic yogurt maintains serum insulin levels
Taghizadeh and Asemi, 2014	To determine the effect of symbiotic food consumption on glycemic status and serum high sensitivity C-reactive protein on pregnant women	Randomized, placebo-controlled clinical trial. Synbiotic food consisted of a probiotic *Lactobacillus sporogenes* and inulin	Synbiotic food consumption decreased serum insulin levels and HOMA-IR and increased QUICKI but did not affect hs-CRP
Preeclampsia			
Brantsaeter et al., 2011a	To test whether consumption of milk-based probiotic products during pregnancy impacted on the development of preeclampsia	Prospective Norwegian mother and child cohort study, n=33 399. Self-reported food frequency questionnaire	Intake of probiotic milk products was associated with reduced risk of preeclampsia
Preterm delivery			
Myhre et al., 2011	To test whether the intake of food with probiotics reduces pregnancy complications and risk for spontaneous preterm birth	Prospective Norwegian Mother and Child Cohort. Self-reported food frequency questionnaire.	Women with habitual intake of probiotic dairy products had a reduced risk of spontaneous preterm delivery
Safety			
Lee et al., 2012	To assess the effects of probiotic supplementation during pregnancy on adverse pregnancy outcomes	Prospective probiotic study of *Lactobacillus* supplementation in early pregnancy	No association was identified between ingestion of *Lactobacillus* in early pregnancy and adverse pregnancy outcomes

Reference	Outcome	Method	Result
Other			
Asemi et al., 2012a	To determine the effects of daily consumption of probiotic yogurt on oxidative stress among pregnant women	Randomized single-blind controlled clinical trial with commercially available product prepared with starter cultures of *Streptococcus thermophilus* and *Lactobacillus bulgarius*, enriched with *Lactobacillus acidophilus* and *Bifidobacterium animalis*	Consumption of probiotic yogurt among pregnant women resulted in increased level of erythrocyte glutathione reductase compared to conventional yogurt
Asemi et al., 2012b	To determine the effects of daily consumption of probiotic yogurt on lipid profiles in pregnant women	Randomized single-blind controlled clinical trial with commercially available product prepared with starter cultures of *Streptococcus thermophilus* and *Lactobacillus bulgarius*, enriched with *Lactobacillus acidophilus* and *Bifidobacterium animalis*	Consumption of probiotic yogurt among pregnant women did not affect serum lipid profiles when compared to the conventional yogurt
Taghizadeh et al., 2014	To evaluate the effects of daily consumption of a synbiotic food on blood lipid profiles and biomarkers of oxidative stress in pregnant women	Randomized, double-blinded controlled clinical trial performed among pregnant women. The synbiotic food consisted of a probiotic viable and heat-resistant *Lactobacillus sporogenes* and inulin	Consumption of synbiotic food decreased serum triacylglyserols and increase in plasma total glutathione compared the control food. No difference was detected in total cholesterol, Low density lipoprotein, High density lipoprotein or plasma total antioxidant capacity

2.3.3 Epigenetics in developmental programming

Recently, it has been suggested that epigenetic gene regulation mechanisms also participate in developmental programming (Desai et al., 2015; Li et al., 2013). The term epigenetics is used to describe the modifications of the genome that do not alter the DNA sequence including the mechanisms such as DNA methylation, histone modifications and noncoding RNAs (Ross and Desai, 2013). Histone modifications and noncoding RNAs and their function as epigenetic mechanisms are not yet very well characterized. Histones are proteins that participate in the packaging of DNA in the nucleus. DNA is wrapped around an octamer of core histone proteins (H2A, H2B, H3 and H4), which form nucleosome. Post-transcriptional modifications such as acetylation, methylation, phosphorylation, ubiquitinylation and SUMOylation at the N-terminal tails of histones contribute to the genomic stability and thereby affect the availability of DNA for transcription (Lee, 2015). Noncoding RNAs, in turn, represent a huge fraction of human genome that are transcribed to short- or noncoding-RNAs but are not translated into proteins. They act mainly to regulate gene expression either at transcriptional or translational level (Desai et al., 2015).

For now DNA methylation is the best characterized epigenetic modification of chromatin (Li et al., 2013). It is a covalent addition of methyl group by methyltransferases to cytosine base at the 5'position of a cytidine preceding a guanosine resulting the formation of 5-methylcytosine in DNA (Gopalakrishnan et al., 2008; Li et al., 2013). The CpG dinucleotide methylation is generally known to lead to transcriptional repression because it inhibits binding of transcription factor and co-activators to DNA. These CpG dinucleotides are often clustered in short stretches of DNA known as CpG islands (Takahashi, 2013). During early embryogenesis both maternal and paternal chromosomes undergo progressive demethylation which erases most of the epigenetics marks in the zygote (Li et al., 2013). After implantation, global embryonic methylation patterns are reestablished and then supposed to be maintained throughout the life in the somatic cells (Deaton and Bird, 2011; Li et al., 2013). The dynamic methylation reprogramming process is essential for fetal development since it is involved in various normal biological processes such as cell differentiation, organogenesis and genomic imprinting (Gopalakrishnan et al., 2008; Li et al., 2013; Takahashi, 2013). Emerging evidence, however, suggest that maternal lifestyle factors as well as other environmental factors during embryogenesis and childhood may affect epigenetic processes that lead to epigenetic alterations (Takahashi, 2013). These epigenetic alterations may lead to embryonic lethality, developmental malformations, or influence long-term health (Takahashi, 2013). During postnatal and adult life, DNA methylation is susceptible to both intrinsic and extrinsic factors and with aging there is global loss of DNA methylation (Desai et al., 2015).

Dietary factors may induce epigenetic changes, which again may permanently alter the phenotype in the adult organism (Chango and Pogribny, 2015). In genomic DNA methylation, the methyl group originates from the universal methyl donor, S-adonosyl-

L-methionine (AdoMet). Dietary factors such as amino acids e.g. glycine, histidine, methionine and serine and vitamins such as B_6, B_{12} and folate play a key role in DNA methylation by donating the methyl group to AdoMet (Chango and Pogribny, 2015; Ji et al., 2015). Methyl group deficiency resulting from dietary methyl source inadequacies, and /or other lifestyle factors (e.g. alcohol, tobacco and stress), can lead to global and/or specific DNA methylation changes (Chango and Pogribny, 2015). One well-known example comes from Agouti mice (Wolff et al., 1998). The mouse agouti alleles, A^w and A, regulate the alternative production of black and yellow pigment in hair follicles. Transcription of this gene occurs only in the skin during the short period at the beginning of each hair growth cycle. When pregnant black agouti dams were fed with methyl-supplemented diet, their offspring's coat color was changed to yellow as a consequence of altered epigenetic regulation (Wolff et al., 1998).

Dietary folate is one of the main sources of methyl groups required for DNA methylation: therefore, various studies have been conducted to analyze the effects of folic acid supplementation to DNA methylation. Increased folate intake during pregnancy is necessary to accommodate both fetal development and placental functionality (Chango and Pogribny, 2015), but still results concerning optimal folic acid supplementation are still conflicting. Experiments in mice have suggested that high gestational folic acid supplementation may alter expression of imprinted genes (Barua et al., 2015). Further, maternal high folic acid supplementation in mice has promoted glucose intolerance and insulin resistance in male offspring (Huang et al., 2014). In contrast, it has been suggested that chronic dietary lack of methyl donors in mice during the developmental period may affect learning, memory and gene expression on the hippocampus (Tomizawa et al., 2015). Also, human studies have thus far produced rather confusing results. Folate deficiency has been connected with increasing neural tube defect risk (De-Regil et al., 2015) and associated with lung cancer (Tastekin et al., 2015). Moreover, maternal folate status has influenced the risk of autism spectrum disorders in offspring (DeVilbiss et al., 2015). High dose folic acid supplementation, in turn, has been associated with altered epigenomes in human sperm (Aarabi et al., 2015). The generation R study from the Netherlands evaluated the effects of vitamins B_6 and B_{12}, folate, folic acid and methionine for measures of growth and body composition at six years of age. They concluded that early high folic acid intake was associated with a lower body weight and BMI at the age of 6 years. In contrast, early high methionine intake was associated with unfavorable body composition at the same age. Another study with population-based birth cohort from the Netherlands studied the effect of maternal folic acid supplementation to the methylation status of newborn. An association was observed between maternal folate deficiency and lower newborn DNA methylation (van Mil et al., 2014). A deficiency of vitamin B12, in turn, was recently suggested as an important metabolic risk factor due to its ability to epigenetically regulate cholesterol biosynthesis pathways in human adipocytes (Adaikalakoteswari et al., 2015).

In addition to methyl-donors, recent studies have evaluated the role of other dietary and lifestyle factors in DNA methylation. An American study evaluated the effect of lifestyle modification on DNA methylation. In particular, one study aimed to achieve weight loss by encouraging physical activity and reducing caloric intake. Global DNA methylation and LINE 1- (Long interspersed element 1) gene methylation were followed. Researchers found significant elevation in LINE-1 methylation both at 6 and 12 months and changes in body fat percentage and plasma glucose concentrations were positively associated with LINE-1 DNA methylation. The changes in global DNA methylation were detectable at 6 and 12 months after the intervention was started (Delgado-Cruzata et al., 2015). A recent animal study, in turn, evaluated the effects of maternal high-lipid, high-energy diet on the DNA methylation patterns in mice offspring. They concluded that global DNA methylation profile in adult offspring livers was changed by the maternal diet during gestation and lactation (Yu et al., 2015).

Very few human studies have aimed at modifying DNA methylation via intervention during early life. Lee et al. aimed to test whether prenatal dietary supplementation with omega-3-PUFA during pregnancy would modulate epigenetic states in the infant immune system. They found no differences in global methylation status of promoter region methylation. Instead, they found that omega-3 PUFA supplementation was associated with changes in methylation levels in LINE1 repetitive elements in infants of mothers who smoked during pregnancy (Lee et al., 2013). Moreover, it was studied the effect of prenatal dietary supplementation of docosahexaenoic acid (DHA). The study found that methylation level in IGF 2 gene promoter 3 was significantly higher in the DHA group compared to control group of preterm infants. They also found a positive association between DNA methylation levels of IGF2/H19 imprinted regions and maternal body mass index (Lee et al., 2014a). Lind et al., in turn, conducted a randomized trial in which nine months of infants received fish oil or sunflower oil for 9 months. In a subset of 12 children, genome-wide differences in methylation between the groups were analyzed. The study revealed that 43 CpG-sites appeared to be modified. The methylation levels at these sites were associated with phenotypic changes mainly in blood pressure (Lind et al., 2015).

Taken together it seems that the developmental programming phenomenon leads to increased obesity risk which results from multiple different mechanisms that all may be affected by a complex set of maternal and environmental factors. Maternal overweight / obesity, diet, and gut microbiome may all cause metabolic and/or epigenetic changes that contribute to offspring's obesity risk. So far it has remained obscure whether dietary interventions during pregnancy may endorse healthy developmental programming and stop the vicious cycle of obesity. It is also not known whether obesity risk is associated with the DNA methylation of certain genes.

3. AIMS OF THE STUDY

The main objective of the present study was to evaluate the impact of maternal diet, dietary counseling and the use of probiotics during pregnancy in endorsing favorable developmental programming. In particular, leptin concentration was used as a marker for obesity-associated low-grade inflammation, antioxidant vitamin status as an efficiency marker for dietary counselling, and epigenetic DNA methylation of obesity-related genes as a marker for probiotics influence. Moreover, leptin concentration was followed to evaluate the efficacy of probiotics to decrease obesity-related low-degree inflammation.

The specific aims in this thesis were:

1. To analyze the extent to which obesity-induced inflammation as manifested by leptin concentrations is related with aberrations in glucose metabolism during pregnancy (I).

2. To analyze the factors (dietary counseling, probiotic intervention, dietary intake, maternal characteristics) explaining leptin concentrations in women and their children (II).

3. To analyze the dietary counseling's potential for improving women's dietary intake of antioxidant vitamins and analyze explaining factors for vitamin status of women and their children (III).

4. To analyze if probiotics modify the epigenetic methylation of obesity-related genes in women and their children (IV).

4. SUBJECTS AND METHODS

4.1 Subjects and study design

Altogether 256 pregnant women were recruited to a prospective, randomized mother and infant nutrition and probiotic study (NCT00167700; section 3, http://www.clinicaltrials.gov) which was conducted in Southwest Finland between April 2002 and November 2004 (Figure 1). Criteria for inclusion: mother must be over 18 years old and in the early stages of pregnancy (less than 17 weeks) with no chronic diseases (except allergy). Women were informed about the study in the maternal welfare clinics and interested women contacted the study nurse for an appointment at the study clinic in the Turku University Hospital. At the first study visit the women were randomized into three parallel groups. One group received dietary counseling with probiotics, another group received dietary counseling with placebo, and the third group was control with placebo. Randomization was conducted via computer-generated block randomization and it was based on the randomization list which was drawn up by statistician, who was not involved in the study visits. The sample size calculations were based on infant atopic sensitization studies (Kalliomaki et al., 2001). The randomization code was opened after all the infants had completed the 12-month follow-up. Women in the dietary counseling groups received individualized nutritional advice during the study visits. In addition they received in a double-blind manner either probiotic capsules or placebo. Women in the control group received placebo capsules in a single-blind manner. Participants visited the study clinic at the first, second and third trimester of pregnancy and again with their infants at one, six and twelve months of age.

A subsample of 85 pregnant women and their children from the control group was taken to participate in the first leptin study (study I). To increase the amount of overweight participants, additional participants (n=18) were recruited from maternal welfare clinics in the Southwest Finland area and from the prenatal outpatient ward at Turku University Hospital. The inclusion criteria for overweight women recruited were pregnancy at third trimester and no metabolic diseases (Figure 1). A subsample of 89 women was taken in the consecutive order to participate in vitamin study (III). Of these women, 44 were from the dietary counseling group and 45 from the control group. Both groups received placebo. The sample size was determined based on the current literature at the time. A subsample of 15 women and their children was taken in a consecutive order to participate in DNA methylation study. Only mother-child pairs with blood samples from both mother and child at the same time point when the child was from 6 to 12 months of age were included. All women received dietary counseling; seven with probiotic supplementation and eight with placebo.

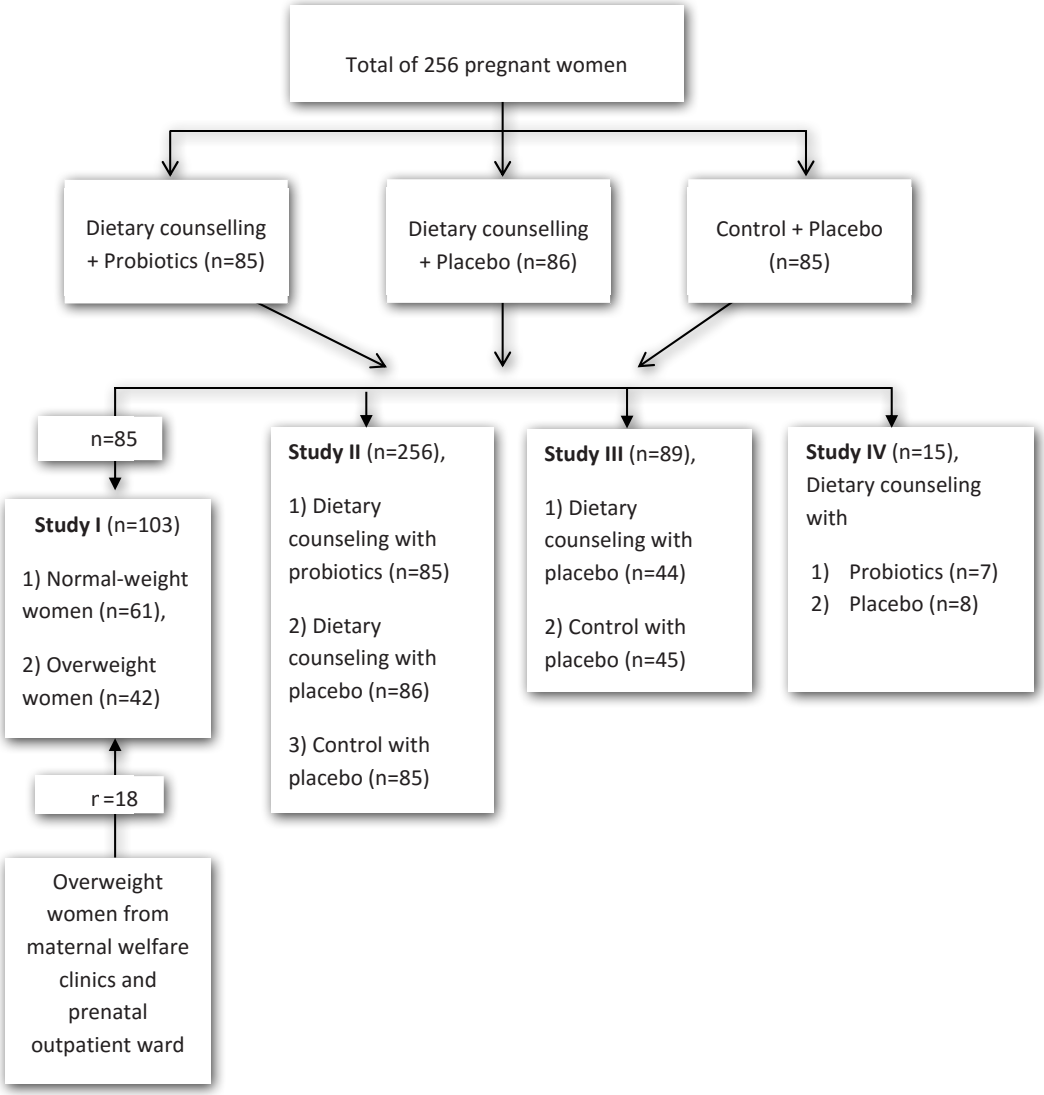

Figure 1. Study design

4.2 Clinical evaluation

Clinical data was collected during study visits at the study clinic. Information on age, parity, smoking, education and breastfeeding were obtained by interview at the first study visit. Self-reported weight prior to pregnancy was recorded at the first visit to a maternal welfare clinic and used in calculation of pre-pregnancy BMI as weight (kg) divided by the square of height (m^2). A study nurse measured weight during pregnancy at every study visit. Height was measured at the first study visit. Weight before delivery was recorded at the last prenatal visit to a maternal clinic or in hospital within one week before delivery and was

used to determine total weight gain during pregnancy. In study I, women were identified as normal weight, if pre-pregnancy BMI was less than 25, or overweight if pre-pregnancy BMI exceeded 25. In study II, women's weight gain during pregnancy was classified as normal, excessive, or low. Normal weight gain was defined to be 12.5-18.0 kg for women with pre-pregnancy BMI below 19.9, 11.5-16.0 kg for those with pre-pregnancy BMI from 19.8 to 26.0, and 7-11.5 kg for those with BMI above 26.0 prior to pregnancy. Infants' weights at birth were obtained from hospital records. At one, six, and twelve months of age, the study nurse measured the infants' weight.

4.3 Maternal dietary and probiotic intervention

In studies II-IV the dietary counseling groups received detailed individual dietary counseling at every study visit. Dietary counseling was aimed to modify the women's dietary intake to comply with the recommended intake for pregnant women. Specifically, counseling aimed to affect the type of fat used and to increase the amount of fiber in the women's diets. Furthermore, women were encouraged to consume more vegetables, fruits, wholegrain bread, cereals, leaner meat products, low-fat cheese, and milk products as well as vegetable oils and margarines. Fish was recommended as one of the main meals twice a week.

The participants in the dietary counseling group were advised about healthy options to consume at home. Products with favorable fat and fiber contents were given as examples as part of the recommended diet and to motivate mothers to make healthier food choices. These products were normally available on the market (e.g., rapeseed oil-based spreads including plant stanol ester spreads, salad dressing and fiber-enriched pasta, muesli and cereals). The consumption of these products was fully voluntary. Women were given a recommendation on the amount of the products to be used daily, but it was not followed how much participants used them. Dietary counselling was provided at every study visit, and products were provided until the mother discontinued exclusive breastfeeding (maximum time 6 months).

Women in the probiotic group in the studies II and IV received in a double-blind manner probiotic capsules that contained *Lactobacillus rhamnosus GG* (American Type Culture Collection 53 103; Valio Limited) and *Bifidobacterium lactis Bb 12* (Chr.Hansen), 10^9 colony-forming units/d each. Women in the control groups received placebo capsules that contained microcrystalline cellulose and dextrose anhydrate but no nutrients in a single-blind manner. Probiotic supplementation started at the first study visit and continued until the mother stopped the exclusively breastfeeding or at a maximum of 6 months postpartum.

4.4 Food records

Food and nutrient intakes were evaluated using 3-day food records that included one weekend day. The records were collected at the first and third trimester of pregnancy and

one month postpartum. Daily energy and nutrient intakes were calculated using the Micro-Nutrica computerized programme version 2.5 (Reseach Center of the Social Insurance Institution, Turku, Finland). The programme was continuously updated.

4.5 Sampling and analytical methods

4.5.1 Sampling

In study I blood samples were taken at the third trimester of pregnancy and one month postpartum after overnight fasting and measured for glucose, insulin and leptin concentration levels. In study II venous blood samples were taken from women after overnight fasting at the first and third trimester of pregnancy and at one month postpartum. Children's blood samples were taken at one month of age without fasting. Serum samples were stored at -70°C until the leptin measurements. In study III venous blood samples were taken at the first and third trimester of pregnancy and one month postpartum after overnight fasting. Colostrum samples were collected on the maternity ward and mature milk samples were collected at home one month postpartum. For vitamin C analysis plasma samples were collected in a tube with 5% metaphosphoric acid and then frozen immediately at -20°C. All other samples for vitamin concentration measurements were transferred to - 70°C within one week from sampling and stored there until the analysis of vitamins. In study IV blood samples were taken without fasting from mothers and their children at the same time point when children were at the age from 6 to 12 months of age. Whole blood samples were stored in EDTA at -70°C until the analysis of DNA methylation.

4.5.2 Leptin concentration measurements

In study I serum leptin concentrations were measured using I-radioimmunoassay (Human leptin RIA kit, 125 tubes LINCO Research Inc. St Charles, MO, USA). Measurements were carried out at the Department of Pharmacology, Drug Development and Therapeutics at the University of Turku. Analyses were performed according to manufacturer's instructions, with appropriate quality controls included.

In study II leptin analyses were carried out in the NIHR Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory. Analyses were performed with an in-house, two-site microtiter plate-based, time-resolved fluorometric (dissociation-enhanced lanthanide fluorescent immunoassay, DELFIA) assay (Perkin-Elmer) using DELFIA multibuffer as an assay buffer and as a diluent. Assays were run in duplicate in 20 µl of sample per well and dilution series of human recombinant leptin was used as a calibrator. The lower limit of detection was 0.1 ng/ml, and between-batch imprecision was 7.1% at 2.7 ng/ml, 3.9% at 14.9 mg/ml and 5.7% at 54 ng/ml. Antibodies and standards were purchased from R&D Systems Europe.

4.5.3 Glucose and insulin concentration measurements

In study I glucose and insulin concentrations were measured out in TYKSLAB of Hospital District of Southwest Finland at Turku University Hospital according to the manufacturer's instructions, with appropriate quality controls included. The glucose concentrations were measured from plasma on the day of sampling with Modular P800 automatic analyzer (Roche Diagnostics GMbH, Mannheim, Germany). An enzymatic method utilizing hexokinase was applied. Insulin measurements from serum samples (stored at -70° C) were carried out with an immunoelectrochemiluminometric assay on a modular E170 automatic analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

4.5.4 Insulin sensitivity and insulin resistance determinations

In study I insulin sensitivity was determined using the quantitative insulin check index (QUICKI) (Katz et al., 2000) and insulin resistance by homeostasis model assessment (HOMA) (Matthews et al., 1985). QUICKI was determined using the formula QUICKI=1/ ((LG10 (insulin concentration)) + (LG10 (fasting glucose concentration X 18 / 1.1))). HOMA was determined by using the formula HOMA= (fasting insulin concentration X fasting glucose concentration)/22.5).

4.5.5 Vitamin concentration measurements from blood samples

In study III serum concentrations of vitamins A, C, D, E, E /cholesterol and beta-carotene were carried out in United Laboratories Ltd, Helsinki within one month from sampling.

The serum concentrations of vitamins A and E were measured after deproteinization with ethanol and extraction with hexane. The hexane phase was evaporated and the residue reconstituted with diethyl ester and methanol. After that HPLC analysis was performed by isocratic elution with methanol. Symmetry C 18 columns (Waters, Milford Massachusetts, USA) were used with a detection wavelength 290 nm. Retinol acetate and tocoferol acetate were used as internal standards.

The vitamin C concentrations were measured from samples that were collected in a tube with metaphosphoric acid. After rapid centrifugation the supernatant was injected into HPLC via auto sampler vial. The column used was a Discovery RP Amide C16 (Supelco, Bellafonte, Pennsylvania, USA). Detection wavelength used was 254 nm and quantification was performed by external standard method.

Serum vitamin D measurements were performed with the DiaSorin 25-hydroxyvitamin D assay. The samples were measured after the extraction with acetonitrile using an equilibrium radioimmunoassay procedure with 25-hydroxyvitamin D125 iodine.

Serum carotenoid concentrations were measured after extraction with ethanol and hexane. Separation of carotenoids was performed with Nova-Pak C18 column (Waters, Milford, Massachusetts, USA) with a detection wavelength of 436 nm. Carotene was quantified using ethyl β-apo-8'carotenoate as internal standard.

All HPLC analyses were made using a Hewlett-Packard HP 1100 high-performance liquid chromatograph with a diode-array detector. Data acquisition, peak integration and spectral analysis were performed with an Agilent ChemStation data system.

To determine the serum concentration of fat-soluble vitamin E, the total plasma cholesterol concentration was measured with an enzymatic method utilizing cholesterol esterase in a Modular P800 automatic analyzer.

4.5.6 Vitamin concentration measurements from breast milk

In study III breast milk concentrations of vitamins A, C, E and beta-carotene were measured in United Laboratories Ltd, Helsinki within one month from sampling. Vitamins A, E and beta-carotene were measured after sonification, saponification and liquid-liquid extraction with hexane. Butylated hydroxytoluene was used as antioxidant and ethyl β-apo-8'-carotenoate as an internal standard. The vitamins were resolved with a reverse-phase column Discovery RP Amide C16 (Supelco, Bellefonte, Pennsylvania, USA) with the detection wavelength for retinol 325 nm, for tocopherol 294 nm and for beta-carotene and ethyl β-apo-8'-carotenoate 436 nm. Quantification was done by the internal standard method and comparison of the UV-spectra of vitamin peaks with those of pure vitamins diluted in ethanol. Vitamin C concentrations were measured in a similar way than in the case of blood samples.

All HPLC analyses were made using a Hewlett-Packard HP 1100 high-performance liquid chromatograph with a diode-array detector. Data acquisition, peak integration and spectral analysis were performed with an Agilent ChemStation data system.

To analyze the concentration of fat-soluble vitamin E, total triacylglycerol were analyzed by gas chromatography (PerkinElmer, AutoSystem, Norwalk, CT) with a DB-23 column (Agilent Technologies, Palo Alto, CA). The concentration was determined using triheneicosanoin (Sigma Chemical Co.–Aldrich) as internal standard

Non-homogenized milk was used in the preparation of standards.

4.5.7 DNA methylation profiling

In study IV, DNA methylation profiling was carried out at the Finnish Microarray and Sequencing Center (FMSC, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University). For DNA methylation profiling, the genomic DNA was extracted from the EDTA blood sample with a QIAamp DNA Blood Maxi kit (Qiagen). From each sample, 5 µg of genomic DNA was sheared with a Covaris S2 sonicator for 10 min (Duty cycle 10; Intensity 5; Cycles/burst 100) into an average fragment size of 150 bp, as determined with an Agilent 2100 Bioanalyzer and Bioanalyzer High Sensitivity DNA kit. The methylated DNA was enriched with a MethylMiner™ Methylated DNA Enrichment kit (Invitrogen) by following the high-salt (2 M NaCl) single elution workflow as described in the kit manual. For the

next-generation sequencing, 500 ng of enriched methylated DNA was processed with a SOLiD Fragment Library Construction kit (Life Technologies) according to the kit manual. The double-stranded DNA fragments were subjected to end-repair, which was followed by adaptor ligation, nick-translation and PCR amplification. The SOLiD™ Fragment Library Barcoding Kit Module 1–16 (Life Technologies) was used for multiplexing the samples. The libraries were purified with AMPure XP beads (Agencourt) and size selected from 1% agarose gel to collect 150 - 300 bp fragments. A qiaquick gel extraction kit (Qiagen) was used to purify the size-selected libraries. The size distribution of the libraries was determined with a Bioanalyzer DNA 1000 kit. The quantity of the libraries was measured with both a Qubit and SOLiD Library TaqMan Quantitation Kit (Life Technologies). Equal amounts of barcoded libraries were pooled for multiplexed sequencing. The bead preparation was carried out according to the SOLiD4 System Templated Bead Preparation Guide. A SOLiD™ EZ Bead™ System was used for automated templated bead preparation. The libraries were run with a SOLiD4 or SOLiD 5500XL Sequencer (Life Technologies) with 50 bp chemistry.

4.5.8 DNA methylation data analysis

In study IV, the raw sequence data was mapped to hg19 reference genome sequence with Life Technologies Bioscope (version 2.0) software using the default parameters which yielded on average 41.8M mapped reads per sample (stdev 8.84M reads). The read counts for proximal promoters (region between 1000 bp upstream and 500 bp downstream TSS, coordinates derived from Refseq gene annotations) were calculated using bedtools (version 2.17.0). Functional enrichment analysis toward the GO and KEGG databases was carried out using the topGO and GOstats packages in R/Bioconductor. Alterations in the DNA methylation status of 623 obesity and 433 weight gain associated genes were visualized and functional associations were examined with the Ingenuity Pathway Analysis Tool (Ingenuity Systems).

4.6 Statistical analysis

Characteristics of the women and their children are shown as means with range or as proportion of study subjects. Independent samples t-test was used to compare the differences between the groups and paired samples t-test when comparing changes between time points. Chi-Square or Fisher's test was used to analyze categorical variables. Correlations were assessed using Pearson's correlation coefficients. Univariate analysis ANOVA and multivariate analysis of variance MANOVA were used to compare the groups in the DNA methylation study.

Dietary intakes of energy and nutrients are shown as the intake during pregnancy (mean of first, second, and third trimester) and after pregnancy (mean of 1 and 6 months postpartum). ANOVA was used to analyze the differences between the groups. ANCOVA was used to compare the differences in the women's serum leptin concentrations between

the groups. Baseline leptin concentration at the first trimester of pregnancy was included as a covariate, and results are shown as geometric means with 95% CI. ANOVA was used to compare children's serum leptin concentration and results are given as geometric means with 95% CI. ANCOVA for repeated measures for women and ANOVA for repeated measures for children were used to study within subject changes in serum leptin concentrations. To identify explanatory factors for serum leptin concentrations, Pearson's correlations were first used. Then ANOVA was used to study differences in women's characteristics between the groups. The associations between dietary intake and leptin concentration were studied by dividing women into tertiles according to their intakes of energy and nutrients. ANOVA was then used to study differences between the tertiles. Thereafter multivariate forward stepwise regression models were applied. Interventions were forced to the models but for other factor the criterion for entry was $P<0.10$ (study II).

ANCOVA for repeated measures was used to compare the differences in dietary intakes and maternal serum vitamin concentrations between the groups. The baseline dietary intake or vitamin concentration was included as a covariate. Results are given as baseline-adjusted marginal means with 95% CI. ANOVA for repeated measures was used to study within-subject changes in women's serum vitamin concentrations. ANOVA was used to compare vitamin concentrations in children, in colostrum and in mature milk between the groups. Values were logarithmically transformed before analysis. Results are given as geometric means with 95% CI and due to logarithmic transformation the rations intervention/control were used for group comparisons. To study the associations between maternal characteristics and serum vitamin concentrations further and identify potential explanatory factors for serum concentrations in women and children, the Pearson's correlations and ANOVA were used. Thereafter the multivariate forward stepwise regression models were applied. The intervention for forced to all models but for other factors the criterion for entry to the model was $P<0.10$ (study III).

Statistical analysis for comparing differentially methylated promoter regions between sample groups was carried out using R/Bioconductor limma package on TMM normalised and voom transformed count values as suggested in the limma manual. The promoters with an absolute fold-change above 2 and modified t-test p-value below 0.05 were listed as significantly differentially methylated (study IV).

4.7 Ethics

All applicable institutional and governmental instructions concerning the ethical use of human volunteers were followed during this research. The study is registered as clinical study (NCT00167000; section 3, http://www.clinicaltrials.gov) and it follows the format and requirements set for clinical trials. All studies were approved by the Ethical Committee of the Hospital District of Southwest Finland, and written informed consent was obtained from all participants before enrollment.

5. RESULTS

5.1 Clinical characteristics of the women and their children

The clinical characteristics of the women and their children are presented in Table 3. All women were Caucasian and in good health. The majority of the women were university or college educated and gave a birth after the normal pregnancies. Study populations in sub-studies represent the whole study population in the nutrition and probiotic study, except in study I where women's pre-pregnancy BMI was higher. Clinical characteristics in study groups in sub-studies have been shown in original publications (Vahamiko et al., 2010; Vahamiko et al., 2013a; Vahamiko et al., 2013b). In general there were no differences in clinical characteristics between study groups in sub-studies except in study I where there was a significant difference in pre-pregnancy weight and BMI between the groups.

Table 3. Clinical characteristics of the women and their children in Studies I-IV (mean with range)

	Whole study population in nutrition and probiotic study n=256	Obese mothers recruited from maternal welfare clinics and prenatal outpatient ward in study I n=18	Study population in study I n=103	Study population in study II n=256	Study population in study III n=89	Study population in study IV n=15
Mother						
Age at the recruitment	30.0 (17.6-44.2)	30.1 (22.9-41.6)	30.3 (18.3-42.2)	30.0 (17.6-44.2)	30.4 (18.5-44.2)	29.0 (21.5-35.5)
Pre-pregnancy BMI, kg/m^2	23.6 (17.4-40.8)	32.1(23.1-44.8)	25.2 (18.0-44.8)	23.6 (17.4-40.8)	23.7 (18.2-34.9)	23.2 (18.6-32.7)
Pregnancy weight gain, kg	14.9 (-0.60-26.8)	10.3 (-1.7-20.0)	14.1 (-1.7-26.1)	14.9 (-0.60-26.8)	15.2 (5.7-26.5)	14.4 (6.9-22.0)
Children						
Sex, male (%)	53.2	46.7	54.3	53.2	57.5	53.3
Birth weight, kg	3.6 (1.6-4.8)	3.480 (1.925-4.340)	3.6 (1.9-4.7)	3.6 (1.6-4.8)	3.6 (1.6-4.7)	3.8 (3.0-4.4)
Birth height, cm	51.0 (44.0-57.0)	50.39(44.0-54.0)	50.9 (44-57)	51.0 (44.0-57.0)	51.0 (44.0-55.0)	51.7 (48.0-55.0)

5.2 Women's dietary intake of energy and nutrients

Food diaries were used to evaluate women's dietary intakes in all studies. In study I, participants did not receive dietary counseling, but a portion of the women in studies II and III and all of the women in the study IV received detailed individual dietary counseling. Women's dietary energy intake and energy yielding nutrients have been reported elsewhere (Piirainen et al., 2006) and are shown also in the original publications (Vahamiko et al., 2010; Vahamiko et al., 2013a; Vahamiko et al., 2013b). The intakes of methyl-donors in study IV are shown in Table 4. There was no significant difference in the intakes of methyl-donors between the groups.

Table 4. Dietary intake of methyl-donors in women (modified from original publication IV)

			Placebo (n=8)	Probiotics (n=7)
			Mean (range)	Mean (range)
Folate	µg	1st tri	296.2 (231.6 – 360.9)	317.6 (248.5 – 386.7)
	3rd tri	287.7 (251.0 – 324.5)	299.1 (259.8 – 338.4)	
	1 month pp	284.3 (214.0 – 354.6)	301.7 (226.6 – 376.9)	
Riboflavin	mg	1st tri	2.2 (1.7 – 2.6)	1.9 (1.3 – 2.5)
	3rd tri	2.0 (1.6 – 2.4)	2.1 (1.7 – 2.5)	
	1 month pp	2.3 (1.8 – 2.7)	2.1 (1.6 – 2.6)	
B6	mg	1st tri	2.2 (1.7 – 2.6)	2.3 (1.8 – 2.8)
	3rd tri	2.0 (1.7 – 2.3)	2.3 (2.0 - 2.7)	
	1 month pp	2.6 (1.4 – 3.7)	2.4 (1.2 – 3.7	
B12	µg	1st tri	4.9 (3.7 – 6.2)	6.0 (4.7 – 7.3)
		3rd tri	5.7 (4.8 – 6.7)	6.2 (5.2 – 7.3)
		1 month pp	6.9 (3.6 – 10.2)	7.4 (3.9 – 10.9)

5.3 The impact of being overweight on serum leptin concentration in pregnant women

To determine the potential of leptin concentration for indicating obesity-related inflammation, the serum leptin concentrations were measured in normal-weight and overweight women during and after pregnancy in study I. Pregnancy was found to increase the serum leptin concentration, and it was also found to be significantly higher in overweight women compared to normal-weight women. In normal-weight women, mean leptin concentration in the third trimester of pregnancy was 31.1 (27.8-34.4) ng/ml and one month after the delivery, mean leptin concentration was 16.2 (13.9-18.5) ng/ml. The difference between the time points was statistically significant, $P<0.001$. Also, in overweight women the mean leptin concentration was significantly higher in the third trimester of pregnancy, at 45.3 (39.4-45.3) ng, when compared to the postpartum mean of 31.8 (27.4-36.3) ng/ml, $P<0.001$ (Figure 2).

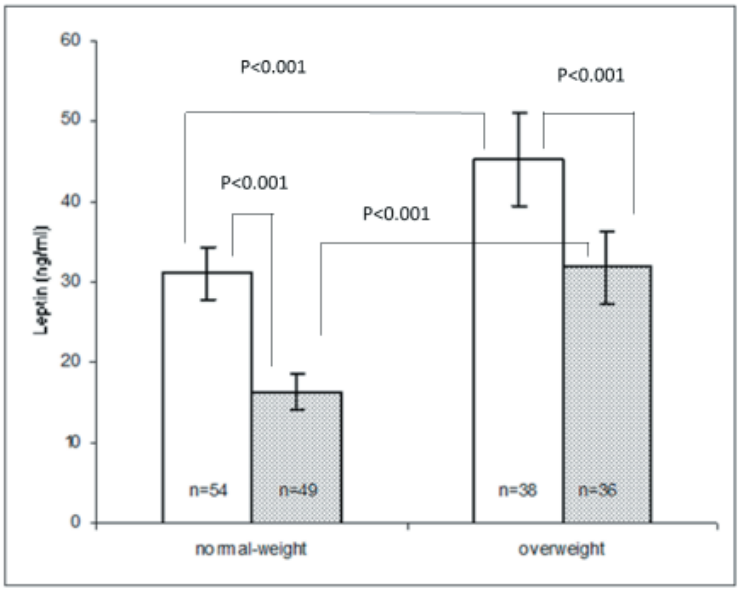

Figure 2. Serum leptin concentrations in normal-weight and overweight women during and after pregnancy. White bar represent third trimester and grey bar one month postpartum. Error bars represent 95% confidence interval for mean (Original publication I).

5.4 The relationship between leptin and glucose metabolism

To analyze the extent to which leptin is related to the aberrations in glucose metabolism during pregnancy, the glucose and insulin concentrations as well as insulin sensitivity and insulin resistance were determined during and after pregnancy in study I. It was found that in normal-weight women mean the glucose concentration was lower during pregnancy, at 4.5 (4.4 - 4.6) mmol/l when compared to postpartum levels of 4.97 (4.9 - 5.0), P<0.001. However, mean insulin concentration was significantly elevated during pregnancy, at 8.3 (7.2 - 9.4) mU/l, compared to postpartum levels of 4.3 (3.6 - 5.0) mU/l, P<0.001. Insulin sensitivity (QUICKI) in turn, decreased, P<0.001, and insulin resistance (HOMA) increased, P<0.001, during pregnancy. On the contrary, in overweight women both mean fasting glucose concentration, 4.8 (4.7 – 5.0) mmol/l, P=0.001, and mean insulin concentration, 15.3 (12.0-18.7) mU/l, P<0.001, were significantly increased during pregnancy compared to normal-weight women. Moreover, QUICKI was lower and HOMA higher in overweight women during pregnancy (Figure 3).

In order to evaluate leptin's role in the regulation of glucose metabolism, the relationships of leptin concentration with insulin concentration, insulin sensitivity, and insulin resistance were evaluated using Pearson's correlations. The results showed that leptin concentration is positively correlated with insulin concentration and insulin resistance and negatively correlated with insulin sensitivity during pregnancy. In postpartum women, leptin

concentration correlated with insulin resistance but not with insulin concentration or insulin sensitivity. Correlations were stronger between leptin and insulin concentrations as well as leptin and HOMA and QUICKI in overweight women (Table 5).

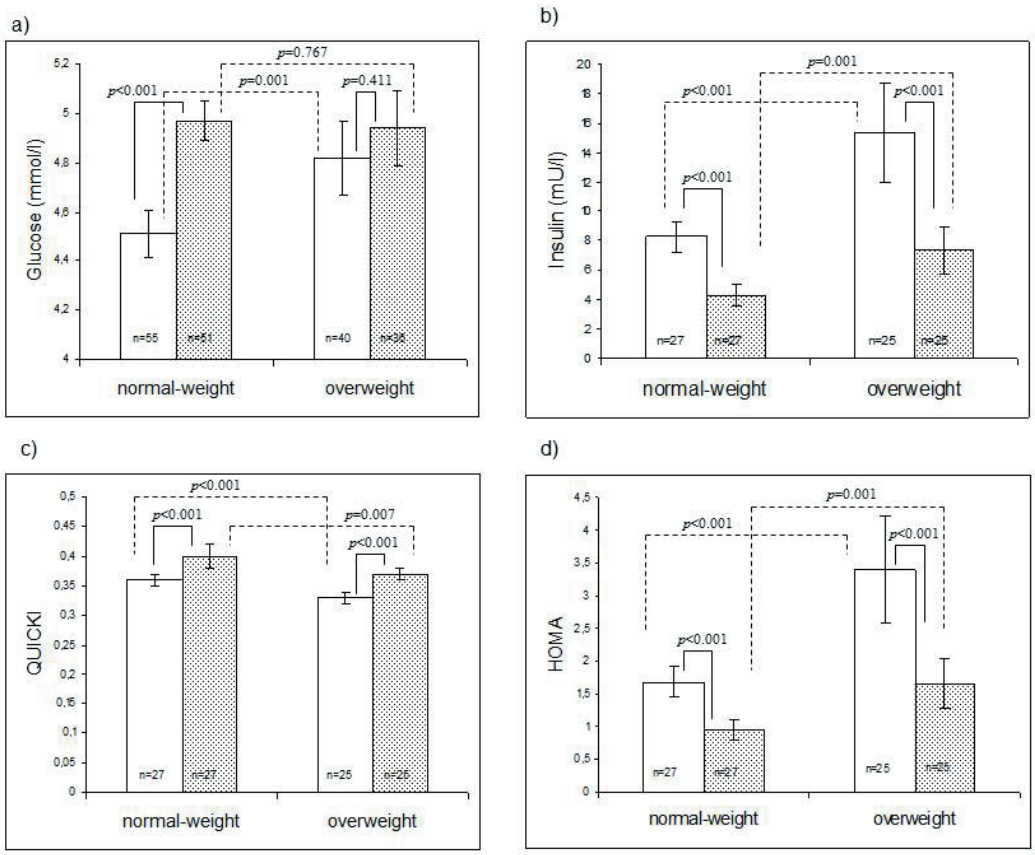

Figure 3. Fasting plasma glucose, serum insulin concentrations, QUICK and HOMA indices at third trimester and one month postpartum in normal-weight and overweight women. White bars represents third trimester of pregnancy and grey bars one month postpartum. Error bars represent 95% confidence interval for mean (Original publication I).

Table 5. Pearson's correlations between leptin and insulin and glucose concentrations as well as HOMA and QUICKI at third trimester of pregnancy and one month postpartum in normal-weight and overweight women (Original publication I)**.**

	Normal-weight women				**Overweight women**			
	third trimester		**postpartum**		**third trimester**		**postpartum**	
	r	P	r	P	r	P	r	P
Insulin concentration	0.561	0.002	0.368	0.065	0.736	<0.001	0.548	0.005
Fasting glucose concentration	0.203	0.141	0.215	0.139	0.244	0.146	0.238	0.161
HOMA	0.568	0.002	0.393	0.047	0.731	<0.001	0.519	0.008
QUICKI	-0.484	0.011	-0.357	0.074	-0.711	<0.001	-0.516	0.008

5.5 Explanatory factors for women's leptin concentration

Study II showed that neither dietary counseling nor probiotic intervention had an influenced on leptin concentrations in women's serum (Table 6). Therefore, other potential explanatory factors were evaluated. Based on the statistical analyses, women's baseline leptin concentration in the first trimester of pregnancy as well as women's pre-pregnancy BMI and weight gain during pregnancy explained women's serum leptin concentrations. Moreover, dietary fiber intake was negatively associated and intake of saturated fatty acids (SAFA) was positively associated with serum leptin concentrations (Table 7). To further study these associations in more detail, mothers were divided into tertiles according to their fiber and SAFA intakes. Analyses showed that in the lowest tertile of fiber intake (as adjusted to energy intake) women's mean leptin concentration was higher, (25.7 nm/ml), than in other tertiles (middle tertile 22.6 ng/ml and highest tertile 19.3 ng/ml), P=0.033. In the case of SAFA no significant differences were found in leptin concentrations between tertiles as quantitatively (T1: 20.8 ng/ml, T2: 23.4 ng/ml and T3: 24.0 ng/ml, P=0.349) or as adjusted to energy intake (T1: 20.4 ng/ml, T2: 22.7 ng/ml, T3: 24.2 ng/ml, P=0.276).

Table 6. Women's serum leptin concentrations in study groups in the first and third trimester of pregnancy and one month postpartum. The results are given as baseline-adjusted marginal means with 95% CI (Modified from original publication II)**.**

Women	Dietary counseling with probiotics		Dietary counseling with placebo		Controls		
	G mean (95%CI)	N	G mean (95% CI)	N	G mean (95% CI)	N	P
First trimester	20.0 (17.5-22.8)	84	23.7 (20.8-27.0)	80	26.0 (23.0-29.5)	78	
Third trimester	26.2 (23.1-29.7)	80	27.5(23.8-31.8)	78	33.1 (29.2-37.4)	77	0.757
Postpartum	12.2 (9.9-14.9)	78	14.3(11.9-17.2)	73	16.7 (14.3-19.6)	73	0.793
Mean of third trimester and postpartum (baseline adjusted)	19.9 (18.1-22.0	78	20.3(18.3-22.6)	73	21.1 (18.9-23.5)	73	0.760

Group comparisons were performed using ANCOVA for repeated measures, where the baseline concentration was included as a covariate.

Table 7. Multivariate regression models explaining the women's (mean of the third trimester and one month postpartum) serum leptin concentrations (Regression coefficients and 95% CI) (Original publication II)**.**

Explaining factor	Regression coefficient B	95% CI	P
Dietary counseling	-0.115	-0.246 to 0.015	0.084
Probiotic supplementation	0.057	-0.070 to 0.185	0.376
Baseline leptin concentration	0.36	0.312 to 0.561	<0.001
Pre-pregnancy BMI	0.052	0.033 to 0.072	<0.001
Weight gain during pregnancy	0.032	0.021 to 0.043	<0.001
Fiber intake	-0.013	-0.021 to -0.004	0.003
Saturated fatty acid intake	0.006	0.000 to 0.002	0.053

In study I, leptin's association with diet was analyzed using correlations in the third trimester of pregnancy. It was found that leptin concentrations were positively associated with sucrose intake both quantitatively (r=0.424, P=0.009) and as a proportion of energy intake (r= 0.408, P=0.012) in overweight women but not in normal-weight women (r=0.156, P=0.264 and r=0.128, P=0.362, respectively). After pregnancy, no significant associations between dietary sucrose and leptin concentration were found in overweight or normal-weight women (Figure 4).

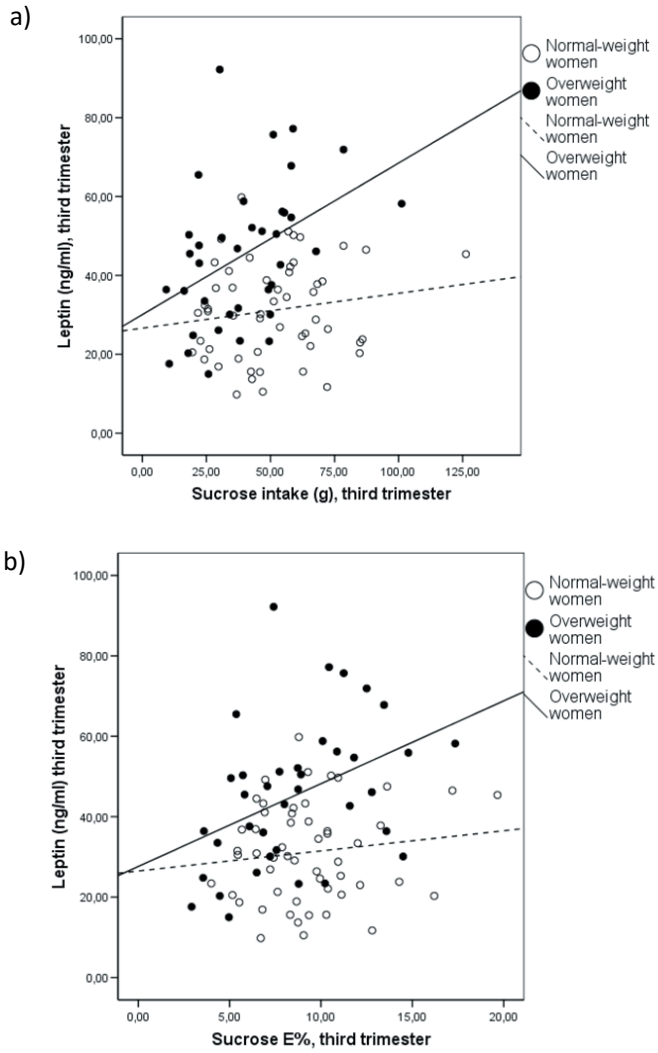

Figure 4. Correlation between leptin concentration and sucrose intake as quantitatively (a) and as a proportion of energy intake (b) at third trimester of pregnancy in normal-weight (r=0.156, p=0.264 and r= 0.128, p= 0.362), respectively and overweight women (r=0.424, p=0.009 and r=0.408, P=0.012, respectively). Regression lines are included separately for normal-weight and overweight women (Original publication I)**.**

5.6 Serum leptin concentration in children

Leptin concentrations were measured from cord blood and from children's serum at one month and six months of age in study II. Cord blood leptin was significantly higher when compared to children's serum at one month of age (P<0.001) whereas no significant difference was found between one and six months of age (P=0.407). Dietary counseling or probiotic intervention during pregnancy did not influenced to leptin concentration either in cord blood or in children's serum (Table 8).

Table 8. Serum leptin concentrations (ng/ml) in children. The results are given as baseline-adjusted marginal means with 95% CI (Modified from original publication II)**.**

	Dietary counseling with probiotics		**Dietary counseling with placebo**		**Controls**		
	G mean(95% CI)	**N**	**G mean**	**N**	**G mean**	**N**	**P[2]**
Cord blood	10.5 (8.5-13.0)	60	10.2 (8.1-12.8)	55	13.0 (10.5-16.2)	48	0.236
One month of age	3.9 (3.2-4.8)	68	2.9 (2.4-3.5)	40	3.7 (2.9-4.6)	40	0.098
Six months of age	3.5 (3.0-4.2)	64	3.4 (3.0-4.0)	69	3.6 (3.0-4.2)	63	0.938
Mean of one and six months	3.9 (3.4-4.5)	55	3.1 (2.6-3.7)	36	3.5(2.9-4.2)	33	0.328

Group comparisons were performed using ANOVA.

Statistical analyses were conducted to evaluate factors explaining both cord blood and children's serum leptin concentrations. In these analyses infant birth weight and sex as well as maternal leptin concentration and dietary protein intake were found to affect cord blood leptin concentration (Table 9). These findings were studied further and it was found that mean cord blood leptin concentration was significantly lower in boys (8.6ng/ml) compared to girls (14.8ng/ml, P<0.001). The effect of birth weight, in turn, was evaluated by dividing infants into tertiles according to their birth weight. It was found that cord blood leptin was lower in the lowest tertile of birth weight (T1: 7.3 ng/ml) compared to other tertiles (T2: 11.8 ng/ml and T3: 15.4 ng/ml, P<0.001). Equally, in the lowest tertile of maternal leptin concentration, cord blood leptin was significantly lower (T1: 7.9 ng/ml) than in other tertiles (T2: 10.9 ng/ml and T3: 13.7 ng/ml, P= 0.002). Interestingly, women's dietary protein intake was inversely associated with cord blood leptin concentration. In the highest tertile of protein intake, the cord blood leptin was significantly lower (T3: 8.2 ng/ml, (compared to lowest tertiles (T2: 13.2 ng/ml and T1: 12.1 ng/ml, P= 0.005).

When modeling explanatory factors for children serum leptin concentrations (mean of one and six months of age) birth weight and infants' sex were found to affect serum leptin concentrations (Table 10). Indeed, boys (3.0 ng/ml) had significantly lower mean serum leptin concentrations at one month of age compared to girls (4.2 ng/ml, P=0.007), but at six months of age no difference was found in serum leptin concentrations between boys (3.2 ng/ml) and girls (3.8 ng/ml, P=0.063). Maternal characteristics or dietary intake did not significantly affect children's serum leptin concentration. Nevertheless, a statistical

multivariate model showed a tendency that maternal use of probiotics during pregnancy may increase infant's leptin concentrations (P=0.090).

Table 9. Multivariate forward stepwise regression model explaining cord blood leptin concentrations (Regression coefficients and 95% confidence intervals)(Original publication II)**.**

Step	Independent variables	Regression coefficient B	95% CI	P
1.	Dietary counseling	-0.195	-0.528 to 0.138	0.248
	Probiotic supplementation	0.051	-0.253 to 0.354	0.742
2.	Dietary counseling	-0.114	0.410 to 0.182	0.447
	Probiotic supplementation	0.102	-0.167 to 0.371	0.455
	Birth weight of the infant	0.001	0.001 to 0.001	<0.001
3.	Dietary counseling	-0.125	-0.398 to 0.148	0.366
	Probiotic intervention	0.114	-0.134 to 0.362	0.366
	Birth weight of the infant	0.001	0.001 to 0.001	<0.001
	Male gender	-0.556	-0.747 to -0.333	<0.001
4.	Dietary counseling	-0.087	-0.354 to 0.180	0.519
	Probiotic supplementation	0.168	-0.076 to 0.413	0.175
	Birth weight of the infant	0.001	0.001 to 0.001	<0.001
	Male gender	-0.540	-0.747 to -0.333	<0.001
	Maternal leptin concentration	0.313	0.110 to 0.516	0.003
5.	Dietary counseling	-0.076	-0.340 to 0.187	0.568
	Probiotic supplementation	0.167	-0.074 to 0.408	0.172
	Birth weight of the infant	0.001	0.001 to 0.001	<0.001
	Male gender	-0.523	-0.727 to -0.318	<0.001
	Maternal leptin concentration	0.276	0.073 to 0.478	0.008
	Maternal protein intake	-0.007	-0.013 to -0.001	0.024

Maternal leptin concentration was taken into account as a mean of first and third trimester concentrations for logarithmic conversion. Maternal protein intake was taken into account as a mean intake during pregnancy.

Table 10. Multivariate regression models explaining children's (mean of one and six months of age) serum leptin concentrations. (Regression coefficients and 95% confidence intervals) (Original publication II)**.**

Explanatory factor	Regression coefficient	95% CI	P
Dietary counseling	-0.132	-0.340, 0.075	0.210
Probiotic supplementation	0.176	-0.027, 0.379	0.090
Male gender	-0.221	-0.388, -0.054	0.010
Birth weight of the infant	0.193	0.004, 0.382	0.045
Maternal energy intake	-0.007	-0.123, 0.110	0.912
Maternal carbohydrate intake	-0.001	-0.004, 0.003	0.713
Maternal fiber intake	-0.002	-0.018, 0.015	0.845

Maternal intake of energy, carbohydrates and fiber as a mean of the third trimester and one month postpartum

5.7 Women's dietary intake of antioxidant vitamins

In study III, dietary counseling increased women's dietary intake of beta-carotene and vitamin E but no other significant changes were detected when compared to the control

group (Table 11). In women, the mean vitamin intakes were in accord or even in excess of the dietary reference values except for vitamin D in which the reference value was reached only when the intake from supplements was taken into account.

Table 11. Antioxidant vitamin intakes (mean of intakes in the third trimester and one month postpartum) in women (Original publication III)**.**

	Dietary counseling	**Control**	**Dietary counseling versus control**	
	Mean (95%CI)	Mean (95% CI)	Mean (95% CI)	p
Vitamin A total intake (RE)	1205 (1017-1393)	1008 (823-1194)	196.7 (-70.7 to 464.1)	0.147
Vitamin A from diet (RE)	1164 (985-1344)	969 (792-1147)	195 (-60 to 451)	0.132
Vitamin A from supplements (µg)	35.6 (5.5-65.7)	42.0 (11.5-72.5)	-6.4 (-49.4 to 36.6)	0.767
Beta-carotene (µg)	3647 (2822-4473)	2263 (1448-3077)	1385 (221 to 2549)	0.020
Vitamin D total intake (µg)	11.6 (10.3-13.0)	10.6 (9.3-12.0)	1.0 (-0.9 to 2.9)	0.295
Vitamin D from diet (µg)	6.9 (5.9-7.8)	6.2 (5.3-7.2)	0.6 (-0.7 to 2.0)	0.337
Vitamin D from supplements (µg)	4.6 (3.6-5.7)	4.4 (3.3-5.4)	0.3 (-1.2 to 1.7)	0.712
Vitamin E total intake (mg)	16.9 (15.1-18.8)	15.6 (13.8-17.4)	1.4 (-1.3 to 4.0)	0.305
Vitamin E from diet (mg)	11.8 (10.9-12.7)	9.9 (9.0-10.8)	2.0 (0.7 to 3.2)	0.003
Vitamin E from supplements (mg)	4.9 (3.2-6.6)	5.7 (4.0-7.3)	-0.7 (-3.1 to 1.6)	0.538
Vitamin C total intake (mg)	179.3 (152.7-205.9)	180.5 (154.3-206.8)	-1.2 (-38.6 to 36.1)	0.948
Vitamin C from diet (mg)	131.2 (111.7-150.8)	133.6 (114.3-152.9)	-2.3 (-29.9 to 25.2)	0.866
Vitamin C from supplements (mg)	51.9 (31.8-72.1)	48.1 (28.0-68.3)	3.8 (-24.7 to 32.2)	0.792

Group comparisons were made using ANCOVA for repeated measures. The baseline (first trimester intake) was included as a covariate. The intake data are given as baseline-adjusted means with 95% confidence intervals.

5.8 Women's antioxidant vitamin concentrations in serum and breast milk

The development of antioxidant vitamin concentrations in women's serum during pregnancy was analyzed in study III. Serum vitamin concentrations of vitamins A, C, D, E, and beta-carotene were changed significantly from the first trimester to one month postpartum. No significant change was detected only in the case of fat-adjusted vitamin E concentration (Figure 5). Vitamins D and E were increased from the first to third trimester whereas concentrations of vitamins A and C and beta-carotene were decreased. From the third trimester of pregnancy to postpartum, the serum concentrations of vitamins C, D and E were decreased while vitamin A and beta-carotene were elevated. The changes in serum vitamin concentrations during the follow-up were nearly similar in the dietary counseling group compared to control group. Only, in the case of beta-carotene concentration the decrease was steeper in the intervention group compared to controls from the first to third trimester of pregnancy (P= 0.023 for interaction).

In the women receiving dietary counseling the beta-carotene concentration was significantly lower than in the control group (Table 12). No other significant differences were detected between the groups.

When analyzing breast milk samples, significantly higher colostrum vitamin A concentrations were found in the dietary counseling group compared to controls but no other significant changes were found in colostrum or in mature breast milk between the dietary counseling and control groups (Table 13).

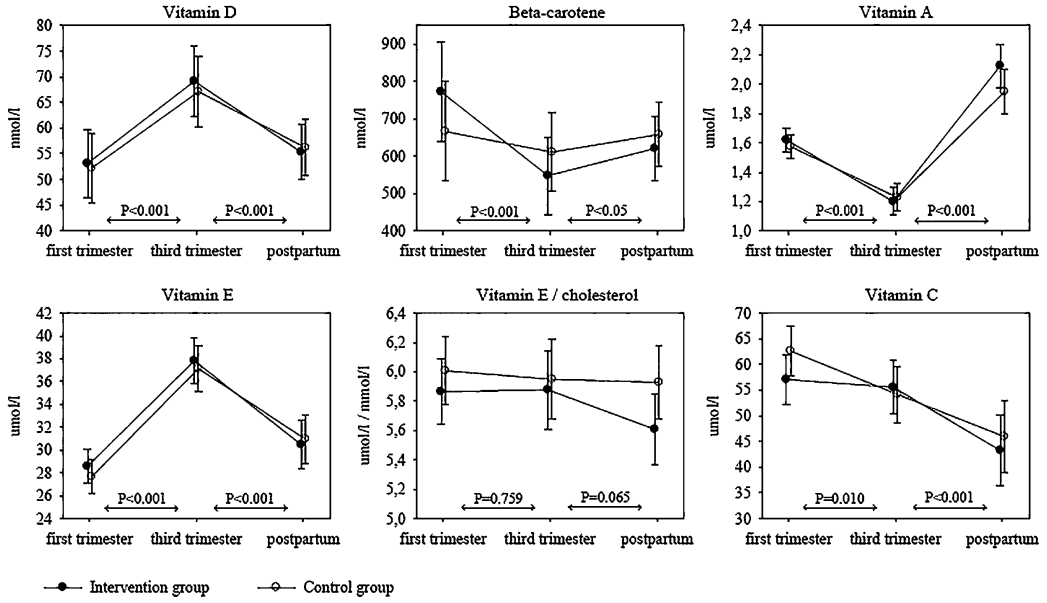

Figure 5. The development of serum vitamin concentrations from the first trimester of pregnancy to one month postpartum. The error bars indicate the 95% confidence intervals. ANOVA for repeated measures was used to test the within-subject changes (P=0.061 for vitamin E /cholesterol and P<0.001 for other vitamins). The p-values in the figure indicate the changes between time points. Significant time x group interaction was detected only in the concentration of beta-carotene in the change from first to third trimester (P=0.023) (Original publication III).

TABLE 12. Antioxidant vitamin concentrations in women's serum (mean of the third trimester and one month postpartum) (Modified from original publication III)**.**

	Dietary counseling		Control		Dietary counseling versus control	
	N	Mean (95% CI)	N	Mean (95% CI)	Mean (95% CI)	P
Vitamin A (µmol/l)	40	1.7 (1.6 - 1.7)	39	1.6 (1.5 - 1.7)	0.04 (-0.08 to 0.16)	0.466
Beta-carotene (nmol/l)	40	561 (498 - 624)	39	658 (594 - 722)	-97 (-187 to -7)	0.036
Vitamin D (nmol/l)	40	62.1 (57.2 - 67.0)	39	61.8 (56.9 - 66.8)	0.2 (-6.7 to 7.2)	0.947
Vitamin E (µmol/l)	40	33.8 (32.4 - 35.1)	39	33.8 (32.4 - 35.1)	-0.7 (-2.6 to 1.3)	0.496
Vitamin E / cholesterol (µmol/mmol)	40	5.8 (5.6 – 6.0)	38	5.9 (5.7 - 6.1)	-0.1 (-0.3 to 0.2)	0.432
Vitamin C (µmol/l)	39	51.0 (46.9 - 55.1)	37	48.4 (44.2 - 52.6)	2.6 (-3.4 to 8.5)	0.390

Group comparisons were made using ANCOVA for repeated measures, where baseline (first trimester) was included as a covariate. Results are given as baseline-adjusted marginal means with 95% confidence intervals.

TABLE 13. Vitamin concentrations in colostrum and in mature milk in dietary counseling and control groups (Original publication III)**.**

	Dietary counseling		Control		Ratio dietary counseling / Control	
Colostrum	N	G mean	N	G mean	Mean	P
Vitamin A (µmol/l)	32	4.8 (4.2-5.4)	33	3.7 (3.0-4.5)	1.3 (1.0 to 1.7)	0.024
Beta-carotene (nmol/l)	32	274 (200-375)	33	213 (155-294)	1.3 (0.8 to 2.0)	0.261
Vitamin E (µmol/l)	32	38.4 (31.8-46.3)	33	30.1 (23.1-39.2)	1.3 (0.9 to 1.8)	0.132
Vitamin E/total TAG (µmol/mg)	31	13.7 (11.1-16.9)	33	13.26 (10.7-16.4)	1.03 (0.8 to 1.4)	0.830
Vitamin C (µmol/l)	31	368 (308-440)	33	394 (330-471)	0.93 (0.7 to 1.2)	0.585
Mature milk	N	G mean	N	G mean	Mean	P
Vitamin A (µmol/l)	36	1.72 (1.5-2.0)	39	1.6 (1.4-1.9)	1.1 (0.9 to 1.3)	0.554
Beta-carotene (nmol/l)	36	33.0 (25.4-42.7)	39	34.2 (28.3-41.3)	0.96 (0.7 to 1.3)	0.818
Vitamin E (µmol/l)	36	8.3 (7.1-9.7)	39	7.9 (6.8-9.2)	1.1 (0.8 to 1.3)	0.684
Vitamin E/total TAG (µmol/mg)	35	3.5 (3.1-4.1)	38	3.3 (2.9-3.7)	1.1 (0.9 to 1.3)	0.404
Vitamin C (µmol/l)	36	228 (187-277)	39	216 (190-246)	1.1 (0.8 to 1.3)	0.644

TAG= triacylglyserols. Group comparisons were made using ANOVA with logarithmically transformed values. Results are given as geometric means with 95% confidence intervals.

5.9 Explanatory factors for women's vitamin status

To clarify the factors determining vitamin concentrations in women's serum, statistical multivariate analyses were conducted in Study III. The effect of dietary counseling was forced into the models and the other potential explanatory factors were added stepwise. Models suggested that the total intake of vitamins explains the corresponding concentrations of vitamins C, D, E as adjusted to cholesterol and beta-carotene (Table 14). Instead, the intake of vitamin supplements (as a dichotomous variable, yes versus no) was not associated with the corresponding concentration. The multivariate model also showed a negative effect of dietary counseling on serum vitamin E concentration but no other associations between dietary counseling and vitamin concentrations were found. However, the models suggested a positive association between women's pre-pregnancy BMI to vitamin A and a negative association with beta-carotene concentration. Moreover, the model proposed a positive association between women's ages and vitamin E concentration.

The associations suggested by multivariate models were studied further by dividing women into two groups according to their pre-pregnancy BMI (<25 BMI ≥25). Indeed, overweight women had significantly lower serum beta-carotene concentration, at 480 (95% CI 407-533) nmol/l, compared to normal-weight women, at 659 (586-732) nmol/l, P=0.004. However, vitamin A concentration did not differed between the normal-weight women, 1.6 (1.5-1.7) µmol/l, and overweight women, 1.6 (1.5-1.8) µmol/l, P=0.429. In the case of vitamin E, women were divided into two groups according to their age (under or over 30 years). Younger women had significantly lower concentrations of vitamin E adjusted to cholesterol, 5.3 (5.3-5.7), than in older women, 6.1 (5.8-6.3), P=0.001.

TABLE 14. Multivariate regression models explaining the women's serum vitamin concentrations (Original publication III)**.**

Dependent variable	Explaining factors	Regression coefficient B	95% CI	P	Adjusted R^2
Vitamin D (nmol/l)	Dietary counseling	-0.09	-7.10 to 6.92	0.980	
	Intake of vitamin	1.63	0.81 to 2.45	<0.001	
	Pre-pregnancy BMI	- 0.85	-1.77 to 0.08	0.071	
					0.152
Vitamin A (µmol/l)	Dietary counseling	0.052	-0.08 to 0.19	0.454	
	Pre-pregnancy BMI	0.018	0.00037 to 0.04	0.046	
					0.035
Beta-carotene (nmol/l)	Dietary counseling	-76.16	-187.9 to 35.6	0.179	
	Intake of vitamin (diet)	0.028	0.007 to 0.050	0.010	
	Pre-pregnancy BMI	-20.55	-34.98 to -6.12	0.006	
	Age	10.64	-0.72 to 21.99	0.066	
					0.160
Vitamin E (µmol/l)	Dietary counseling	-1.440	-4.045 to 1.165	0.275	
	Age	0.348	0.076 to 0.619	0.013	
					0.058
Vitamin E /cholesterol (µmol/mmol)	Dietary counseling	-0.323	-0.626 to 0.021	0.036	
	Age	0.054	0.021 to 0.086	0.001	
	Intake of vitamin	0.026	0.001 to 0.051	0.038	
					0.183
Vitamin C	Dietary counseling	-1.158	-7.411 to 5.095	0.714	
	Intake of vitamin	0.046	0.009 to 0.082	0.015	
					0.049

Total intake of vitamin included both dietary and supplementary intake

5.10 Antioxidant vitamin concentrations in children

In study III children's serum vitamin (A, C, D, E, E / cholesterol and beta-carotene) concentrations were analyzed (Table 15).

Table 15. Antioxidant vitamin concentrations in infant's serum at one month of age in the intervention and control groups (Modified from original publication III)**.**

	N	Dietary counseling Mean (95% CI)	N	Control Mean (95% CI)	Dietary counseling versus control Mean (95%CI)	p
Vitamin A (µmol/l)	38	0.8 (0.7-0.8)	32	0.7 (0.7-0.8)	0.004 (-0.05 to 0.13)	0.347
Beta-carotene (nmol/l)	38	136 (105-176)	32	137 (102-184)	1.00 (0.7 to 1.5)	0.983
Vitamin D (nmol/l)	38	49.1 (43.2-55.1)	32	51.2 (45.3-57.0)	-2.0 (-10.3 to 6.2)	0.626
Vitamin E µmol/l	38	28.7 (26.6-30.9)	32	27.5 (25.6-29.4)	1.2 (-1.6 to 4.1)	0.392
Vitamin E /cholesterol (µmol/mmol)	37	8.3 (7.5-9.1)	32	7.7 (7.2-8.2)	0.62 (-0.4 to 1.6)	0.223
Vitamin C (µmol/l)	39	67.3 (60.7-73.8)	34	64.5 (60.5-68.6)	2.8 (-5.1 – 10.6)	0.484

Group comparisons were made using ANOVA. Results are given as means with 95% confidence intervals and group comparison as ratio dietary counselling / Control (95% CI). The beta-carotene values were logarithmically (log_e) transformed before analysis.

The intensive dietary counseling given during pregnancy to mothers was not reflected in children's serum vitamin concentrations. Multivariate analyses suggested that mother's serum concentrations of vitamins C, D, E and beta-carotene were associated with the corresponding concentration in infants. Vitamin A concentration in children's serum, in turn, was associated with mother's pre-pregnancy BMI. Only children's beta-carotene concentration was related to mother's dietary intake of vitamins (Table 16).

Associations suggested by the multivariate model were studied further by dividing children into two groups according to their mothers pre-pregnancy BMIs. Indeed, it was found that children of overweight women had significantly higher vitamin A concentration, at 0.8 (95%CI 0.7 – 0.9 µmol/l, when compared to children of normal-weight women, at 0.7 (0.6-0.8) µmol/l, P=0.004.

TABLE 16. Multivariate regression models explaining the infant's serum vitamin concentrations at one month of age (Original publication III)**.**

Dependent variable	Explaining factor	Regression coefficient B	95% CI	P	Adjusted R^2
Vitamin D (nmol/l)	Dietary counseling	-2.57	-10.27 to 5.13	0.508	
	Mother's serum vitamin concentration	0.40	0.18 to 0.62	0.001	
					0.145
Vitamin A (µmol/l)	Dietary counseling	0.017	-0.067 to 0.102	0.679	
	Pre-pregnancy BMI	0.019	0.007 to 0.031	0.002	
					0.127
Beta-carotene (nmol/l)	Dietary counseling	-0.027	-0.358 to 0.304	0.871	
	Mother's serum vitamin concentration	0.001	0.001 to 0.002	<0.001	
	Mother's intake of vitamins	0.00007	0.00001 to 0.00014	0.027	
					0.292
Vitamin E (µmol/l)	Dietary counseling	1.573	-1.291 to 4.436	0.277	
	Mother's serum vitamin concentration	0.409	0.150 to 0.667	0.002	
					0.124
Vitamin C (µmol/l)	Dietary counseling	3.518	-4.703 to 11.739	0.396	
	Mother's serum vitamin concentration	0.308	0.014 to 0.602	0.040	
	Maternal atopy	8.088	-0.384 to 16.559	0.061	
					0.057

5.11 Epigenetic DNA methylation alterations in obesity and weight gain related risk genes and signaling pathways by probiotics

It was first examined whether the intake of probiotics affects the DNA methylation status of any known obesity risk gene promoters. The gene promoter of the methionine sulfoxide reductase A (MSRA) gene was affected in both the women and their infants (Table 17) with

decreased DNA methylation in the probiotics group. Further, two other genes were affected in women and another four genes in children (Table 17). Importantly, the DNA methylation of the promoter of the fat mass and obesity-associated (FTO) gene, the strongest known genetic risk factor for obesity, decreased in the women in response to the probiotics intake.

Table 17. DNA methylation changes in the promoters of obesity-associated risk genes in response to the intake of a placebo versus probiotics. Positive fold change = less methylated in the probiotics group, negative fold change = more methylated in the probiotics group (Original publication IV).

		Mothers		Children		
Gene Symbol	**Gene Name**	**Fold change**	**t-test p-value**	**Fold change**	**t-test p-value**	**Genomic location**
FTO	fat mass and obesity associated	3.13	0.021	1.06	0.872	chr16:53,736,875-53,738,375
MC4R	melanocortin 4 receptor	3.47	0.007	1.89	0.107	chr18:58,039,501-58,041,001
MSRA	methionine sulfoxide reductase A	2.59	0.042	2.57	0.016	chr8:9,910,830-9,912,330
MTMR9	myotubularin related protein 9	2.36	0.093	2.31	0.024	chr8:11,141,000-11,142,500
TNKS	tankyrase	1.90	0.180	2.76	0.012	chr8:9,412,445-9,413,945
CTNNBL1	catenin beta like 1	1.63	0.221	2.19	0.044	chr20:36,321,434-36,322,934
BDNF	brain-derived neurotrophic factor	-1.08	0.873	2.02	0.047	chr11:27,743,105-27,744,605

Pathway analysis revealed epigenetic changes in a large set of additional genes that are functionally associated with obesity or weight gain based on the literature. Tables 18 and 19 show the gene promoters that were significantly affected by probiotics in the women and their children. In the women, 37 gene promoters showed decreased levels of DNA methylation in the probiotic group. In addition, the gene promoter of 5-hydroxytryptamine (serotonin) receptor 3D (HTR3D) was more methylated in the probiotic group (Table 18). In the children, 68 gene promoters were found to be significantly affected, all of which were less methylated in the probiotic group (Table 19). In the pathway analysis, five genes were identified to be influenced in both the mothers and infants (insulin-like growth factor binding protein 1 (IGFBP1), complement component 3 (C3), interleukin 5 (IL5), solute carrier family 6 (neurotransmitter transporter), member 5 (SLC6A5) and myosin, heavy chain 11, smooth muscle (MYH11)); all of them were less methylated in the probiotic group in both the mothers and their children.

Table 18. Obesity- and weight gain- related genes with significantly altered methylation in the mothers. Positive fold change = less methylated in the probiotics group, negative fold change = more methylated in the probiotics group (Original publication IV).

Symbol	Gene Name	Fold Change	p-value
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	2.01	0.016
ADCYAP1	adenylate cyclase activating polypeptide 1 (pituitary)	5.38	<0.001
ADRB1	adrenoceptor beta 1	3.13	0.008
ADRB2	adrenoceptor beta 2, surface	2.76	0.029
BBS2	Bardet-Biedl syndrome 2	3.06	0.014
C3	complement component 3	3.51	0.002
CA3	carbonic anhydrase III, muscle specific	2.34	0.036
CAV1	caveolin 1, caveolae protein, 22kDa	3.03	0.013
CXCL11	chemokine (C-X-C motif) ligand 11	2.95	0.020
ESR1	estrogen receptor 1	2.15	0.026
FOXA2	forkhead box A2	3.17	0.013
FTO	fat mass and obesity-associated	3.13	0.021
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	2.35	0.039
GABRB1	gamma-aminobutyric acid (GABA) A receptor, beta 1	3.14	0.018
GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3	2.62	0.031
GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	3.67	0.013
GYS1	glycogen synthase 1 (muscle)	2.41	0.012
HTR1F	5-hydroxytryptamine (serotonin) receptor 1F, G protein-coupled	3.75	0.007
HTR3D	5-hydroxytryptamine (serotonin) receptor 3D, ionotropic	-2.00	0.024
IGF2R	insulin-like growth factor 2 receptor	3.24	0.019
IGFBP1	insulin-like growth factor binding protein 1	4.71	<0.001
IL18	interleukin 18	2.87	0.025
IL1B	interleukin 1, beta	2.48	0.033
IL2	interleukin 2	2.53	0.042
IL5	interleukin 5	3.28	0.015
IRS1	insulin receptor substrate 1	3.00	0.021
LDLR	low density lipoprotein receptor	3.17	0.023
MC4R	melanocortin 4 receptor	3.47	0.007
MYH11	myosin, heavy chain 11, smooth muscle	2.40	0.048
OMA1	OMA1 zinc metallopeptidase	2.46	0.038
PANK1	pantothenate kinase 1	2.74	0.015
POU3F4	POU class 3 homeobox 4	2.12	0.036
PTEN	phosphatase and tensin homolog	2.27	0.014
RGS7	regulator of G-protein signaling 7	3.87	0.001
SLC6A5	solute carrier family 6 (neurotransmitter transporter), member 5	3.28	0.010
SP4	Sp4 transcription factor	2.02	0.021
SPTLC1	serine palmitoyltransferase, long chain base subunit 1	2.68	0.013
SST	somatostatin	3.16	0.002
TIMP2	TIMP metallopeptidase inhibitor 2	2.75	0.021
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	2.23	0.033

Table 19. Obesity- and weight gain- related genes with significantly altered methylation in the children. Positive fold change = less methylated in the probiotic group, negative fold change = more methylated in the probiotics group (Original publication IV).

Symbol	Entrez Gene Name	Fold Change	p-value
ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	2.20	0.043
ADORA2A	adenosine A2a receptor	2.40	0.023
ADRA1D	adrenoceptor alpha 1D	2.09	0.035
APP	amyloid beta (A4) precursor protein	2.39	0.029
ARNT	aryl hydrocarbon receptor nuclear translocator	2.50	0.019
ARRB1	arrestin, beta 1	2.11	0.031
BDNF	brain-derived neurotrophic factor	2.02	0.047
C3	complement component 3	2.51	0.034
CCND3	cyclin D3	2.08	0.026
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	2.37	0.024
CD38	CD38 molecule	2.32	0.030
CGB	chorionic gonadotropin, beta polypeptide	2.23	0.015
CRHR1	corticotropin releasing hormone receptor 1	2.17	0.025
CXCR4	chemokine (C-X-C motif) receptor 4	2.09	0.012
DGAT1	diacylglycerol O-acyltransferase 1	2.14	0.037
DPP4	dipeptidyl-peptidase 4	2.18	0.027
DRD2	dopamine receptor D2	2.06	0.022
FABP2	fatty acid binding protein 2, intestinal	2.65	0.017
GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	2.65	0.005
GABRG2	gamma-aminobutyric acid (GABA) A receptor, gamma 2	2.24	0.041
GAL	galanin/GMAP prepropeptide	3.29	0.002
GAS6	growth arrest-specific 6	3.34	0.003
GNB5	guanine nucleotide binding protein (G protein), beta 5	2.40	0.038
GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2	2.73	0.010
GRIN2C	glutamate receptor, ionotropic, N-methyl D-aspartate 2C	2.29	0.017
HDAC9	histone deacetylase 9	2.77	0.004
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	2.97	0.002
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	2.11	0.027
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	2.81	0.006
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled	2.43	0.008
IAPP	islet amyloid polypeptide	2.19	0.009
IGF1R	insulin-like growth factor 1 receptor	2.02	0.023
IGFBP1	insulin-like growth factor binding protein 1	3.31	0.001
IL5	interleukin 5	2.41	0.017
IL6R	interleukin 6 receptor	2.33	0.019
INSR	insulin receptor	2.15	0.033
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	3.66	0.003
KDM3A	lysine (K)-specific demethylase 3A	3.19	0.003
LCLAT1	lysocardiolipin acyltransferase 1	2.86	0.009
LOX	lysyl oxidase	2.19	0.044
MFSD2A	major facilitator superfamily domain containing 2A	2.98	0.003
mir-103	microRNA 107	2.07	0.029

Symbol	Entrez Gene Name	Fold Change	p-value
MMP11	matrix metallopeptidase 11 (stromelysin 3)	2.19	0.042
MYH11	myosin, heavy chain 11, smooth muscle	2.37	0.008
NHLH2	nescient helix loop helix 2	2.29	0.047
NR4A2	nuclear receptor subfamily 4, group A, member 2	2.06	0.017
PNRC2	proline-rich nuclear receptor coactivator 2	2.22	0.047
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	2.37	0.044
PRL	prolactin	3.17	0.003
RETSAT	retinol saturase (all-trans-retinol 13,14-reductase)	2.77	0.008
SCN3B	sodium channel, voltage-gated, type III, beta subunit	2.72	0.020
SCN9A	sodium channel, voltage-gated, type IX, alpha subunit	2.69	0.006
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	3.10	0.006
SIRT2	sirtuin 2	2.11	0.033
SLC17A6	solute carrier family 17 (vesicular glutamate transporter), member 6	2.55	0.011
SLC4A10	solute carrier family 4, sodium bicarbonate transporter, member 10	2.68	0.006
SLC6A4	solute carrier family 6 (neurotransmitter transporter), member 4	2.31	0.022
SLC6A5	solute carrier family 6 (neurotransmitter transporter), member 5	2.20	0.033
SPTLC2	serine palmitoyltransferase, long chain base subunit 2	2.77	0.005
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	2.07	0.009
STC1	stanniocalcin 1	2.34	0.029
STC2	stanniocalcin 2	2.13	0.021
TACR1	tachykinin receptor 1	2.83	0.003
TLR5	toll-like receptor 5	2.45	0.036
TP53INP1	tumor protein p53 inducible nuclear protein 1	2.91	0.004
TRPC1	transient receptor potential cation channel, subfamily C, member 1	2.76	0.010
UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	2.17	0.022
UGCG	UDP-glucose ceramide glucosyltransferase	2.29	0.009
VEGFA	vascular endothelial growth factor A	2.75	0.004

6. DISCUSSION

6.1 Leptin as an obesity-related inflammation marker

The primary aim in the present study was to endorse favorable developmental programming and to decrease the risk for the inheriting of obesity and its comorbidities from mother to offspring by modifying the intrauterine environment. The first objective was to decrease the obesity-related low-grade inflammation through dietary means. Previous studies had suggested that maternal BMI is positively associated with both maternal and cord blood leptin concentrations (Lemas et al., 2015) and further that cord blood leptin levels would predict weight gain in infancy (Mantzoros et al., 2009; Parker et al., 2011). Therefore it was first tested whether leptin concentration could be used as a marker for obesity-related low-grade inflammation and other co-morbidities during pregnancy. In agreement with previous studies (Catov et al., 2007; Schubring et al., 1998), leptin concentration was highly elevated in overweight pregnant women. Moreover, leptin concentration was associated with aberrations in glucose metabolism. Specifically, positive associations were observed between leptin concentration and insulin concentration and insulin resistance, whereas a negative association was found between leptin concentration and insulin sensitivity.

To confirm the suitability of leptin as a marker for obesity and related inflammation and other co-morbidities, further statistical analyses were conducted. These aimed to identify which maternal and environmental factors determine leptin concentrations in women during pregnancy and in their offspring during early life. These experiments revealed that maternal pre-pregnancy BMI and weight gain during pregnancy mostly explain women's serum leptin concentrations. Maternal weight did not have a direct influence on leptin concentration in cord blood or in infant serum, but maternal leptin concentration was associated with the infant's leptin concentration. Taken together, these results suggest that maternal weight control both before and during pregnancy is the most important way to modify the leptin concentration both in women and their children. Further, these results confirm that leptin concentration is a useful marker for obesity-related low-grade inflammation and other co-morbidities.

6.2 Diet and dietary counselling as developmental programming factors

Dietary counseling did not affect leptin concentrations in women or their children. However, further studies revealed that in overweight pregnant women, dietary sucrose intake was positively associated with leptin concentration, which means that increased sucrose intake may increase leptin concentration. This finding is supported by a previous cross-sectional

study of nearly 8000 individuals (Lana et al., 2014), which found that consumption of sugar-sweetened beverages is positively associated with higher plasma leptin concentrations. The present study also showed an inverse relationship between serum leptin concentration and dietary fiber intake which suggests that high fiber intake from diet may decrease leptin concentration. This finding was congruent with a recent randomized crossover clinical trial that noticed a favorable change in leptin concentrations in overweight girls after the six weeks of consuming of whole-grain products (Hajihashemi et al., 2014). Another earlier study showed an inverse association between dietary fiber intake and serum leptin levels in male adolescents (Parikh et al., 2012). Further, the present results suggest that saturated fat may have a potential detrimental impact on leptin concentrations. Specifically, it was observed that increased intake of saturated fat may increase leptin concentration. This result is supported by an experiment in mice, which showed that leptin levels were reduced by n-3-PUFA compared to highly saturated fat (Wang et al., 2002). These results are also congruent with current Finnish dietary recommendations, which advise higher intake of fiber and whole-grain products and low intake of saturated fatty acids and sugar. Additionally, the current results suggest that maternal high protein intake during pregnancy may decrease leptin concentration in children. This was supported by a previous experiment in rats, which showed that protein restriction during pregnancy alters leptin physiology in offspring (Zambrano et al., 2006). However, studies evaluating the effects of maternal protein intake on gestational weight gain, birth weight, or offspring's later risk for obesity have yielded contradictory results (Chong et al., 2015; Maslova et al., 2014; Tielemans et al., 2016). Altogether the present results suggest that maternal dietary intake during pregnancy may affect obesity-associated low-degree inflammation as measured by leptin concentration. These results support the theory that diet may be utilized to modify the intrauterine environment towards favorable developmental programming.

Because dietary counseling did not affect leptin concentrations, the efficacy of counselling was tested in another experiment by using antioxidant vitamin status as an indicator for efficacy. Antioxidant vitamin status was selected as an indicator because antioxidants have an essential task in defending the body against free radicals (Rodrigo et al., 2007; Wintergerst et al., 2007), acting as a regulator in growth and differentiation of a number of cells and tissues, and in the maturation of the immune systems (Black, 2001; Mora et al., 2008). Moreover, the need for antioxidant vitamins is increased in pregnant women because of increased oxidative stress by pregnancy and accumulation of vitamin reserves in the fetus (Allen, 2005; Hung et al., 2010; Strobel et al., 2007). Here it was found that dietary counseling improved women's vitamins intakes. Specifically, higher intake of beta-carotene and vitamin E were detected. However, the effect of counseling was not directly reflected in women's serum vitamin concentrations. Only lower serum beta-carotene concentration and further higher colostrum vitamin A concentration were found in women receiving counseling. Previously it was suggested that diets high in carotenes are associated

with beneficial health effects such as reduced type 2 diabetes (Sluijs et al., 2015) or lower lipid oxidation (Cocate et al., 2015). However, serum concentrations of carotenoids have previously been shown to be positively associated with fruit and vegetable intake (Souverein et al., 2015) and therefore it would be expected that the increased serum concentration of beta-carotene would be seen in the dietary counselling group. To understand why increased vitamin intake was not reflected as increased serum concentration in the present study, the multivariate regression models were conducted to identify factors that explain women's and their offspring's serum vitamin concentrations. The models revealed that total intake of vitamin explains corresponding serum concentration, but other maternal and environmental factors may also impact the serum concentration levels. Indeed, the absorption and utility of antioxidant vitamins are known to be affected by many factors such as individual vitamin status and the physicochemical properties of the vitamin source (Faure et al., 2006; Tang, 2010). Present results showed the positive association of maternal pre-pregnancy BMI with vitamin A and negative association with beta-carotene concentration. This is congruent with the earlier finding suggesting that adipose tissue constitutes the major beta-carotene storage tissue. Indeed, individuals with abdominal obesity or high BMI have low serum levels of carotenoids (Burrows et al., 2015; Suzuki et al., 2006; Tourniaire et al., 2009). Here it is likely that the higher portion of overweight women in dietary counseling group can explain the detected lower serum beta-carotene concentration. Therefore special attention should probably be paid to overweight pregnant women's antioxidant vitamin status.

Dietary counseling targeted to pregnant women did not directly affect their offspring's serum vitamin concentrations. Instead, elevated vitamin A concentration was seen in colostrum milk. Breast milk contains numerous different nutrients, which are known to act as immune response regulators in newborns (Cunningham-Rundles et al., 2009). When taking into account that it has been proposed that vitamin A influences immune responses (e.g.modulating the Th1-Th2 balance and enhancing the conversion of naïve T-cells into regulatory cells) (Mora et al., 2008), it may be assumed that elevated vitamin A concentration in colostrum may promote the immune maturation during early infancy. Taken together, the present results show that dietary counselling during pregnancy increases the intake of antioxidant vitamins, which is further reflected as increased vitamin concentrations in breast milk. This suggests that dietary counseling is a useful method to modify the intrauterine environment towards favorable developmental programming.

6.3 Probiotics in developmental programming

Probiotic bacteria may modify the risk for metabolic and immunological diseases (Laitinen et al., 2009c; Micallef et al., 2009; Tighe et al., 2010). Therefore, it was first tested here whether probiotic supplementation during pregnancy would impact on serum leptin concentrations in women, cord blood, or children and thereby affect obesity-related

inflammation. The study showed that probiotic supplementation had no statistically significant influence on serum leptin concentration in women. However, the multivariate regression model, which was designed to explain leptin concentration, showed that probiotics tended to increase the infant's leptin concentration. This tendency is congruent with a recent experiment in rat, which showed a positive correlation between the quantity of *Bifidobacterium*, *Lactobacillus* and serum leptin concentration, whereas the number of *Clostridium, Bacteroides and Prevotella* were negatively correlated with serum leptin levels (Queipo-Ortuno et al., 2013). Another experiment with cats showed that number of fecal *Lactobacillaceae* was negatively associated with body weight and positively associated with blood leptin (Hooda et al., 2013). When taking into account that the primary role of leptin is to inform the brain about stored energy and maintain the homeostasis between energy intake and expenditure (i.e. stop eating when no energy is needed) (Zhou and Rui, 2013), and on the other hand that *Lactobacillus rhamnosus* intervention during pregnancy has been shown to modify children's growth patterns by inhibiting of excessive weight gain during the first years of life (Luoto et al., 2010), it is tempting to speculate that leptin may mediate the weight controlling effect of probiotics. However, larger trials with different probiotic species and strains are needed to make profound conclusions about probiotics' impact on weight control and obesity-related low-grade inflammation.

Further the present study aimed to analyze whether probiotic supplementation during pregnancy has an impact on women's and their children's epigenetic DNA methylation in obesity- or weight gain-related genes. First it was examined whether the intake of probiotics affects the DNA methylation status of promoters of any known obesity risk genes and then the pathway analyses were conducted to find affected gene promoters that are functionally associated with obesity. Methylation in one known obesity-related gene promoter, MSRA, was affected both in women and their children being less methylated in probiotic group. This gene encodes a highly conserved protein that carries out the enzymatic reduction of methionine sulfoxide to methionine (Drazic and Winter, 2014). In particular, MSRA protein functions in the repair of oxidative damaged proteins to restore their biological activity. Experiments in mice have revealed that animals lacking the MSRA gene are prone to develop high-fat-diet induced insulin resistance and reduced physiological insulin response compared to wild-type mice (Styskal et al., 2013). The decreased methylation in MSRA promoter by probiotics suggests that transcription of the gene may be up-regulated. This may confer better protection from oxidative damage, and in a light of the animal experiments, may also protect women and their children from obesity-related impaired insulin function.

Additionally two other genes, FTO and melanocortin 4 receptor (MC4R) were affected in women but not in children; both were less methylated in the probiotic group. The FTO region harbors the strongest genetic association with obesity, and it has been linked with body mass index, obesity risk and type 2 diabetes in numerous studies, although the detailed molecular mechanisms have remained obscure. Previous studies have suggested

that increased expression of FTO leads to increased body weight (Berulava et al., 2013). Indeed, mice globally over-expressing FTO were obese. Loss of function FTO mutations, in turn, both in mice and humans have resulted in severe growth retardation (Yeo, 2014). Further the expression of genes regulating adipogenesis were affected in FTO-deficient mice resulting in smaller adipocytes, altered adipokine production and altered response of adipose tissue to high-fat diet (Ronkainen et al., 2015). Here, decreased methylation of FTO suggests higher transcription activity of FTO, which in a light of previous findings may cause increased weight gain. However, it was previously shown in the same NAMI study cohort that probiotic-supplemented dietary counselling during pregnancy decreased the central adiposity defined as waist circumference in women after pregnancy (Ilmonen et al., 2011). The MC4R gene, in turn, encodes a membrane-bound receptor which is known as an important regulator of food intake because it participates in appetite and energy control regulation in the brain (Rovite et al., 2014). MC4R defects have been shown to lead to a clinical phenotype characterized by lack of satiety and early-onset obesity (Fani et al., 2014). In particular, inhibition of melanocortin receptor activity has resulted in increased food intake (Adan et al., 2006). Here the decreased methylation suggests increased expression of the MC4R gene and thereby increased concentration of the melanocortin 4 receptor. However, it is important to notice that the present study did not evaluated the signal transduction activity of this receptor, and thereby it is difficult to estimate health effects of the methylation in MC4R.

Another set of four genes, myotubularin related protein 9 (MTMR9), tankyrase, TRF1-interacting ankyrin related ADP-ribose polymerase (TNKS), catenin, beta like 1 (CTNNBL1) and brain-derived neurotrophic factor (BDNF) were affected in children but not in women. All of the affected genes were less methylated, which suggests increased transcription activity in the probiotic group. MTMR9 encodes a myotubularin-related protein. Recently it was shown that MTMR9 polymorphism is associated with glucose tolerance, insulin secretion, insulin sensitivity, and increased risk of prediabetes (Tang et al., 2014). TNKS and CTNNBL1 are poorly known but their gene regions have been shown to be associated with body weight and the risk of developing obesity. BDNF is a member of the neurotrophin family, which regulates neuronal differentiation and functions. Low levels of BDNF have been related to psychological stress and may potentially play a role in the pathogenesis of depression and cognition disorders. Further plasma BDNF levels have been decreased in the patients with heart failure (Takashio et al., 2015). In general, it could be said that these genes affected in children are not very well characterized.

Pathway analysis revealed a large set of additional obesity and weight gain associated gene promoters with different methylation status by probiotics. IGFBP1, C3, IL5, SLC6A5 and MYH11, were found to be less methylated both in women and their children, which suggests that these genes have higher activity in probiotic group. IGFBP1 belongs to the family of insulin-like growth factor binding proteins and encodes a protein that binds both

insulin-like growth factor 1 and 2. The low concentration of IGFBP1 has previously been associated with insulin resistance and diabetes. Furthermore, animal experiments have suggested that increased IGFBP1 concentrations may be an effective approach to prevent insulin resistance and diabetes (Rajwani et al., 2012). On the other hand, the decreased placental expression of IGFBP1 has been reported in pregnancies complicated with fetal growth restriction (Koutsaki et al., 2011). Here we showed decreased methylation, which in conjunction with previous findings, suggests that the increased activity of the IGFBP1 gene may improve glucose metabolism in women. C3, in turn, plays a central role in the activation of the complement system. Its activation is required for both classical and alternative complement activation pathways. The C3 peptide modulates inflammation and possesses antimicrobial activity, and it has been shown that its concentration is associated with insulin resistance, liver dysfunction, risk of the metabolic syndrome, type 2 diabetes, and cardiovascular diseases (Hertle et al., 2014). Il5 encodes a cytokine that acts as a growth factor and differentiation factor for both B cells and eosinophils. Recent studies have suggested that IL-5 inhibition may be an effective approach for the treatment of asthma (Garcia et al., 2013). SLC6A5 encodes a sodium- and chloride-dependent glycine neurotransmitter transporter and MYH11 encodes a smooth muscle myosin.

Additionally, this study found a set of gene promoter regions that were differently methylated by probiotic supplementation in women or in their children. In women the affected genes include cytokines or other growth factors, enzymes, receptor-molecules, ion channels, kinases, transmembrane proteins and transporters. In children for example, the promoters of signal transducer and activator of transcription 3 (STAT3), Toll-like receptor 5 (TLR5) and Interleukin 6 receptor (IL6R) were less methylated in the probiotics group. All of those genes participate in essential metabolic and immunological processes (Fisman and Tenenbaum, 2010; Ge et al., 2008; Hruz et al., 2010; Vijay-Kumar et al., 2010) and changes in their activity may explain the clinical findings about probiotics, for instance in the prevention and treatment of allergies (Iemoli et al., 2012; Kalliomaki et al., 2001; Singh et al., 2013) and infections (Johnston et al., 2012; Smith et al., 2013), or in the treatment of necrotizing enterocolitis (Jakaitis and Denning, 2014).

Taken together, present results show that probiotic supplementation during pregnancy causes varying changes in DNA methylation of obesity- and weight gain- related genes both in women and their children. This suggest that probiotic supplementation during pregnancy affects the gene activity both in women and their children. When taking into account the present results and various studies suggesting clinical benefits of probiotics (Brantsaeter et al., 2011; Kadooka et al., 2010; Laitinen et al., 2009; Myhre et al., 2011), it may be hypothesized that DNA methylation acts as a potential molecular mechanism for the clinical effects of probiotics. Further, it may be suggested that probiotic supplementation during pregnancy may support healthy pregnancy by modifying the DNA methylation and consequent gene expression. This may further modify the risk for metabolic aberrations

and the risk for pregnancy-related complications in women (Figure 6). In children, it may be suggested that probiotic intervention during pregnancy may endorse favorable developmental programming by modulating DNA methylation and further an individual's risk for obesity and related co-morbidities (Figure 6). However, it is obvious that detected changes in DNA methylation may be dependent on the used probiotic species or genus. Moreover, affected genes likely act in numerous functions and it is not possible to clarify the overall picture about the clinical significance of probiotic supplementation and life-long health effects based on the current results.

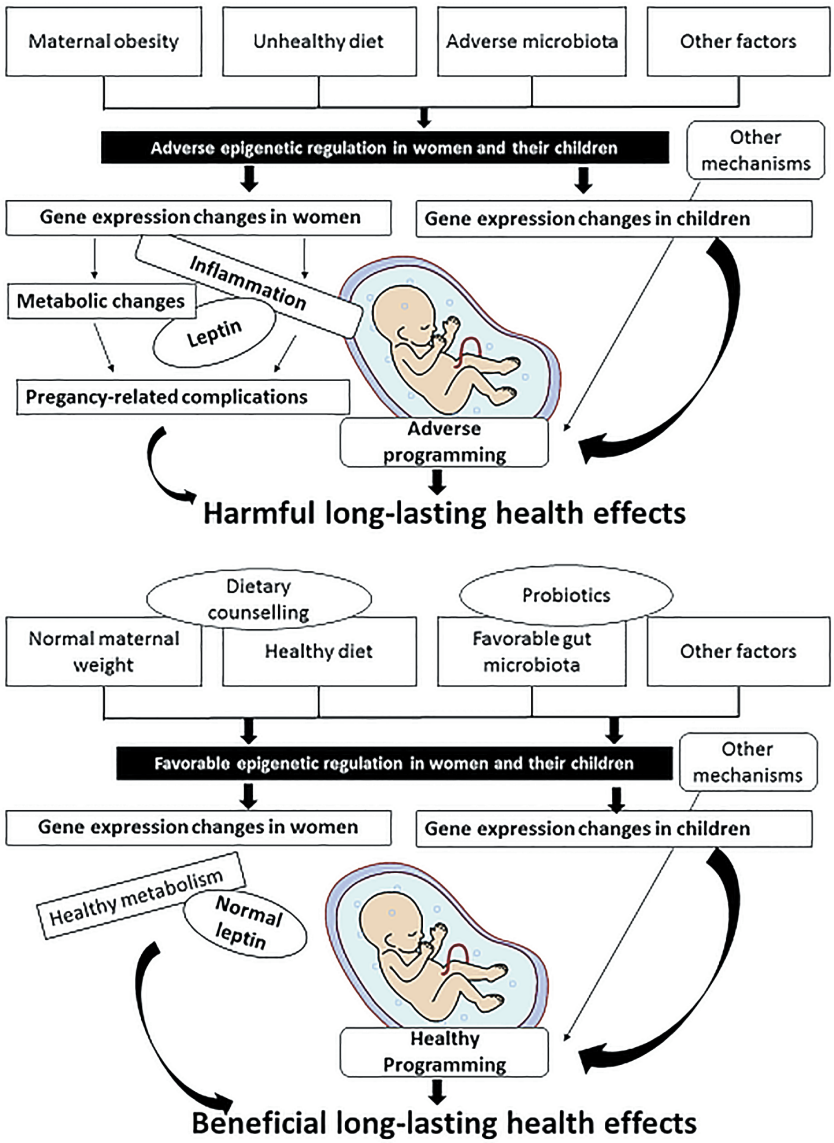

Figure 6. Developmental programming

6.4 Safety aspects of pregnancy-related intervention studies

The safety aspects are of particular importance in studies where the effects are expected to be transmitted from pregnant women to their children (Christian and Stewart, 2010; Fall, 2009). In the present study, it was found that neither dietary counseling nor probiotic supplementation have an effect on women's leptin concentrations. Instead, probiotics tended to increase infant's leptin concentration. Dietary counselling, in turn, affected to the dietary vitamin intake in pregnant women, and some of the changes were also reflected into the breast milk vitamin concentrations. The detected changes seemed relatively small, and it would be expected that they would either have no significant effects or they provide clinical benefits to both women and their children.

Evaluation of the effects of probiotics at the level of DNA methylation revealed that dozens of genes were affected both in women and their children. A more detailed analysis of the affected genes showed that these genes carry out a wide variety of different functions. Further, several of these genes have been linked with known diseases. The analysis of DNA methylation is still quite a new, expensive and uncommon way to evaluate the influences of environment and results are more specific and profound when compared to earlier studies. This raises the question whether detected changes in DNA methylation are safe for women and in particularly for their children. This study followed participants for only a short time period during pregnancy and in early life; therefore it is impossible to evaluate long-term health consequences of the detected changes in DNA methylation. For now, it is not known if these modifications in DNA methylation are temporary or permanent. It is also not known whether the changes in methylation are reflected to the protein level and what the "ideal level" of DNA methylation in specific genes would be at a certain age or environment. In the case of children, it should be evaluated whether changes in DNA methylation during embryogenesis or in early life promote life-long health consequences and whether they are beneficial or detrimental. Interestingly, some recent studies have suggested the intergenerational transmission of DNA methylation in offspring (Yehuda et al., 2015). However, it will take decades before we are able to rigorously evaluate the long-term impact of transgenerational epigenetic changes in human. Because of these aspects the deep and careful evaluation of safety in pregnancy-related interventions is needed.

6.5 Proposals for improvements

The present study consisted of four different sub-studies with a relatively limited number of participants. Moreover, women recruited into the present study were well educated, and their diets were rather healthy even before the dietary counselling sessions. To improve the quality of the study, more participants may have been recruited and study population should have been more heterogeneous.

Dietary intake was determined in the present study using food diaries, which is not a rigorous method for evaluating the quantity and quality of consumed food. Self-reported measures such as food diaries and frequency questionnaires have earlier been shown to underestimate energy intake when compared to objectively measured energy expenditure (Subar et al., 2003). Further, underestimations have been shown to be greater in overweight and obese individuals compared with normal weight individuals (Scagliusi et al., 2009). In addition to reporting error, daily variation in the diet also makes it difficult to make comprehensive conclusions about diet. However, food diaries are an easy, user-friendly, and inexpensive method for evaluate dietary intake. In the future the use of digital photography-based systems may improve the accuracy of the evaluation of dietary intake evaluation (Naska et al., 2016).

DNA methylation changes were detected in the probiotic intervention group. The measurements of corresponding RNA or protein concentrations would have increased the value of the study.

6.6 Future aspects

The present study showed that probiotic supplementation during pregnancy influences on the DNA methylation in dozens of obesity- and weight gain-related genes both in women and their children. It would be very important to follow these mother-child pairs regularly to evaluate if detected changes in DNA methylation are permanent and whether these changes are reflected in the later health of the women or children.

In this study, only obesity- and weight gain-related genes were evaluated. However, it is likely that probiotics also influence other genes. To get a complete picture about probiotics' influence on the DNA methylation, it would be important to also analyze the other genes with other known functions.

Further large clinical studies are needed to understand the detailed interactions between dietary components and developmental programming of obesity risk.

7. SUMMARY AND CONCLUSION

Pregnancy is considered as a window for opportunity for decreasing the risk of obesity and related co-morbidities in the offspring of obese mothers. Therefore, the present study aimed at evaluating the means that would modify the early life environment towards favorable developmental programming and ultimately stop the vicious cycle of obesity. Specifically, dietary counselling and probiotic intervention were tested here by using leptin concentration as a marker for obesity-associated low-grade inflammation, antioxidant vitamin status as a marker for efficiency of dietary counselling, and epigenetic DNA methylation as an indicator of probiotics' effect in women and their children.

Neither dietary counseling nor probiotic supplementation affected leptin concentration in women in the present study. Instead, weight control both before and during pregnancy is the best way to modify leptin concentration and obesity-related inflammation in women. Moreover, dietary modifications such as increased fiber intake and decreased intake of sugar and saturated fatty acids may help to decrease leptin concentration in women. However, probiotic supplementation during pregnancy tended to increase leptin concentration in children, whereas dietary counselling had no effect.

The present study also shows that dietary counselling may be utilized as an inexpensive and useful method to increase the dietary intake of antioxidant vitamins in pregnant women and further to improve the antioxidants concentrations in breast milk. Nevertheless, it is important to take into account that other women's characteristics such as weight and age impact on the women's vitamin status.

Based on the current results, probiotic supplementation during pregnancy modifies DNA methylation patterns of dozens of obesity- and weight gain-related genes both in women and their children. This suggests that probiotics affect the transcription activity of these genes which may further confer health effects to both mother and child. However, it is likely that detected changes in DNA methylation are dependent on the probiotic species or genus used and therefore, more specific clinical trials are needed to illustrate the complete picture about probiotics' effect on DNA methylation. Nevertheless, these results are an encouraging starting point for the studies evaluating the probiotics' potential in favorable developmental programming and ultimately stopping the vicious cycle of obesity.

Taken together, the present results suggest that dietary factors during pregnancy impact on the metabolism and gene activity of women and their offspring. Thereby pregnancy-related interventions such as dietary counselling and probiotic supplementation may be used to modify the early life environment to endorse favorable developmental programming and decrease the risk for obesity and related co-morbidities in the offspring of obese mothers.

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