



**Maijaliisa Erkkola**

# **Diet in Early Life and Antibody Responses to Cow's Milk and Type 1 Diabetes Associated Autoantigens**

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Department of Department of Epidemiology  
and Health Promotion  
National Public Health Institute  
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**Maijaliisa Erkkola**

DIET IN EARLY LIFE AND ANTIBODY  
RESPONSES TO COW'S MILK AND TYPE 1  
DIABETES ASSOCIATED AUTOANTIGENS

ACADEMIC DISSERTATION

*To be presented with the permission of the Faculty of Medicine,  
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*and*

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Äiti leipoo hymyellen. –  
Kelle leivot, äiti, kellen?  
Vielä kysyt - kelles muille:  
pienoisille piimäsuille.  
Tää on kakku pikku Annin,  
tuo on Heikin, tää on Hannin.  
Tämän saapi pikku Asta,  
joll' on yksi hammas Vasta.

Immi Hellén  
(Lasten runokirja, 1930)

Äidilleni ja Isälleni kiitokseksi vahvoista juurista &  
Amandalle, Adalmiinalle, Eemelille ja Taaville  
jokapäiväisen kasvun mahdollisuudesta

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## ABSTRACT

This study evaluated diet during pregnancy and infancy and assessed the relation between the early dietary exposures and the humoral immune responses to cow's milk and type 1 diabetes associated autoantigens in the offspring. Research questions were assessed in three different populations. Altogether 113 pregnant women in the city of Oulu between August 1995 and April 1996 participated in a validation study and 111 in a reproducibility study. In the framework of the validation study, the diet of 118 women for whom we had complete food records was assessed. Humoral immune responses to cow's milk in the offspring were assessed among 97 infants, born between 1995 – 1997 randomized to receive hydrolysed infant formula in the Trial to Reduce Insulin dependent diabetes mellitus in the Genetically at Risk (TRIGR) study. The diet during infancy and humoral immune responses to type 1 diabetes associated autoantigens in the offspring was assessed among a cohort of infants in the Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition Study (children born between 1996 – 2001) in Oulu and Tampere University hospital areas in Finland. Subjects in TRIGR and DIPP studies have a genetically determined increased risk for type 1 diabetes.

The first aim of the present doctoral thesis was to evaluate the validity and reproducibility of a self-administered food frequency questionnaire developed to be used as a dietary instrument, which could be administered after delivery to effectively study the putative effects of the maternal diet during pregnancy on the development of type 1 diabetes. The validity and reproducibility of our 181-item food frequency questionnaire were found to be reasonably good. On average, 70% of the foods and 69% of the nutrients fell into same or adjacent quintiles, according to the food frequency questionnaire and the food record as a comparative method. Pregnancy seemed to be an incitement for dietary overreporting. Our study represents an internationally important contribution to the methodological field of studies into diet during pregnancy.

The second aim was to measure food consumption and nutrient intake during pregnancy, and to evaluate dietary habits and nutrient intake of pregnant women. The results showed that a balanced diet met the increased nutrient requirements during pregnancy, except for vitamin D, folate, and iron. An increase in the consumption of

whole grain cereals, vegetables, and fish, and a decrease in the consumption of foods rich in sugar and saturated fatty acids represent the strategy for improving the intake of critical nutrients: essential fatty acids, dietary fibre, vitamin D, folate, and thiamine. This is also a strategy for reducing the risk of overweight. Our findings suggest that the use of dietary supplements increases during pregnancy, but unfortunately their use is focused on the wrong nutrients. Supplementation is needed to ensure an overall adequate intake of iron and vitamin D. Young, less well educated and smokers were less likely to adhere to dietary recommendations.

The third aim was to assess whether maternal consumption of milk and milk products could affect the development of cow's milk antibodies in infants. In a unique intervention setting, in which the infants did not receive any intact cow's milk proteins during the first 6 months of life, few relationships were established. Protein intake from raw milk products and cheese tended to slightly inhibit the humoral immune responses to cow's milk proteins in the offspring: cheese during the first 6 months and raw milk later, close to 2 years, with the impact being stronger during lactation than pregnancy.

The fourth aim was to investigate infant feeding patterns during the first two years of life, and to study the effects of breastfeeding and age at introduction of complementary foods on the development of type 1 diabetes associated autoantibodies. The results on infant feeding indicated that national and international goals have not been achieved. The median duration of exclusive breast feeding among 3565 infants was 1.5 months, and only every second child was still receiving breast milk at the age of six months. Of the children, 63% were introduced to complementary foods (including cow's milk formula) before the age of 4 months. The diet during infancy was strongly influenced by sociodemographic factors; maternal age and education had a positive and infant's male gender and maternal smoking exhibited an inverse association with the duration of breast feeding and the age at introduction of supplementary foods.

In the largest cohort series so far reported, an early introduction of fruits and berries was related to the risk of advanced  $\beta$ -cell autoimmunity. The finding of the independent association of fruits and berries with  $\beta$ -cell autoimmunity is novel. The next step will be to assess whether these findings can be replicated, and if so, whether they are proxies for other life style characteristics, or reflect a causal relationship.

**Keywords:** diet; nutrition; pregnancy; infant; breast feeding; complementary feeding; cow's milk; food frequency questionnaire; reliability and validity; type 1 diabetes; autoimmunity; autoantibodies; etiology; socioeconomic factor

Maijaliisa Erkkola, Ravinto raskauden aikana ja imeväisiässä ja lapsen vasta-aineet lehmänmaidolle ja tyypin 1 diabetekseen kytkeytyville autoantigeneille

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## TIIVISTELMÄ

Väitöskirjatyössä arvioitiin raskausajan ja imeväisiän ruokavaliota ja niiden yhteyttä lapsen humoraalisiin immuunivasteisiin lehmänmaidolle ja nuoruustyyppin diabetekseen kytkeytyville autoantigeneille. Väitöskirjatutkimus tehtiin kolmessa eri aineistossa. Äidin raskauden ja imetyksen aikaisen ruoankäytön selvittämiseksi kehittämämme kyselylomakkeen validointitutkimukseen (viitemenetelmänä 2 x 5 vrk:n ruokapäiväkirja) osallistui 224 raskaana olevaa oululaisäitiä elokuun 1995 ja huhtikuun 1996 välisenä aikana. Validointitutkimuksen puitteissa selvitettiin tarkemmin 118 tutkittavan raskaudenaikainen ruokavalio. Äidin raskauden ja imetyksen aikaisen maidon kulutuksen yhteyttä lapsen immuunivasteisiin maitoproteiineille selvitettiin lapsuusiän diabeteksen ravintoperäisen ehkäisy tutkimuksen (TRIGR) immunologisen esitutkimusaineiston interventoryhmältä. Tutkittavat 97 vastasyntyntä (syntymävuodet 1995 – 1997) olivat nuoruustyyppin diabeetikoiden 1. asteen sukulaisia, joilla on lisääntynyt diabetesriski HLA-DQB1-tekijöiden perusteella. Tutkimus oli satunnaistettu kaksoissokkokoe, jossa verrattiin tavanomaista äidinmaidonkorviketta ja hydrolysaattia (Nutramigen). Äitien ravitsemusta selvitettiin kehittämällämme frekvenssikyselyllä. Kolmannen aineiston muodostivat kaikki 1996 – 2001 Oulun ja Tampereen yliopistollisissa sairaaloissa syntyneet perinnöllisen diabetesalttiuden omaavat lapset, joiden vanhemmat suostuivat Tyypin 1 diabeteksen ennustaminen ja ehkäisy (DIPP) -tutkimukseen. Lapsen ravitsemusta, menetelminä ruokapäiväkirja, kyselylomake ja lisäruoan aloitusikälomake, infektioita sekä diabetekseen kytkeytyvien autovasta-aineiden ilmaantumista seurattiin 3-12 kuukauden välein.

Väitöskirjatyön ensimmäinen tavoite oli arvioida kehittämämme ruoankäytön frekvenssikyselylomakkeen luotettavuutta ja toistettavuutta ja täten sen käyttökelpoisuutta raskaudenajan ruoankäytön retrospektiivisenä mittausmenetelmänä DIPP -tutkimuksessa. Lomakkeen luotettavuus ja toistettavuus osoittautuivat kohtuullisen hyviksi. Keskimäärin 70 % ruoka-aineista ja 69 % ravintoaineista sijoittui samaan tai vierekkäiseen viidennesluokkaan verrattaessa 181-kohtaisen lomakkeemme ja vertailumenetelmänä käytetyn ruokapäiväkirjan saantimääriä toisiinsa. Raskaus näyttäisi lisäävän ruoankäytön yliparantointia. Tutkimuksemme tuotti kansainvälisestikin merkittävää tietoa raskaudenajan ruoankäytön tutkimisen metodologiasta.

Toisena tavoitteena oli arvioida suomalaisnaisten raskaudenaikaista ruoankäyttöä ja ravinnonsaantia. Monipuolisesti ja tasapainoisesti koostettu ruokavalio tyydyttää raskauden ajan lisääntyneen tarpeen D-vitamiinia, folaattia ja rautaa lukuun ottamatta. Täysjyväviljatuotteiden, kasvien ja kalan käytön lisääminen sekä runsaasti sokeria ja kovaa rasvaa sisältävien ruoka-aineiden kulutuksen vähentäminen on suositeltava tapa kohentaa ruokavalion ravintokoostumusta kriittisten ravintoaineiden osalta. Ravintovalmisteiden käyttö on runsasta, mutta osittain väärin painottunutta. Nuorten, vähän koulutettujen ja tupakoivien äitien ruokavalio oli kauimpana suosituksista. Äitiyshuollon ravitsemusneuvonnan haasteita ovat riskiryhmien seulominen, paino-ongelmien ehkäisy ja hoito sekä erityisruokavaliota noudattavien äitien neuvonta.

Väitöskirjatyön kolmantena tavoitteena oli selvittää vaikuttaako äidin raskauden- ja imetyksenaikainen maitotuotteiden käyttö lapsen immuunivasteisiin maitoproteiineille. Ainutlaatuisessa tutkimusasetelmassa, jossa lasten ruokavaliosta puuttuivat kaikki lehmänmaitoaltisteet ensimmäisen puolen vuoden aikana, havaittiin muutama yhteys. Proteiinin saanti juustoista oli yhteydessä lapsen lievästi alentuneisiin immuunivasteisiin maitoproteiineille ensimmäisen puolen elinvuoden aikana ja proteiinin saanti käsittelemättömistä maitotuotteista vastaavasti lähempänä kahta ikävuotta. Äidin imetyksenaikainen maitotuotteiden käyttö oli raskaudenaikaista käyttöä selkeämmin yhteydessä lapsen immuunivasteisiin maitoproteiineille.

Neljäs tavoite oli arvioida suomalaislasten imeväisruokintaa kahden ensimmäisen ikävuoden aikana ja selvittää vaikuttaako lapsen imeväisiän aikainen ravitsemus diabeteksen immunologisten merkki-ominaisuuksien perusteella määräytyvän diabeteksen esiasteen kehittymiseen lapsilla. Imeväisruokinnan kansalliset ja kansainväliset suositukset ovat Suomessa vielä saavuttamatta. Yksinomaisen imetyksen kesto 3565 tutkitulla lapsella oli vain 1.5 kk, ja vain joka toinen lapsi sai vielä puolen vuoden iässä äidinmaitoa. Lapsista 63 % sai lisäravintoa (tavanomainen korvike huomioitu) ennen suositeltua neljän kuukauden ikää. Perheiden sosioekonomiset erot heijastuvat vahvasti imeväisruokintaan; äidin ikä, koulutus ja tupakointi sekä lapsen sukupuoli olivat vahvasti yhteydessä imetyksen keston ja lisäruokien aloitusikäen. Aikainen altistus marjoille ja hedelmille oli yhteydessä diabetekseen kytkeytyvien autovasta-aineiden ilmaantuvuuteen laajimmassa tähän saakka raportoidussa syntymäkohortissa. Marjojen ja hedelmien itsenäinen yhteys  $\beta$ -solujen autoimmunitettiin on täysin uusi tulos. Seuraava askel on tutkia löydöksen toistettavuus isommalla aineistolla sekä arvioida sen kausaalisuutta.

Avainsanat: ravinto, ruokavalio, raskaus, imetys, lisäruoka, lehmänmaito, frekvenssikyselylomake, imeväisruokinta, diabetes, vasta-aine, autoimmunitetti, sosioekonomiset erot

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## ABBREVIATIONS

BABYDIAB Baby Diabetes Study

BLG Beta-lactoglobulin

BSA Bovine serum albumin

CAS Alpha-casein

CI Confidence interval

DAISY Diabetes Autoimmunity Study in the Young

DASP Diabetes Autoantibody Standardization Program

DIPP Type 1 Diabetes Prediction and Prevention Project

DNA Deoxyribonucleic acid

ELISA Enzyme-linked immunosorbent assay

en% Energy percentage (percentages of total energy intake)

EU European Union

FAO Food and Agriculture Organization

FFQ Food frequency questionnaire

FR Food record

GADA Autoantibodies to the 65-kD isoform of glutamic acid decarboxylase

HLA Human leukocyte antigen

HR Hazard ratio

IAA Insulin autoantibodies

IA-2A Autoantibodies to the protein tyrosine phosphatase-related IA-2 molecule

ICA Classic islet cell antibodies

IgA Immunoglobulin A

IgG Immunoglobulin G

IU International Units

MJ	Mega Joule
OR	Odds ratio
SD	Standard deviation
TRIGR	Trial to Reduce Insulin dependent diabetes mellitus in the Genetically at Risk –study
T1D	Type 1 diabetes
UK	United Kingdom
UNICEF	United Nations Children’s Fund
US/USA	United States/United States of America
WHO	World Health Organization

## List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals (I – V):

- I. Erkkola M, Karppinen M, Javanainen J, Räsänen L, Knip M, Virtanen SM. Validity and reproducibility of a food frequency questionnaire for pregnant Finnish women. *Am J Epidemiol* 2001;154:466-476.
- II. Erkkola M, Karppinen M, Järvinen A, Knip M, Virtanen SM. Folate, vitamin D, and iron intakes are low among pregnant Finnish women. *Eur J Clin Nutr* 1998;52:742-748.
- III. Erkkola M, Kronberg-Kippilä C, Savilahti E, Kenward M, Salonen M, Ilonen J, Knip M, Åkerblom HK, Virtanen SM. Maternal consumption of dairy products during pregnancy and lactation and the development of cow's milk antibodies in the offspring. *Acta Paediatrica* 2005;94:696-704.
- IV. Erkkola M, Pigg H-M, Virta-Autio P, Hekkala A, Hyppönen E, Knip M, Virtanen SM. Infant feeding patterns in the Finnish type 1 diabetes prediction and prevention nutrition study cohort. *Eur J Clin Nutr* 2005;59:107-13.
- V. Virtanen SM, Kenward MG, Erkkola M, Kautiainen S, Kronberg-Kippilä C, Hakulinen T, Ahonen S, Uusitalo L, Niinistö S, Veijola R, Simell OG, Ilonen J, Knip M. Age at introduction of new foods and advanced beta-cell autoimmunity in young children with HLA-conferred susceptibility to type 1 diabetes. (Submitted).

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## 1. Introduction

Type 1 diabetes (T1D) is perceived as a chronic immune-mediated disease with a subclinical prodrome characterized by selective loss of insulin-producing  $\beta$  -cells in the pancreatic islets in genetically susceptible persons (Atkinson & Eisenbarth 2001, Knip 2002a). It is diagnosed by the presence of elevated blood glucose levels, usually before the age of 15 years. T1D is the second most common chronic disease among Finnish children after allergies and asthma and the incidence of this disease in Finland is the highest in the world; more than 40 new cases per 100,000 person-years in 0- to 14-year old children (Karvonen et al. 2000). T1D requires long-term medical attention and it is an expensive disease; the costs to the health service in Finland are on average 6 600 EUR/year per person with complications and 600 EUR/year per person with no complications (Kangas 2002). Large geographic differences, a globally reported conspicuous increase in incidence, and the relatively low concordance in monozygotic twins are factors favouring a major role for environmental factors in the etiology of T1D (Barnett et al. 1981, Bingley & Gale 1989, Kaprio et al. 1992, Green & Patterson 2001, Onkamo et al. 1999, Gale 2002).

Evidence for a role of dietary factors in the development of T1D is inconsistent (e.g. Dahlquist 1994, Gerstein 1994, Norris & Scott 1996, Åkerblom & Knip 1998, Vaarala et al. 1999, Virtanen & Knip 2003). Cow's milk protein has been proposed as being a potential initiating factor in the autoimmune process leading to the destruction of pancreatic  $\beta$  -cells and subsequent development of T1D. The major shortcomings of the human studies performed into diet-diabetes relation so far, using most often the case-control design, are that only single dietary exposures have been assessed at single time points, and that inadequate attention has been paid to confounding factors as well as to the limitations of the dietary methods used. Due to lack of knowledge on the relevant period of exposure, and the relatively low incidence of this most likely multifactorial disease, the long-term diet needs to be evaluated if one wishes to study diet-based relations of T1D.

There is clear evidence to suggest that the environmental processes influencing the propensity to disease in adulthood operate during the periconceptual, fetal, and infant phases of life (Barker 1995). This emphasizes the need for consistent and thorough assessments of women's diet throughout pregnancy and children's diet in early infancy. There have been very few methodological studies conducted in pregnant women and infants (Suitor et al. 1989, Greeley et al. 1992, Forsythe & Gage 1994, Brown et al. 1996, Robinson et al. 1996, Wei et al. 1999, De Vriese et al. 2001, Fawzi et al. 2004, Andersen et al. 2003; 2004). Despite the frequent use of food frequency questionnaires in epidemiological studies, there is a need to develop and evaluate questionnaires that extend the range of ages, populations, settings, and dietary factors to be studied (Willett 1994).

Dietary data on Finnish pregnant women and children is dispersed, scanty and out-of-date. The latest survey on the diet of pregnant Finnish women dates from the early 1970s (Pietinen 1974). According to more recent surveys, the intakes of folate, thiamine, and vitamin D are low among Finnish women of childbearing age (Alfthan et al. 2003, Männistö et al. 2003). Increasing obesity is the main nutritional problem in Finnish women of childbearing age (reviewed by Lahti-Koski & Sirén 2004) and in Finnish pregnant women (Kinnunen et al. 2003). In the recently published Finnish nutrition recommendations for infants and young children (Hasunen et al. 2004), exclusive breastfeeding is recommended until the age of 6 months, complementary feeding starting individually at the latest at the age of six months. A short duration of breastfeeding and introduction of complementary foods at an early age is claimed to be associated with the development of several adverse health outcomes, such as impaired neurocognitive development and development of chronic diseases and obesity (Anderson et al. 1999, Chandra 2002, Ivarsson et al. 2002, Jain et al. 2002, Owen et al. 2002; 2003; 2005, Virtanen & Knip 2003). Promoting the health and nutrition of females of reproductive age and children in early infancy could be seen as one element for the prevention of health problems in the coming generation.

This thesis aims to evaluate diet of pregnant women and infants, and to assess the relation between the early dietary exposures to the development of cow's milk antibodies and T1D associated autoantibodies. Accordingly, one specific objective was to evaluate the validity and reproducibility of a self-administered food frequency questionnaire (FFQ) developed to measure food consumption and nutrient intake during pregnancy.

## **2. Review of the literature**

### **2.1 Assessment of diet during pregnancy and infancy**

#### **2.1.1 Pregnancy**

The assessment of dietary intake in pregnant women is complicated by various factors depending on the phase of the pregnancy. The obtained overall picture of the diet during pregnancy is, therefore, most probably influenced by the timing of the dietary assessment. Nausea and vomiting are the most common symptoms experienced in early pregnancy, with nausea affecting between 70 and 85% of women and about half of women vomit during pregnancy (Jewell & Young 2003). Accordingly, approximately two thirds of pregnant women develop heartburn, this occurring most frequently during the last two trimesters of pregnancy (Richter 2003). In general, most food cravings cause an increase in calcium and energy intakes, whereas food aversions lead to decreases in the intakes of alcohol, caffeine, and animal protein (King 2000). Food cravings and aversions do not necessarily have any deleterious effect on the quality of the diet, but appetite fluctuations and nausea may influence the long-term diet reports (Wirfält 1998).

The ability of women to recall their diet during pregnancy and the accuracy of the information they do recall has rarely been assessed. Changes in food intake during pregnancy tend to be relatively small and, therefore, difficult to detect by using the rather imprecise dietary assessment methods currently available (King 2000). Evidence exists that people whose dietary habits are relatively stable are more likely to be able to successfully recall past diet (Cade et al. 2002). However, based on a few studies, diet during pregnancy is recalled with similar accuracy as, or perhaps even a slightly lower accuracy than the adult diet in general (Bunin et al. 2001). In the Tecumseh Diet Methodology Study (Thompson et al. 1990), greater total diet reproducibility was found among normal weight women who did not report consuming any special diet and among women reporting no medication.

### **2.1.2 Infancy**

Diet during childhood tends to be highly variable from day to day (at least twice as great as that of the adults), this being especially true after the age of one year when most of the complementary foods are introduced (e.g. Miller et al. 1991, Thompson & Byers 1994). Due to the dramatic changes in food patterns during the first years of life with significant individual variation in the timing of these changes, the food consumption and nutrient intake vary extensively among 1-2 years old children, as has been demonstrated in previous Finnish studies among infants (Räsänen & Ylönen 1992, Simell et al. 2000). Food records of 7 to 8 days seemed to be a reasonable way of assessing current individual intake of energy and macronutrients in a child population aged 5 to 14 years, though a proper assessment of the intakes of some vitamins would have required keeping a diary for more than 20 days (Miller et al. 1991). Simple, valid, and reliable tools to measure infant feeding are lacking (Ruel et al. 2003). The problem of measurement arises primarily because infant feeding practices encompass a series of age-specific, interrelated behaviours that are difficult to summarize into one or even a few variables. Piwoz and colleagues (2003) offer three indicators to be used to measure and define dietary diversity, quality and frequency of complementary feeding both in developed and developing countries. It is recommended that the proportions of children fulfilling the defined indicators should be described in studies on infant feeding.

When assessing diet in infancy and early childhood, all information by necessity has to be obtained by surrogate reporters. An individual who spends most of the time with the child is thus the best surrogate reporter. The accuracy of surrogate information does not seem to differ between mothers and fathers (Eck et al. 1989). Factors that lessen the time that the parents spend with the child, such as employment outside of the home and larger family size, could possibly lead to less accurate reporting of the child's diet (Willett 1998). The process of food registration does not affect the appetite or food preferences of the infant. However, it is possible that parents want to idealize what they actually give to their infants or report having given them. In the well conducted Norwegian validation studies among 12 and 24 months old children, the food items underreported were typical

unhealthy foods like cake, soft drinks and sweets, while the overreported foods were more healthy foods like bread, fruit and potatoes (Andersen et al. 2003; 2004). It was also found that the parents with more than one child seemed to provide more valid data of their child's diet than the parents with only one child.

Another important question is whether the child's health affects the parents' recall. Emotional and behavioural impacts have been associated with islet autoantibody-positive status, some individuals even reporting lifestyle or health behaviour changes in an effort to delay or prevent type 1 diabetes (T1D) onset (Bennett Johnson & Tercyak 1995, Bennett Johnson et al. 2004, Hummel et al. 2004b). In the longitudinal investigation of the impact of newborn genetic screening for T1D, low level of maternal education, having a child of female gender, being a single mother and having a first degree relative with T1D were the characteristics related to difficulties in understanding the T1D risk and to experience greater anxiety in response to risk notification (Bennett Johnson et al. 2004). However, in most of the cases the initial anxiety appears to dissipate to normal levels over time, and long-term behavioural changes will not be likely to occur.

## **2.2 Methodological issues in dietary assessment in long-term studies**

### **2.2.1 Food frequency questionnaire (FFQ)**

*= a questionnaire in which the respondent is presented with a list of foods and is required to say how often and in what quantity each is eaten in broad terms such as x times per day/per week/per month (Margetts and Nelson 1997).*

In the 1940s Burke (1947) developed a detailed dietary history including a checklist of foods consumed over the preceding month. This checklist represents the forerunner of the more structured dietary questionnaires in use today. During the 1950s a FFQ was further developed and its role in dietary assessment was evaluated (reviewed by Willett 1998). After the failure to observe any correlations with serum cholesterol in the Tecumseh Heart Study (Nichols et al. 1976), interest in the FFQ waned. However, as a result of initiation of several large cohort studies in nutritional epidemiology in the 1980s, there has been a new upswing in the popularity of FFQ and it is now

considered as the primary dietary assessment method in nutritional epidemiology (Willett 1998, Cade et al. 2002). Despite the current frequent use of the FFQ, there are still target groups whose special characteristics as FFQ users are not well evaluated. Additional effort must be given to developing and evaluating questionnaires that extend the range of ages (especially for children), populations, settings, and dietary factors that are studied (Willett 1994). Recently published validation studies among 12-24 month old Norwegian children claimed that FFQ was able to rank small children according to intakes of nutrients and food items (Andersen et al. 2003; 2004). The average proportions of subjects appearing in the same quartile of nutrient intake estimated by the FFQ and weighed records were 38% among 1 year old and 36% among 2 years old. Despite that, the FFQ seemed to represent a valuable tool for measuring average intakes of macronutrients and several food items if estimates were adjusted for energy intake.

Developing an FFQ on the basis of common foods may not explain the between-person variation required for ranking individual intake in diet-disease studies (Cade et al. 2002, Shai et al. 2004), and careful attention must be given to choice of foods as well as the format of the frequency response section. It is preferable in aetiological studies to have a comprehensive food list enabling computation of the full range of nutrients rather than a restricted list to determine the intakes of a few nutrients. A comprehensive food list is also a prerequisite for energy adjustment. The median number of food items in the questionnaires analyzed in the comprehensive review of Cade et al. (2002) was 79 (range 5 – 350). There is a rapidly decreasing marginal gain in the amount of information obtained with increasingly detailed questionnaires (Pietinen et al. 1988a; 1988b). Particularly in a self-administered format, approximately 130 food items may be approaching the limit after which fatigue and boredom can impair concentration and accuracy, and increase the risk of over reporting (Willett 1998). Additional questions producing qualitative information would improve the validity of some estimates; however, energy adjustment has a greater impact on the validity of fat estimates than can be obtained by asking additional questions (Wolk et al. 1998). In

long-term cohort studies, the FFQ may need to be continuously modified due to new foods becoming available over the duration of the study and changes in dietary patterns.

Foods consumed near the time of FFQ administration may prime the memory, such that FFQ responses emphasize recently consumed foods. The FFQ data obtained from 74,958 women in the Shanghai Women's Health Study was consistent with that theory (Fowke et al. 2004). However, it was concluded that the season of FFQ administration does not alter dietary exposure category assignments sufficiently to impact on the interpretation of most epidemiologic studies. The FFQ requires respondents to present rather complex cognitive tasks, which could increase the possibilities of bias and lead to a more selected group of subjects (Flegal 1999).

### **2.2.2 Assessing reproducibility and validity of the FFQ**

The reproducibility of FFQs has generally been assessed by administering them at two time points to the same group of people and testing the association, most often via a correlation coefficient, between the two responses (Willett 1998, Cade et al. 2002). The use of the correlation coefficient has been criticized because it measures only the degree to which the two measurements are related; not the agreement between two administrations of the questionnaire unlike the more comprehensive method created by Bland and Altman (1995; 1999). The time interval between two responses could affect the reproducibility. A very short interval may lead an overestimation of the true reproducibility due to the respondents' ability to remember their previous answers. The interval between repeat measurements should be chosen to minimize changes over time and recall of previous answers, and will depend on the reference period of the FFQ (Cade et al. 2002). For individual food items, a lower initial reproducibility, infrequent consumption, and a larger difference in seasonal intake have been shown to be associated with a greater reduction in reproducibility over time (Tsubono et al. 1995). Consequently, the risk ratios based on exposure data obtained from food frequencies could be markedly affected by misclassification and temporal changes in responses (Hashimoto et al. 1997). However, Willet (1998) does not consider the

difficulty in separating variation due to questionnaire performance from a true change in diet extremely serious from the standpoint of evaluating measurement error; both sources of variation realistically contribute to misclassification of long-term dietary intakes.

Validation studies assess the degree of the FFQ to measure those aspects of diet that it was intended to measure (Willett 1998). A valid FFQ accurately reflects typical food consumption over a designated period of time, being undistorted by behavioural patterns or false memory. Due to the fact that all dietary assessment methods involve some degree of measurement error, the relative validity, rather than absolute validity, is measured. Among the feasible comparative methods available for validating FFQ, food records are likely to have the smallest correlated errors and have, therefore, been commonly used for this purpose. A reasonable size for a validation study is from 100 to 200 persons assuming that a sufficient number of days of dietary information are obtained to reasonably describe an individual's true diet over the reference period (Willett 1998). In most settings, the optimal study design will rarely require more than four or five diet records per subject (Stram et al. 1995). The number of days needed depends also on the within-person variability for the nutrients of interest (e.g. Willett 1998). The days should be randomly dispersed over the time period of interest to include short- and long-term sources of variation. Biochemical markers are potentially useful but often expensive and available only for certain nutrients.

There is no consensus on the best statistical method to be used for assessing the validity of FFQ (Cade et al. 2002, Flood et al. 2004). The use of a variety of statistical approaches guided by the purpose of measuring diet with the FFQ is recommended (Burley et al. 2000, Cade et al. 2002). Two recent papers have compared several different statistical methods for assessing the relative validity of FFQ with 4-day weighed diet records (Masson et al. 2003, Flood et al. 2004). Different methods of analysis provide different information, and measures of agreement differ also between nutrients and gender. None of the methods is fully comprehensive when used alone, and the need to

use several methods was demonstrated in both studies. Masson et al. (2003) concluded that with Spearman correlation coefficients above 0.5, more than 50% of subjects correctly classified and less than 10% of subjects grossly misclassified into thirds, and weighted kappa values above 0.4 are recommended for nutrients of interest in epidemiological studies. However, Willett (1998) discouraged the use of the kappa statistic for comparing ordinal nutrient intakes due to its considerable disadvantages.

### **2.2.3 Under- and over reporting**

In addition to a selection bias of better-off populations, a systematic bias due to under- or over reporting is a major source of bias in dietary surveys. It is evident that bias in reporting total energy intake is associated with variable bias in estimated nutrient intake. There are three main determinants of variation in energy intake between individuals: body size, physical activity, and metabolic efficiency (e.g. Willett 1998). Epidemiological studies of diet and disease should be principally directed to the effects of the nutrient composition of the diet independent of total energy intake. Adjustment for total energy is appropriate to control for confounding, reduce extraneous variation, and predict the effect of dietary interventions (Willett et al. 1997).

Subjects commonly tend to report their food intake in a socially desirable way, by reporting less frequently the foods which are considered unhealthy or fattening, whereas foods considered healthy are reported more frequently (Andersen et al. 2003; 2004, Scagliusi et al. 2003). Body mass index over 25 kg/m<sup>2</sup>, female gender, older age and high educational level are among the most common predictors for underreporting (reviewed by Livingstone & Black 2003), and also noted in a Finnish survey (Hirvonen et al. 1997). Though it has been thought that there is an inverse association with socio-economic status, Black and Cole (2001) found that biased over- or underreporting is characteristic of some persons and not overtly dependent on socio-economic differences. In the European Prospective Investigation of Cancer (EPIC) Norfolk –study (Bingham et al. 2001), which

is one of the largest epidemiological studies of nutrition in the United Kingdom, there were significant differences in mean intake of all nutrients measured by two different methods, FFQs and diet records, in women but less so in men. In validation studies, FFQs have generally overestimated absolute intakes when compared with food records or 24-hour recalls (Willett 1998, Cade et al. 2002). However, overestimation is not necessarily problematic in epidemiological studies if the ranking of the persons according to their dietary intake is valid (Willett 1998).

#### **2.2.4 Quality of food composition database**

Assessment of a long-term diet requires a continuously and properly updated food composition database (Cade et al. 2002, Kronberg-Kippilä et al. 2003). Changes over time in the content of prepared and processed foods, variations in recipes and cooking practices, and geographic and environmental variability may all contribute to a difference between the actual nutrient value of a specific food and the value calculated from a food database (Flegal 1999). For most nutrients and most populations, the groups at risk are those in the upper or lower percentiles of nutrient consumption. The lack of database updating and inadequate sampling underpinning the estimates of mean nutrient concentrations will create the greatest errors in the regions of greatest risk (Stewart 1997). Cowin and Emmett (1999) studied the effect of missing data in McCance and Widdowson's food tables and supplements (Royal Society of Chemistry 2003) used for calculating nutrient intake of 3-day dietary diaries completed for 1026 children aged 18 months. The underestimation of nutrient intake was largest for vitamins E and D (13.8% and 14.7% of total intake, respectively). The effect of missing data on calculated nutrient intakes was proportionately greater at the bottom end of the nutrient intake distribution. Even though missing data had a fairly small effect on calculated mean daily intakes, it could result in some individuals being misranked within a nutrient intake distribution.

Collection of data on supplement use is often limited by costly and time-consuming process of setting and updating a reliable database due to expanding and highly changeable market in these

products. Estimates of consumption of specific supplements may provide sufficient precision to correctly classify individuals into either users or non-users of those supplements. However, precise details of the products consumed are required in order to rank individuals into categories of nutrient intakes (Cade et al. 2002).

The quality of research data is also affected by how skilful and well trained the research personnel are and by how standardized are the data collection and entering methodologies. Validating and standardizing the dietary methods is very important in long-term cohort studies, in which the research personnel may change over the course of the study. In the comparison of surveys and in international surveys of food consumption and nutrient intake, it is essential that the dietary data are comparable when different databases and calculation programs are used. In the comparison between Swedish and Finnish food databases, differences between estimated intakes reflected either actual differences in foods between the two countries or methodological differences in the assessment of nutrient intakes (Hakala et al. 2003).

## **2.3 Nutrition in pregnancy**

### **2.3.1 Influence on fetal growth**

Nutrition is the major intrauterine environmental factor that alters expression of the fetal genome and may have lifelong consequences (e.g. Wu et al. 2004). Perinatal nutrition is recognized to have a profound and persistent influence on neurologic development and cognitive functions (e.g. Larque et al. 2002). It has been shown in animal studies that both maternal undernutrition and overnutrition reduce placental-fetal blood flow and can stunt fetal growth. Fetal growth seems to be most vulnerable to maternal dietary deficiencies of nutrients during the pre-implantation period and also during the period of rapid placental development, i.e. the first trimester of gestation (Hobel & Culhane 2003). The early stages of fetal nutrition and growth are to a large extent determined by the nutritional state of the mother at the time of conception (Jackson 1996). Therefore, the nutritional status, and body composition of a woman at the time she becomes pregnant are at least as important

as her nutrient intake during the course of the pregnancy itself for the growth of her fetus. Fetal development can be affected by nutritional variation within the normal range of western diets (Godfrey et al. 1996). A large body of epidemiological evidence supports the association between several nutritional deficiencies before or during pregnancy and maternal morbidity, length of pregnancy or fetal growth (Kramer 2003). It is unlikely that any specific nutrient on its own can prevent the major health problems encountered in pregnant women and developing fetuses. The complex relationship between maternal nutritional and birth outcomes emphasizes the need for consistent and thorough assessments of women's diets throughout the duration of their pregnancy.

### **2.3.2 Fetal origins of diseases**

Epidemiological observations have led to the hypothesis that certain conditions, most likely nutritional in origin, “program” the fetus for the development of chronic diseases in adulthood (Waterland & Garza 1999, Rasmussen 2001, Bateson et al. 2004, Gluckman & Hanson 2004a; 2004b). The extensive literature on research in evolutionary biology, developmental biology, and animal and human physiology provides support for this idea and suggests that environmental processes influencing the propensity to disease in adulthood operate during the periconceptual, fetal, and infant phases of life. Since the first evidence for programming obtained in birds over 100 years ago (Spalding 1873), some of the most ground-breaking observational studies in humans were done by Barker (1992) in the late 1980s. He and his colleagues showed that small size at birth or in infancy is associated with an increased propensity to adverse health outcomes in adulthood. Based also on later additional data, Barker and his colleagues proposed that different profiles of poor fetal growth for each trimester are associated with hypertension and/or diabetes and stroke (Barker 1995, Godfrey et al. 1996). The proposed mechanism is related to the regulation of allometric growth with those organs most crucial for continued survival of the organism, in particular the brain, being favoured over organs such as the pancreas, kidney or endothelial cells. This "developmental origins of health and disease" is not yet established to be causal. Only a handful of studies have been able to examine the relationship between maternal nutrition in pregnancy with the health of offspring in

adult life directly. In fact, some studies could find no evidence for maternal intake of nutrients being an important determinant of the long-term health of infants of the relatively well-nourished women in industrialized countries (e.g. Mathews et al. 2004). In fact, an opposite relationship has been observed. Researchers in this field are faced with the methodological difficulties of eliminating biases and adequately adjusting for potentially important confounding factors in retrospective studies; this is known to be a complex task (reviewed by Waterland & Garza 1999).

Gluckman and Hanson (2004a, 2004b) have introduced a model in which the embryo or the fetus develops predictive responses (either disruptive or adaptive) according to environmental stimuli. If the prenatal and postnatal environments match, the physiological settings achieved through the processes of developmental plasticity will leave the organism well prepared for the postnatal environment. Conversely, a mismatch between the prenatal and postnatal environments may be pathogenic. Based on that model, they have emphasized the importance of the need to promote the health and nutrition of females of reproductive age as one element for the prevention of chronic disease in future generations across the globe. Other have claimed that it is too early to use the “metabolic imprinting” hypothesis as a basis for new interventions directed at pregnant women for the purpose of reducing chronic disease in their offspring (Rasmussen 2001). However, such interventions could be important in populations that experience high rates of low birth weight (Waterland & Garza 1999).

### **2.3.3 Transfer of nutrients and antibodies to the fetus**

The transfer of nutrients to the fetus depends on the maternal nutritional status and on the adequacy of uterine blood flow. Fat-soluble vitamins cross the placenta by simple diffusion, carbohydrates by facilitated diffusion, and amino acids, water-soluble vitamins, and some minerals by active transport (McGanity et al. 1994). Placental transport mechanisms and placental metabolism play major roles, independently and together, in providing for the nutritional requirements of fetal growth and metabolism. For some nutrients, the infant’s status is maintained at the expense of the

mother; for other nutrients mother and offspring compete more evenly, and for others the infant can suffer more severe consequences due to deficiency than the mother (Garza 1993). The neonatal immune system is functionally immature and undergoes a period of extensive differentiation and maturation once exposed to foreign proteins and other dietary components (Hughes 1998). There is a transfer of anti-idiotypic antibodies from the mother to the fetus (Malek et al. 1996). Newborns have predominantly maternal IgG in their serum, but after 3 months, most of the serum IgG has been produced by the infant (Sarvas et al. 1993).

In utero fetal exposure to an allergen from around 22 weeks of gestation may result in primary sensitisation to that allergen, leading to positive proliferative responses, at birth (Jones et al. 1996). However, the placenta seems to protect the fetus against the effect of maternal food antigens, and specific sensitization has been demonstrated only occasionally in newborn infants (Lovegrove et al. 1994, Zeiger 2000). The latest Cochrane database review concluded that prescription of an antigen avoidance diet to a high-risk woman during pregnancy is unlikely to reduce substantially her child's risk of atopic diseases; such a diet may even adversely affect maternal and/or fetal nutrition (Kramer & Kakuma 2003).

#### **2.3.4 Dietary recommendations during pregnancy**

The latest national nutrition recommendations for infants and young children as well as pregnant and breastfeeding mothers were published in Finland in 2004 (Hasunen et al. 2004). The dietary guidelines are based on the Finnish Nutrition Recommendations issued by the National Nutrition Council (Valtion ravitsemusneuvottelukunta 2005) and the latest research. The main message concerning nutrition during pregnancy is simple; healthy mothers with a balanced diet do not need to make any specific changes in their diet during pregnancy and breastfeeding. Some specific quantitative guidelines are given for fruits and vegetables (5-6 portions per day), and liquid milk products (8 dl per day), but the given overall picture is that dietary guidelines may be achieved with

a wide range of foods. However, further clarification is given for the use of several of the food items listed in table 1.

**Table 1. Specific dietary recommendations for pregnant women (based on Hasunen et al. 2004)**

<b>Food to be restricted/avoided</b>	<b>Basis for the given advice</b>	<b>Literature</b>
Liver and liver products	High content of vitamin A and heavy metals → increased risk of malformation and preterm birth.	Rothman et al. 1995, Miller et al. 1998, Elintarvikevirasto et al. 2004
Pike and high use of freshwater fish species: perch, pike-perch and burbot, in large sizes. Baltic salmon and herring longer than 17 cm may be eaten only a few times a month.	Contain neurotoxic agents such as methylmercury → a range of effects varying from severe cerebral palsy to subtle developmental delays.	Castoldi et al. 2001
Vacuum packed fish, roe, un-pasteurized milk products and soft cheeses.	Foodborne illnesses like L. monocytogenes infection → abortion, stillbirth or severe neonatal infection.	Smith 1999, Doganay 2003, Rocourt et al. 2003,
Artificial sweeteners	Saccharin and cyclamate → a bladder cancer-inducing effects had been reported from animal studies in rats.	Weihrauch & Diehl 2004
Licorice and salmiac	High glycyrrhizin content → increases risk of preterm delivery.	Strandberg et al. 2002
Alcohol	Teratogenic effect → fetal alcohol syndrome.	Jones & Smith 1973, Kaskutas & Graves 2001, Cogswell et al. 2003, O’Leary 2004, Mukherjee et al. 2005
Caffeine	Is a methylated xanthine that acts as a mild central nervous system stimulant → preterm birth and low-birth-weight infants, but the findings are contradictory. Increased risk of fetal death in a recent Danish cohort study.	Hinds et al. 1996, Christian & Brent 2001, Leviton & Cowan 2002, Bech et al. 2005

### **2.3.5 Nutrient intake in pregnancy**

The estimated energy requirement during a full-term pregnancy, in excess of a woman's nonpregnant need, is estimated to be  $\approx 335$  MJ (FAO/WHO/UNU 1985). Well-nourished women use different strategies to meet the energy demands of pregnancy including reductions of diet-induced thermogenesis or activity energy expenditure, increases in energy intake, and deposition of less body fat mass than anticipated (Kopp-Hoolihan et al. 1999), and therefore, the use of a single recommendation for increased energy intake in all pregnant women is not justified. Butte and his colleagues (Butte et al. 2003, Butte et al. 2004) have recently questioned the common thinking that the energy cost of pregnancy could be met without any increase of food intake by economy of activity; their studies showed that appropriate, but not excessive, gestational weight gain is needed to optimize infant birth weight and to minimize maternal postpartum fat retention. They also showed that weight gain above the recommendations based on prepregnancy basal metabolic indexes tends to consist of fat, which confirms the belief that such weight gain is undesirable.

The fetal pattern of nutrient demand varies in detail at each stage of gestation. Nutritional deficiencies and infections among pregnant women are rather prevalent in many developing countries as well as in inner-city populations of industrialized countries. Nutrients are provided to pregnant women as supplements to food either to increase the intake of those individuals with a deficiency or to obtain a pharmacological, perhaps non-nutritional, effect in individuals with an adequate intake of the nutrient; a borderline situation in between those two extremes being sometimes fluctuating and critical during pregnancy. Several nutritional interventions have been shown to be effective during pregnancy including iron and folate supplements in reducing anemia, and calcium supplementation in lowering the risk of hypertension (Fall et al. 2003, Villar et al. 2003). There has been an increasing interest in the role of essential fatty acids during pregnancy during the last two decades. The essential fatty acids requirements are especially high during the last trimester of pregnancy because of the rapid synthesis of brain tissue (Al et al. 2000). However,

more studies are still needed to clarify their functional benefits for mothers and children. Studies in developed countries have indicated that folate, vitamin D, and iron are the most critical nutrients in terms of deficiencies during pregnancy (Table 11; Ortega et al. 1994, Henriksen et al. 1995, Rogers & Emmett 1998, Rogers et al. 1998, Giddens et al. 2000, Hess et al. 2001, French et al. 2003, Turner et al. 2003) and they are therefore reviewed in more detail below. The only data on dietary habits and nutrient intakes of Finnish pregnant women dates from the early 1970s (Pietinen 1974). According to the Findiet 2002 –study (Männistö et al. 2003), the intakes of folate, thiamine, and vitamin D were low in Finnish women of childbearing age.

Due to the rather low energy costs during pregnancy, the increased requirements of vitamins and minerals during pregnancy have to be met by enhancing the nutrient density of the diet. The use of some dietary supplements is recommended for all pregnant women (Table 2) (Nordic nutrition recommendations 2004, Hasunen et al. 2004, Valtion ravitsemusneuvottelukunta 2005).

**Table 2. Recommendations on nutrient supplementation during pregnancy (Hasunen et al. 2004, Valtion ravitsemusneuvottelukunta 2005)**

Nutrient	Dose	Target group
Vitamin D	10 µg	All women during October – March.
Iron, Fe <sup>2+</sup>	50 mg	If needed; from 12. pregnancy week if Hb < 110 g/l and later immediately if Hb < 100 g/l.
Folic acid	400 µg	Selected groups: <ul style="list-style-type: none"> <li>- women on very unbalanced diet, and</li> <li>- women with certain chronic illnesses (such as celiac disease, diabetes) and women using certain drugs (e.g. epilepsy treatment).</li> </ul>
	4 mg	Women with an increasing risk of neural tube defects in the offspring.
Calcium, Ca <sup>2+</sup>	500 – 1000 mg	Women having a low intake of milk and milk products, or calcium fortified foods.
Multivitamin and mineral supplementation		Women on very unbalanced diet. Overlapping use of supplements is not advisable.

## *Folate*

During the last decade, folate has become a topic of considerable interest and there is an intense, on-going debate on the folate needs of women of childbearing age if one wishes to reduce the risk of babies born with neural tube defect (e.g. Lewis et al. 1999). A new interest of research is the relation between folate and homocysteine intakes and the risk of cardiovascular disease (Boushey et al. 1995, Rasmussen et al. 2000). Due to the increased demands that are placed on the supply of folate during pregnancy for the synthesis of DNA and other one-carbon transfer reactions, pregnant women are at a higher risk of developing folate deficiency than non-pregnant women (Bailey 2000). Spina bifida (neural tube defect) results from failure of fusion of the caudal neural tube, and is one of the most common malformations of human structure (Mitchell et al. 2004). As many as 70% of spina bifida cases can be prevented by maternal, periconceptional folic acid supplementation, first demonstrated in the trials of Smithells and colleagues (1980). The mechanism underlying this protective effect is unknown, but it is likely to include the genes that regulate folate transport and metabolism. Inadequate folate intake and low serum folate concentrations have also been shown to be associated with other poor pregnancy outcomes, including a greater risk of preterm delivery and infant low birth weight (Scholl et al. 1996, Siega-Riz et al. 2004).

The main dietary sources of folate in Finnish diet are vegetables 12%, wholemeal ryebread 11%, fruits 10%, and potato 10% (Alfthan et al. 2003). Folic acid supplements taken with food are calculated to be 1.7 times more available than the folate occurring naturally in foods (Bailey 2000). The losses of folate by oxidation and during heat treatments have to be taken into account when evaluating the actual intake from food (Bergström 1994). It is also assumed that nutrient databases currently provide underestimates of the folate contents of foods due to methodological problems in the analytic assays used to measure food folate (Bailey 2000).

In the Finnish study of Alfthan et al. (2003), the mean dietary folate intake in women was 205 µg/day (33 µg/MJ), which is half of the recommended daily intake (400 µg/day) during pregnancy. Furthermore, the dietary folate intake is also inadequate for those who want to become pregnant; a finding which has been noted in many other countries as well (Brussaard et al. 1997). Pregnant women are advised to increase their consumption of foods rich in folate (Hasunen et al. 2004). However, the international retrospective cohort study covering more than 13 million births (Finland included) detected no detectable change in rates of neural tube defects associated with recommendations to consume more folic acid (Botto et al. 2005). The authors recommended rapid integrated food fortification with greater implementation of recommendations on supplements; a strategy which is also supported by other studies on the same field (e.g. Langley-Evans & Langley-Evans 2002). Lack of awareness of the importance of folate seems to be the most common reason given for choosing not to use folic acid supplements before pregnancy (French et al. 2003). However, the knowledge about folate seems not to be related to its intake.

### *Vitamin D*

Unlike many other nutrients used in the fortification of foods, the purpose of vitamin D is to correct for an environmental deficit (less ultraviolet exposure) and not to correct for lack due to classical nutritional reasons (Vieth 1999). Our evolution has effectively adapted human beings to live in the presence of far more vitamin D than the levels present today for much of the world's population, yet there is no consensus about optimal or safe vitamin D intake. The recommended dietary allowance of vitamin D (National Academy of Sciences 1989) as well as the Nordic (Nordic Nutrition Recommendations 2004) and the Finnish nutrition recommendation (Valtion ravitsemusneuvottelukunta 2005) during pregnancy is 10 µg (400 IU). The scientific basis for this recommendation is not well defined; nor is the appropriate dose of vitamin D during pregnancy. The recommended dose has been criticized as having little or no effect in women (Vieth 1999, Hollis & Wagner 2004). Several studies have indicated that doses exceeding 25 µg (1000 IU)

vitamin D per day during pregnancy and lactation are required to achieve a robust normal concentration of circulating 25(OH)D (reviewed by Vieth 1999, Hollis & Wagner 2004). Studies reviewed by Vieth (1999) support the belief that total-body sun exposure can easily provide the equivalent of 250 µg (10 000 IU) vitamin D/day, suggesting that this is a physiologic limit. The exact amount of vitamin D required to induce toxicity is unknown in humans. In the well known case series by Adams and Lee (1997) four individuals taking several dietary supplements containing vitamin D presented with hypercalciuria that moderated after vitamin D intake was terminated. Since vitamin D is potentially toxic, intake of > 25 µg (1000 IU)/day has not been recommended even though there is no evidence of adverse effects on serum 25(OH)D concentrations which do not exceed 140 nmol/L, to exceed that level is thought to require a total vitamin supply of 250 µg (10 000 IU) (reviewed by Vieth 1999). In comparison to the toxic amounts in animal models, millions of units of vitamin D would have to be ingested to achieve the same results in humans.

Adequate vitamin D concentrations during pregnancy are necessary to ensure appropriate maternal responses to the calcium demands of the fetus, which are estimated to be up to 250 mg/day during the last trimester (Widdowson 1981). Vitamin D insufficiency and deficiency during pregnancy is reflected in lower maternal weight gain and in extreme situations, reduced bone mineralization, rickets and fractures in the infant (reviewed by Pawley & Bishop 2004). The vitamin D status of the human fetus and neonate is totally dependent on the vitamin D stores of the mother (Hollis & Pittard 1984). Observational studies and vitamin D supplementation trials among pregnant women at high risk of vitamin D deficiency showed improved neonatal handling of calcium with improved maternal vitamin D status (reviewed by Specker 2004). However, results concerning the effects of vitamin D on maternal weight gain and fetal growth were conflicting and inconclusive. There was no evidence to indicate any beneficial effects of vitamin D intakes in excess of the amounts routinely recommended during pregnancy to prevent vitamin D deficiency (Specker 2004).

In the updated Finnish nutrition recommendations (Valtion ravitsemusneuvottelukunta 2005) the recommended daily intake for vitamin D for people aged 2 – 60 years has increased to 7.5 µg (vs. the old recommendation of 5 µg) to try to combat the decrease in the circulating 25(OH)D concentration occurring during the winter months and in order to prevent the risk of some chronic diseases. Since February 2003, most Finnish liquid milk products have been supplemented with vitamin D and the dose in margarines has been increased (KTMa n:o 917/2002; Kauppa- ja teollisuusministeriö 2002). Due the fortification, the Ministry of Social Affairs and Health updated its recommendation on vitamin D supplementation (Sosiaali ja terveyministeriö 2003), though the recommendation on supplementation during pregnancy remained unchanged. Populations at risk for vitamin D deficiency are those for which, for environmental, cultural, or medical reasons, exposure to sunlight is poor and the dietary intake of vitamin D is low. The Findiet 2002 Study (done before the new vitamin D fortification) showed that especially in the youngest age group, the intake of vitamin D in Finnish women was below the recommendation (Männistö et al. 2003). The main dietary sources of vitamin D were fish dishes (about the half of the total intake) followed by fortified margarines. In the simulated risk assessment of vitamin D fortification, two modelled scenarios (1: all potential foods in question were fortified; 2: only part of food brands were fortified) showed that foods potentially fortified with vitamin D were mainly safe in the adult population (Hirvonen et al. 2004). The real impact of the recent vitamin D fortification on the total dietary intake of vitamin D or vitamin D status in Finns has yet to be demonstrated.

### *Iron*

A considerable proportion of pregnant women in both the developing and industrialized countries become anemic during pregnancy (Mungen 2003). In Europe, iron deficiency is considered to be one of the main nutritional deficiency disorders affecting large proportions of the population, particularly those that are at peak rates of growth, namely, infants, children, and pregnant women (Hercberg et al. 2001). Moreover, women, especially adolescents consuming low-energy diets,

vegetarians and vegans are at high risk of suffering iron deficiency. The necessity of routine iron supplementation during pregnancy has been widely debated in industrialized countries and routine supplementation is not universally practiced in all countries. Routine iron supplementation results in a substantial reduction of women with haemoglobin levels < 100 g/L in late pregnancy (Villar et al. 2003). The major problem with iron supplementation during pregnancy is compliance. Despite many studies, the relationship between maternal anemia and adverse pregnancy outcome is unclear. Existing data suggest that severe iron deficiency anemia (haemoglobin concentration of 90 – 100 g/L) may be associated with both impaired fetal and maternal health, including preterm delivery and higher maternal mortality (Allen 1997).

The Nordic Council of Ministers has issued a statement that the physiological need for iron during the second and third trimester of pregnancy cannot be supplied solely through the diet, and the statement is also followed in Finland (Nordic Nutrition Recommendations 2004, Valtion ravitsemusneuvottelukunta 2005). Iron supplementation during pregnancy is therefore recommended (Table 2). It is assumed that targeting intervention programs to the prenatal period may be of greater benefit than relying on intervention during the late pregnancy, when the real window of opportunity for a positive impact on fetal growth and development has possibly passed (Beard 2000). Large iron stores may represent a health hazard since they can promote the production of free radicals and evoke oxidative damage; most evidently increasing cardiovascular disease risk (Schumann 2001). Gastrointestinal distress is primarily observed in individuals who have consumed high levels of supplemental iron on an empty stomach.

There are clear relationships between iron requirements, bioavailability of dietary iron, and amounts of stored iron. A reduction in iron stores and a decrease of hemoglobin iron had the same increasing impact on iron absorption, suggesting that the control of iron absorption is mediated from a common cell (most likely the hepatocyte), which may register both the size of the iron stores and

haemoglobin-derived iron deficit (Hallberg & Hulthen 2002). Dietary recommendations during pregnancy should include all means for increasing the content of dietary factors enhancing iron absorption or reducing the content of factors inhibiting iron absorption.

### **2.3.6 Obesity in pregnancy**

The rising prevalence of obesity as well as the high proportion of women who gain weight in excess of recommendations during pregnancy are significant health problems in the Western World (Siega-Riz et al. 2004). Different weight gain targets are recommended during pregnancy according to prepregnancy BMI so that recommendable weight gain is 12,5 – 18; 11,5 – 16; and 7 – 11,5 kg in BMI-classes 18,5 – 19,9; 20,0 – 25,9; and > 26, respectively (Scientific Committee for Food 1993, Stakesin perhesuunnittelun ja äitiyshuollon asiantuntijaryhmä 1999). Among women of childbearing age, one potential pathway for the development of obesity has been attributed to the retention of gestational weight gain (Rössner & Ohlin 1995). The mean age and prepregnancy body mass index of all Finnish pregnant women increased from the 1960s to the year 2000 (from 26.5 to 29.6 years, from 21.9 to 23.7 kg/m<sup>2</sup>) (Kinnunen et al. 2003). Also the mean pregnancy weight gain in all body mass index categories has increased in Finland since the 1960s; on average from 13.2 to 14.3 kg (adjusted for mother's age, body mass index and parity).

According to the results of a Swedish follow-up study (Linne et al. 2004), targeting to the weight gain during pregnancy is more important than focusing on weight control programs for women who are obese already before pregnancy. High weight gainers during pregnancy seem to retain more weight over the long-term, whereas women who are overweight before pregnancy do not have a higher risk of postpartum weight retention than normal weight women. However, women who are overweight or obese before pregnancy breastfeed for a shorter time than normal-weight women (e.g. Baker et al. 2004). Higher pre-pregnancy body mass index is also associated with increased risk of neural tube defects due to some unknown mechanism (Mojtabai 2004).

The largest retrospective analysis to date among 287 213 pregnant London women demonstrated that many adverse outcomes of pregnancy are associated with maternal obesity, and the risk increases with the degree of obesity (Sebire et al. 2001). Increasing maternal body mass index was associated with increased magnitude of gestational diabetes, proteinuric pre-eclampsia, induction of labour, delivery by emergency caesarean section, postpartum haemorrhage, genital and urinary tract infections, wound infection, birthweight above the 90<sup>th</sup> centile, and intrauterine death. However, social class was not used as a covariate in that study, despite the fact that social class has been shown to have an association with some of the outcome variables. In Finland, as well as in most Western countries, the least well educated sector of the community has the greatest risk for obesity (reviewed by Lahti-Koski & Sirén 2004).

## **2.4 Diet in infancy**

### **2.4.1 Breastfeeding**

The benefits of breastfeeding are probably one of the most frequently stated claims in the scientific nutritional literature and the last decades have produced an enormous amount of new information on this issue. More than 100 years ago Ehrlich (1892) demonstrated that milk contains protective factors and although it was realized that some of these factors would be antibodies, it was not until 1961 (Hanson 1961) that the main human-milk immunoglobulin secretory immunoglobulin A (SIgA), was characterized immunologically and isolated. Anti-idiotypic antibodies as well as T and B lymphocytes are transferred via milk and seem to actively stimulate the immune system of the offspring (reviewed by Hanson 1998; 1999). The ability of the mother to secrete antibodies directed against specific antigens that she and the infant encounter in the environment would appear to confer on human milk an environmental specificity and a significant protective potential, possibly with several long-term positive effects (Hanson 1998). Another unique character of human milk is its rich diversity of approximately 130 different oligosaccharides which are involved in a number of functions, such as protecting the breast-fed infant from pathogens and providing substrates for bacteria in the infant colon (Miller & Mc Veagh 1999).

Although breastfeeding is universally accepted as the optimal feeding method for newborns, opinions and recommendations are still divided on its optimal duration. Exclusive breastfeeding is recommended worldwide for infants during the first 4-6 months (Statement of the Standing Committee on Nutrition 1994, Høst et al; The European Society for Paediatric Allergology and Clinical Immunology and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition 1999) or until the age of 6 months (Position of the American Dietetic Association 2001, WHO 2002; 2003, Gartner et al; American Academy of Pediatrics 2005) based on scientific evidence of health benefits in breastfed infants, as well as the advantages for the mothers, the health system and society (Forsyth 1995, Heinig & Dewey 1996, Heinig & Dewey 1997; Anderson et al. 1999, Lutter 2000, Yngve & Sjöström 2001a, Kramer & Kakuma 2002; 2004). In the previous version of the Finnish nutrition recommendations for infants (Hasunen et al. 1997), exclusive breastfeeding was recommended for 4-6 months. The recently published updated version (Hasunen et al. 2004) took into consideration the WHO recommendations (WHO 2003) and the duration of exclusive breastfeeding is now recommended until the age of 6 months, complementary feeding starting individually at the latest at the age of 6 months (Table 3). Breastfeeding should continue partially until the end of the first year.

Insufficient prenatal breastfeeding training, short postpartum hospital stay, insufficient professional support, maternal employment, lack of broad societal support, and powerful marketing of infant formulas are some of the barriers to breastfeeding (Position of the American Dietetic Association 1997, Rogers et al. 1997a, Dennis 2002, Gartner et al. 2005). A short duration of breastfeeding and introduction of complementary foods at an early age can be associated with impaired neurocognitive development (Anderson et al. 1999, Jain et al. 2002), obesity (Arenz et al. 2004, Owen et al. 2005), and chronic diseases, such as celiac disease (Ivarsson et al, 2002), T1D (Virtanen & Knip 2003), and cardiovascular diseases (Owen et al. 2002; 2003). A multidisciplinary review of infant feeding mode in relation to allergy concluded that breastfeeding seems to protect

from the development of atopic disease (van Odijk et al. 2003). However, the immunomodulatory properties of breast milk may vary according to the maternal diet; a maternal diet low in saturated fat and rich in natural sources of vitamin C during breastfeeding was associated with a lower risk of atopic sensitisation and dermatitis in the Finnish prospective birth cohort study and in a follow-up study (Hoppu et al. 2000; 2005). The long-term effect of breastfeeding on atopic diseases has been claimed to double-edged: in children with atopic heredity, breastfeeding protects against atopy, whereas in children without atopic heredity, breastfeeding could increase the risk of atopy (Siltanen et al. 2003).

Results from a Finnish national survey performed in 2000 (Hasunen 2002) indicated that 68% of infants younger than 1 month were exclusively breastfed, but the target of breastfeeding exclusively up to the age 4 to 6 months was rarely achieved (14% and <1%, respectively). The proportions of exclusively breastfed infants at the age of 6 months in local descriptive breastfeeding surveys done in central Finland and Vantaa were 8% and 6%, respectively (Tepora et al. 1999, Sihvola 2001). Since 1995, the length of breastfeeding has also been reported in two Finnish child targeted surveys in Turku; Special Turku Coronary Risk Factor Intervention Project for Babies (STRIP) (Simell et al. 2000) and in some studies of the Nutrition, Allergy, Mucosal immunology and Intestinal microbiota (NAMI) Research group (Hoppu et al. 2000; 2005). Only the length of total breastfeeding was reported among the STRIP subjects, being on average 5.3 months in the intervention and 4.9 (SD 3.7 for both groups) months in the control group (Simell et al. 2000). The inclusion criteria of NAMI studies (Hoppu et al. 2000; 2005) have included the certain length of breastfeeding and the reported breastfeeding rates are therefore, not directly comparable with other Finnish surveys.

**Table 3. Dietary recommendations in infancy (based on Hasunen et al. 2004)**

	<b>Recommended earliest age at introduction (months)</b>	<b>Not suitable among the food group before the age of 1 year</b>
Infant formula if breast milk not available	0	Soy, oat, rice, sesame, and almond based drinks as substitutes for infant formula
First supplementary food	4-6 <sup>(1)</sup>	
Potato / carrot	4-6	New potatoes in small sizes
Fruit / berries	4-6	Canned fruits, rhubarb
Vegetables	4-6	Peas, beans, and cabbages in large portions; mushrooms; nuts, almonds and seeds; sprouts
Gluten-containing cereals (wheat, rye, barley, oat)	5-6	
Other cereals (rice, maize, etc.)	5-6	
Meat	5-6	Liver and liver products
Fish	6	
Egg	5-6	
Dairy products (other than supplementary milk feeding and food containing hydrolyzed milk protein)	10-12	Flavoured milk products containing a great variety of food additives
Vegetables high in nitrates / nitrites	12	Swede, turnip, beetroot, spinach, and Chinese cabbage
Sausages	12	All sausages
Others		Salt, ready-to-eat-foods, cacao, tea, honey

<sup>(1)</sup> Exclusive breastfeeding is recommended until the age of 6 months, complementary feeding starting individually at the latest at the age of 6 months.

Breastfeeding is heavily influenced by cultural considerations and its prevalence varies widely between societies (Rogers et al. 1997a). Among Europeans, high rates of breastfeeding initiation are found in Sweden, Norway and Greece; and low rates in Ireland, France and United Kingdom (The Euro-Growth Study, Freeman 2000, Yngve & Sjöström 2001a, Lande et al. 2003). Only 25% and 15% of Euro-Growth infants were breastfed exclusively up to the ages of 3 and 4 months. In comparison with other European countries, the rate and duration of breastfeeding in Finland have been quite high (Yngve & Sjöström, 2001a). The results of studies on breastfeeding are somewhat difficult to interpret due to the different breastfeeding definitions used (e.g. Aarts et al. 2000). WHO (2002) has developed a set of definitions, in which only drops of vitamins or minerals are allowed if the term 'exclusive' breastfeeding is used. In keeping with the WHO definitions, it is now stated in the new Finnish nutrition recommendations for infants (Hasunen et al. 2004) that only tastes of water are recommended for exclusively breastfed infants. However, due to the old habit and recommendation of giving water to breastfed infants, the possibility that infants classified to the exclusive category in the Finnish studies have been given more than tastes of water, cannot be ruled out.

#### **2.4.2 Weaning**

Infants are at the mercy of their parents regarding feeding from the very beginning. Flavours from the mother's diet during pregnancy are transmitted to the amniotic fluid and swallowed by the fetus (Mennella et al. 2001). Consequently, the cultural-specific flavour principles may be experienced by the infants before their first exposure to complementary foods (Birch 1998). During the first year of life, patterns of food intake change dramatically. The infant moves from a completely milk-based diet to consumption of a wide range of foods and there is the potential for significant variation in how this change is achieved. This early learning of eating is constrained by the child's genetic predispositions, which includes the unlearned preference for sweet and salty tastes, and the rejection of sour and bitter tastes (Birch 1998, Birch & Fisher 1998). Children usually learn to prefer energy-dense foods and foods offered in positive contexts (Birch 1998). Feeding milestones, based on physiologic and neurologic maturation, with a significant impact on weaning behaviors include taking food from a

spoon, chewing foods, self-feeding with fingers, independent feeding from a cup, and using utensils (Pridham 1990). Persistent eating behaviours and particular dietary patterns, which are strongly influenced by social, demographic and lifestyle factors relating to the family, particularly to the mother, could be seen by the age of 2 to 3 years (Nicklas et al. 1991, Birch 1998, North & Emmett 2000). Some of these patterns began to emerge as early as 9 to 11 months of age in a recent survey among infants in the US Feeding Infants and Toddlers Study (FITS), (Fox et al. 2004).

The optimal age for the introduction of complementary foods in infants is poorly known, and recommendations given by nutritionists, health professionals, and the baby food industry may differ greatly, which can easily confuse parents. The scientific basis for the current weaning recommendations relies on the current knowledge on nutritional need, physiologic maturation, and the behavioural and developmental aspects of infants (reviewed by Hendricks & Badruddin 1992). However, more research is needed in a number of areas before the recommendations can include more precise guidelines. WHO issued a global public health recommendation in 2002, stating that being exclusively breastfed for the first 6 months of life, infants should receive nutritionally adequate and safe complementary foods while breastfeeding continues for up to 2 years of age or beyond. Similarly to the EU recommendations (Scientific Committee for Food 1990) in Finland it is advised to start complementary feeding individually at the latest at the age of 6 months (Hasunen et al. 2004). The recommended age when different foods can be introduced at the earliest are shown in Table 3 (Hasunen et al. 2004). In the developed countries, very early complementation is usual, not because of nutritional need, but because of other determinants including social pressures, availability of convenient alternatives, and maternal employment (reviewed by Wright & Schanler 2001).

Accustoming infants to complementary foods is a lengthy process, and this has been shown to take longer the younger the infant is at introduction (Hörnell et al. 2001). Repeated exposure to new foods, especially those that are not sweet, is critical for acceptance (Birch & Fisher 1998). In

contrast to other complementary foods, formula is usually given in large amounts already on the first occasion, since it is viewed as a replacement for breast milk (see e.g. Michaelsen 1997). In a Danish cohort study (Michaelsen 1997) about 80% of the infants received regular infant formula during the first 3 days after delivery, despite the fact that all of the infants were healthy and full-term. With a few exceptions, all of the infants were exclusively breastfed at discharge from the maternity ward. No benefits, in terms of growth, of introducing complementary foods between 4 and 6 months were demonstrated in 20 studies included in the meta-analysis of Kramer & Kakuma (2002; 2004). The only exception was improved iron status in one developing country setting (Dewey et al. 1998). It is assumed that prolonged breastfeeding increases resistance to weaning, and that later feeding problems can be reduced if chewable foods are introduced at an appropriate age (Paine & Spegiorin 1983), but this hypothesis has been strongly questioned (e.g. Hörnell et al. 2001). However, most researchers seem to agree that foods requiring chewing should be introduced at about the ages of 6 to 8 months to avoid later problems with the acceptance of lumpy food (Paine & Spegiorin 1983). Infants are not passive receptacles for flavoured foods; parents offering a variety of foods will provide both a nutritious, well-balanced diet, as well as an opportunity for their children's own personal preferences to develop (Birch & Fisher 1998, Mennella & Beauchamp 1998).

The appropriate time for the introduction of complementary feeding varies considerably in different sociocultural settings and among different socioeconomic groups (Underwood & Hofvander 1982). Complementary foods are introduced early to European infants; more than half of the children in the Euro-Growth Study were consuming some complementary food by the age of 3 months (Freeman et al. 2000). Fruits (73%) and cereals (51%) were the first foods used for most infants. Duration of breastfeeding was significantly correlated with age of introduction of complementary foods. As many as, 93% of infants had received complementary foods before the age of 4 months in a longitudinal study in UK (Savage et al. 1998). Early introduction of complementary foods (mean age 3.4 months) was also reported recently in Lithuanian cohort, and it was assumed that such

patterns can be attributed to the former Soviet recommendations of introducing strained fruit at 1.5 months (Vingraite et al. 2004). Conversely, a rather positive trend has been found in Scandinavia; only 34% and 21% of the infants had been introduced to complementary foods before the age of 4 months in Sweden and Norway, respectively (Hörnell et al. 2001, Lande et al. 2003). However, since the data was obtained from a retrospectively completed FFQ covering the time from birth up to 6 months of age, this could have introduced some recall bias in the Norwegian study. Being a preterm infant seems to increase the risk for early weaning: data collected from a series of prospective trials in UK showed that over three quarters of preterm infants were receiving complementary food by 12 weeks corrected age, the mean age at weaning being less than 4 months in term infants (Fewtrell et al. 2003). This is, however, not supported by Finnish studies in which being a preterm infant was a risk factor for initiation and shorter duration of breastfeeding, but not for earlier introduction of complementary foods (other than infant formula), (Räisänen et al. 1998, Hasunen 2002). In a Danish national birth cohort among 3768 mother-infant pairs prepregnant obesity (BMI  $\geq$  30), short durations of breastfeeding, and earlier introduction of complementary food were associated with 0.7 kg of additional weight gain during infancy (Baker et al. 2004). Recently, some steps have been taken to improve complementary feeding worldwide (Piwoz et al. 2003) by applying some methods used in the successful promotion of breastfeeding by the statements of the Ten Steps to Successful Breastfeeding (WHO/UNICEF 1989).

Infant diet has changed during the last decades in Finland as well as in many other countries (Whitehead & Paul 1984, Hasunen et al. 1996). With the growing popularity of breastfeeding, the transition to a diet including complementary foods has shifted to a later age; from the mean age of 2 months in 1976 (Virkkunen 1978) to the age of more than 3 months in 2000 (Hasunen 2002). There have also been marked changes in the composition of infant formula, in the consumption of ready-made commercial infant food, and in the advice on what constitutes a weaning diet. A particularly high consumption of ready-made commercial infant foods, averaging more than 100 kg/a child/year

(Europeaus 2005), is a noteworthy characteristic of the diet of the Finnish infants. There has been a huge, about a fivefold, increase in their consumption between 1985 and 1998 (Viinisalo 2005).

Unmodified cow's milk is known to be unsuitable for infants until 12 months of age, because they will receive little iron, linoleic acid, and vitamin E and have excessive intakes of sodium, potassium, and protein from cow's milk (e.g. Hasunen et al. 2004). However, in the Euro-Growth Study (Freeman et al. 2000) many children were exclusively fed cow's milk from the age of 2 months at the earliest. At the ages of 6 and 9 months, 9% and 18% of infants, respectively, were given only cow's milk to drink. Quite similar percentages were found in the Glasgow longitudinal infant growth study (Savage et al. 1998). Median introduction of cow's milk was as early as 8 months in a recently published Lithuanian study (Vingraite et al. 2004). Nonetheless, there has been a major improvement in Europe regarding the use of unmodified cow's milk for infants. Feeding cow's milk earlier than recommended is most likely among women with low education, low income and high parity (North et al. 2000, Vingraite et al. 2004).

Clinically significant nutritional problems are unusual in healthy infants and toddlers, particularly if growth is normal (Aldous 1999). Inadequate energy and protein intake and deficiencies of iron, zinc, vitamin A, and vitamin D are the most commonly observed nutrient deficiencies occurring during infancy (reviewed by Hendricks & Badruddin 1992). A relatively high fat intake should continue throughout infancy to ensure adequate energy intake to provide substrates for central nervous system growth. Structural lipid comprises 60% of the human brain, and docosahexaenoic acid and arachidonic acid are major lipid components (Martinez 1992). Breast milk provides the nutrients required for rapid development of the immature brain (Jensen 1999). If infants are exclusively breastfed beyond 6 months, they are at increasing risk of developing iron deficiency anaemia (Krebs 2000). Iron concentrations in human milk are low (0.2 – 0.4 mg/l), but an efficient bioavailability partly compensates for its low concentrations. Accordingly, several studies show that

increased meat intake by half of one year old breastfed infants would adequately support both iron as well as zinc requirements (reviewed by Krebs 2000). Due to poor exposure to sunlight, Finnish infants need to receive D vitamin supplements until the age of three years (Hasunen et al. 2004).

### **2.4.3 Impact of weaning on infant's growth**

There are many factors determining the achievement of the genetically determined growth potential of normal infants and children, one of the most important factors is the availability of adequate amounts of energy and nutrients. Anthropometric indices are commonly used as the principal criteria for assessing the adequacy of nutrition in early childhood. The pattern of growth seems to differ depending on the feeding method in infancy in the developed countries (Dewey et al. 1995, Michaelsen 1997, Dewey 1998, Rogers et al. 1997b, Haschke et al. 2000, Kramer et al. 2004). The most typical pattern is for exclusively breastfed infants to grow rapidly during the first 2 to 3 months. After that time, they become lighter than formula-fed infants and have markedly lower adiposity. There is some evidence of a slightly lower rate of linear growth over the first year of life, but any differences in weight and height do not persist beyond this point, but show a relative deceleration thereafter. However, a meta-analysis based on nine studies from developing countries and 11 from developed countries (all observational studies, except for two controlled trials from Honduras) found no evidence that infants who continue to be exclusively breastfed for 6 months exhibited deficits in weight or length gain from 3 to 7 months and thereafter (Kramer & Kakuma 2002; 2004). Data from a large (n = 17 046) cluster-randomized trial in the Republic of Belarus by Kramer and colleagues (2002) came to a similar conclusion. To assess the potential of bias (including confounding, reverse causality, and selection bias) in observational studies of breastfeeding, the same data was also analyzed as if conducting an observational study. The observational analysis produced similar growth patterns as found in other observational studies. It was concluded that the findings reflect either some unmeasured confounding differences or a true biological effect of formula feeding. Based on the evidence for different growth patterns, WHO is currently developing a new international growth reference based on infants who follow WHO

recommendations (WHO 2000, WHO 2002). It has been questioned whether the first complementary foods should be different in the two groups; complementary foods of breastfed infants should contain greater amounts of certain nutrients (such as iron and zinc), which formula-fed infants receive from infant formula (Wharton 2000).

## **2.5 Social inequalities in maternal and infant health**

Social class differences in diet and health are seen in all ages including pregnancy and infancy. Risk factors including lack of breastfeeding, smoking, physical inactivity, obesity and unhealthy diet are clustered in the lower socioeconomic groups (e.g. Dubois & Girard 2003). The diet of lower socioeconomic groups provides cheap energy from foods such as high fat meat and milk products, butter, sugars, preserves, and potatoes, but is low in fruit, vegetables, low-fat milk, and soft vegetable margarines (Laitinen et al. 1995, Roos et al. 1996, James et al. 1997, Groth et al. 2001, Hulshof et al. 2003, Lopez-Azpiazu et al. 2003). However, cheap foods could differ between countries, and the impact of price on consumption most likely changes with time and between cultures. Higher socioeconomic groups do not necessarily follow current national dietary guidelines any better than lower socioeconomic groups (Roos et al. 1996). Education seems to be the most important social variable to account for social differences in dietary habits.

Social inequality in perinatal health outcomes (5 indicators including number of preterm births, mean birthweight, numbers of low birthweight and small for gestational age children, and perinatal deaths) exist in Finland, but does seem to have diminished in the 1990s (Gissler et al. 2003). The data based on the Finnish Medical Birth Register detected socioeconomic differences in all perinatal health indicators. Maternal smoking explained up to half of the excess risk for adverse perinatal outcome in the lowest socioeconomic group. In the study of Forssas et al. (1999), the Medical Birth Register based data on all newborns in Finland born between 1991 and 1993 found eight maternal characteristics increasing the risks for perinatal death: in-vitro fertilization, earlier stillbirth, higher

maternal age, maternal diabetes, lower socioeconomic status, smoking during pregnancy, being single mother, and first birth.

Social factors determining the initiation and length of breastfeeding are often studied separately. In the industrialised countries, young and less well educated mothers are less likely to breast feed than other mothers (Michaelsen 1997, Rogers et al. 1997a, Riva et al. 1999; Scott et al. 1999, Tepora et al. 1999, North et al. 2000, Dennis 2002, Dubois & Girard 2003, Lande et al. 2003, Vingraite et al. 2004). The impact of family size is not consistent (Yngve & Sjöström, 2001b, Lande et al. 2003). Male infants are more likely to be weaned earlier than females (Pande et al. 1997, Scott 1999, Scott et al. 1999, Lande et al. 2003), and also noted in Finnish surveys (Sihvola 2001, Hasunen 2002). Having a first baby and single marital status of the mother are other important negative determinants of breastfeeding (Rogers et al. 1997a, Yngve & Sjöström, 2001b). Maternal employment outside of the home is a risk factor for initiation and shorter duration of breastfeeding (Dennis 2002).

Studies on social class differences in childhood health are controversial partly because of different methods of collecting data, limited sample sizes and the use of limited numbers of health indicators. Children of well-educated and older mothers came closer to meeting the recommended intakes for most of the critical nutrients and maternal age and educational level seem to influence the food habits of children (Savage et al. 1998, North et al. 2000, Dubois & Girard 2003, Fewtrell et al. 2003, Lande et al. 2003, Navia et al. 2003, Vingraite et al. 2004).

Some gender differences in infant feeding have been found in previous Finnish studies. The energy adjusted nutrient intake of one- to two-year-old boys and girls, calculated by means of 3-day food records, differed from each other with regard to vitamin A, iron and copper (higher for boys), and lactose and selenium (higher for girls), (Räsänen & Ylönen 1992). Overall, the boys had a greater

food consumption and, thus, higher energy intake than the girls. The impact of the intervention was slightly stronger on the diet of the boys compared to the girls in the Finnish STRIP Study (Simell et al. 2000). Girls had higher odds of having a timely introduction to complementary foods compared with boys in a Norwegian study, but the opposite trend was found for the odds of vitamin D supplementation (Lande et al. 2003). A tendency towards stronger effects of infant gender was found in the lowest educational group compared with those in the highest educational groups.

The presence of older siblings is thought to affect the introduction of some foods. The use of fruit juices was significantly associated with the presence of older siblings among 1000 infants in the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) (North et al. 2000). Also the feeding of cow's milk as a main drink at 8 months was most likely to occur in the group of mothers with two or more children. The study of 2103 children in the Longitudinal Study of Child Development in Québec (LSCDQ 1998-2002) noted that adherence to the different recommendations was interrelated, pointing to an accumulation of adverse nutritional circumstances in the children of families having low socio-economic status (Dubois & Girard 2003).

In a study on social class differences in health among the Finnish birth cohort 1987 (health measured as mortality and morbidity in terms of a cumulative disease index, the cumulative incidence of asthma, diabetes, epilepsy and intellectual disability, hospitalisations, disease-related welfare benefits and special education), Gissler and colleagues (1998) found that the health of the children in the lowest social class was the poorest. After adjusting for confounders, the children in the lowest social class had the highest risk for poor health outcome both in the perinatal period and in childhood, and had the most intellectual disabilities, the highest mean number of hospitalisation days, and received most special education. Background variables, such as maternal age and social class, explained the difference in mortality among the Finnish children followed up until the age of

7 years (Gissler et al. 2000). However, they did not explain the regional health differences found for all health indicators except for diabetes.

## **2.6 Epidemiology of type 1 diabetes (T1D)**

### **2.6.1 Autoimmune process and genetic susceptibility**

T1D is viewed as a chronic immune-mediated disease with a subclinical prodrome characterized by selective loss of insulin-producing  $\beta$ -cells in the pancreatic islets in genetically susceptible persons (Atkinson & Eisenbarth 2001, Knip 2002a). The appearance of T1D associated autoantibodies is the first noticeable sign of  $\beta$ -cell destruction, which is thought to be T-cell mediated. B-cell autoimmunity may be induced in any individual at any time, even prenatally in some individuals. In the Finnish DIPP Study population, the first antibodies appeared already before the age of 3 months (Kimpimäki et al. 2001b). This prediabetic period offers an opportunity to identify those individuals who are likely to develop clinical T1D. Data from the Finnish DIPP Study also indicate that a higher proportion of the population develop signs of  $\beta$ -cell autoimmunity rather than clinical T1D. The slight male predominance in the incidence of T1D among Caucasian children becomes more obvious in those diagnosed after puberty (Karvonen et al. 2000). A similar male majority was reported with signs of humoral  $\beta$ -cell autoimmunity among first-degree relatives older than 10 years of age (Williams et al. 2002).

There are four disease-related autoantibodies which have been shown to predict overt T1D (Knip 2002b): classic islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies to the 65-kD isoform of glutamic acid decarboxylase (GADA), and autoantibodies to the protein tyrosine phosphatase-related IA-2 molecule (IA-2A). T1D associated autoantibodies can fluctuate from positivity to negativity, and may occur transiently. The number of detectable autoantibodies is related to the risk of progression to clinical T1D. Positivity for single autoantibody specificity represents in most cases harmless nonprogressive  $\beta$ -cell autoimmunity. Positivity to three to four antibodies is associated with a risk of developing clinical T1D in the range of 60 – 100% over the

next 5-10 years (Bingley et al. 1997, LaGasse et al. 2002, Barker et al. 2004, Hummel et al. 2004a). T1D associated autoantibodies can be used as surrogate markers of clinical T1D in individuals with increased genetic disease susceptibility (Kimpimäki et al. 2000).

A substantial part of the transnational variation in the incidence of T1D in Europe is explained by variations between populations in the distribution of particular DQ genotypes which confer a high risk of T1D in the general population (Ronningen et al. 2001). Nearly 20% of the Finnish population have an increased HLA-conferred genetic predisposition to T1D, only 0.5% of those actually developing the clinical disease (Ilonen et al. 1996). The genetic susceptibility to T1D can be mostly found in the MCH complex on the short arm of chromosome 6. The T1D susceptibility genes are localized near to or are identical to genes encoding class II HLA molecules (Redondo & Eisenbarth 2002). In Caucasian populations, T1D is strongly associated with the serologically defined HLA-DR3 and –DR4 antigens. There is evidence that DQ rather than DR alleles primarily influence susceptibility to T1D. In the Finnish population, the alleles significantly associated with susceptibility (DQB1\*0302, 02) and protection (DQB1\*0301, 0602, 0603) can be defined with only four DQB1 specific oligonucleotide probes. Of Finnish subjects with T1D, 60-70% have a genotype indicating high (DQB1\*02/0302) or moderate (DQB1\*0302 alone in the absence of protective alleles) risk, whereas in the general population, the prevalence of these genotypes is only 14% (Ilonen et al. 1996).

### **2.6.2 The role of environmental factors**

The environment factors include all influences which alter and modify the status and relationship between the living systems and the outside world. Large geographic differences, the globally reported conspicuous increase in disease incidence (Green & Patterson 2001, Onkamo et al. 1999, Gale 2002, Bingley & Gale 1989), and the relatively low concordance in monozygotic twins (Barnett et al. 1981, Kaprio et al. 1992) are factors that favour a crucial role of environmental factors in the etiology of T1D. There is a more than 350-fold worldwide difference in incidence

rates, within Europe these range during the period 1989–1994 from 3.2 cases per 100,000 person-years in the Republic of Macedonia to more than 40 new cases per 100,000 person-years in Finland, and in Finland the incidence has increased more than fourfold since the early 1950s (Karvonen et al. 2000, Green & Patterson 2001, Ronningen et al. 2001). Such a steep increase most likely reflects changes in lifestyle and environment. However, a shift to younger age at diagnosis seems to explain some of the increasing incidence of childhood T1D (Pundziute-Lycka et al. 2002).

Environmental factors are thought to act as the exogenous triggers inducing the immune-mediated process leading to extensive  $\beta$ -cell destruction and ultimately to the clinical manifestation of T1D (Knip & Åkerblom 1998). Viruses and dietary factors have been considered as being major environmental factors (Hyöty & Taylor 2002, Virtanen & Knip 2003). Among the viruses associated with T1D, enteroviruses and rubella seem to be most implicated the role of other viruses not being clear. Enterovirus infection accompanies or precedes the onset of T1D in many children.

The increase in maternal age at delivery over the past two decades could partly account for the increase in the incidence of T1D. Several case-control/referent studies and one cohort study have shown that children born to older mothers have an increased risk of T1D (Blom et al. 1989, Dahlquist & Källén 1992, Patterson et al. 1994, Dahlquist et al. 1999, McKinney et al. 1997, Stene et al. 2004), most of them indicate that the increased risk is limited to the offspring of mothers aged more than 35 years at delivery. The intrauterine environment has been shown to be related to the risk of T1D in terms of preeclampsia, excessive weight gain, amniocentesis, complicated delivery, Caesarean section, higher birth order, and maternal-child blood group incompatibility (Patterson et al. 1994, Wadsworth et al. 1997, Tai et al. 1998, Dahlquist et al. 1999, McKinney et al. 1999, Elfving et al. 2003, Sipetic et al. 2004, Stene et al. 2004) but the findings are not consistent (see e.g. Stene et al. 2003). Symptoms of maternal infections (mostly respiratory or gastrointestinal) in pregnancy are believed to lower the risk of islet autoimmunity (Stene et al. 2004). The influences of

socio-economic status (Sipetic et al. 2004) and maternal smoking during pregnancy (e.g. Stene et al. 2004 vs. Dahlquist & Källén 1992) remain controversial. In contrast to some earlier findings on parental education (e.g. Virtanen et al. 1998, Sipetic et al. 2004), in a recent Swedish prospective population-based series of 4 400 one year old children, poor paternal education was associated with T1D -related autoimmunity in the child, independent of any family history of diabetes (Sepa et al. 2005). The development of T1D has been linked to improved hygiene and to decreased or changed exposure to infections. Decreased exposure to common infections during infancy (Gibbon et al. 1997, The EURODIAB Substudy 2 Study Group 2000, Sipetic et al. 2003) was associated to increased risk of T1D, and day care attendance (The EURODIAB Substudy 2 Study Group 2000, McKinney et al. 2000) with reduced risk, but again inconsistent findings have been reported as well (Dahlquist et al. 1999, Hummel et al. 2000). The adoption of hygiene practices is influenced to some degree by social, lifestyle, and environmental factors (Sherriff et al. 2002).

Psychoimmunology has being offered as one possible pathway (Hagglof et al. 1991, Soltesz et al. 1994, Thernlund et al. 1995, Sepa et al. 2002). Psychosocial stress in families may affect children negatively since there may be a link between hormonal levels and nervous signals that in turn influence both insulin sensitivity/insulin need and the immune system. In a recent Swedish prospective population-based series of 4 400 children aged 1 year, high parenting stress, experiences of a serious life event, foreign origin of the mother and low paternal education were associated with T1D -related autoimmunity in the child, independent of family history of T1D (Sepa et al. 2005). However, psychosocial stress is a risk factor for a variety of health problems beginning with preterm birth and low birth weight (Hobel & Culhane 2003) and may be established in a form for which the individual has genetically or environmentally highest susceptibility.

### 2.6.3 Dietary factors in the development of T1D

Current knowledge on potential nutritional factors in the etiology of T1D has relied greatly on animal studies and ecologic comparisons (Åkerblom & Knip 1998, Virtanen & Knip 2003). Among the first concepts for possible dietary causes in humans was the work of Baum et al. (1975) in which it was observed that children who later developed T1D had an accelerated weight gain during infancy compared with control children. Since that first finding, the following nutritional factors have been linked to the development of T1D: breastfeeding, nicotinamide, zinc, and vitamin C, D, and E as possible protective factors, and N-nitroso compounds, cow's milk, increased linear growth, and obesity as possibly increasing the risk. One major shortcoming of the most studies conducted so far is that only single dietary exposures have been assessed at single time points (Virtanen & Knip 2003). Putative nutritional and other confounding factors have received little attention as have the limitations of the dietary methods used. Given its relatively low prevalence and the fact that the disease is most likely multifactorial, knowledge about the long-term diet is crucial if one wishes to examine diet-based contributions to T1D. Several studies suggest that intra-uterine factors may cause  $\beta$ -cell destruction already during the fetal period (e.g. Lindberg et al. 1999, Elfving et al. 2003). The mechanisms of action of the different nutritional constituents that may play a role in the development of  $\beta$ -cell autoimmunity are largely unknown and they may differ according to life stage (Virtanen & Knip 2003).

#### *N-nitroso compounds*

Nitrate is a naturally occurring compound in vegetables; more than 90% of dietary nitrate is derived from vegetables, including potatoes (Dich et al. 1996). Nitrate and nitrite are both used as food additives, and they are also found in soil and water due to the use of fertilizers (WHO 1998). Nitrate is reduced in the gut to nitrite, which can further be transformed to the N-nitroso compounds (Slorach 1981). The dietary antioxidants may inhibit and thiocyanate ions can accelerate the formation of N-nitroso compounds (Leaf et al. 1989). The main sources of nitrite in the Finnish diet

are sausages and cold-cut meat products (Dich et al. 1996, Salminen & Penttilä 1999). Nitrite intake by children in the age group 13 months to 5 years who ate these food items frequently exceeded the ADI-value in the survey of National Food Administration (Salminen & Penttilä 1999).

One of the most interesting early finding in the research in humans on the etiology of T1D came from an Icelandic ecological observation published in the early 1980s (Helgason & Jonasson 1981). A prenatally acting seasonal factor, the N-nitroso-compound content of processed mutton traditionally consumed in Iceland around Christmas time lead to a preponderance of T1D in boys born in October. The association between N-nitroso compounds and nitrite, and T1D have been confirmed later in animal experiments, ecological comparisons, and case-control studies in humans (reviewed by Åkerblom & Knip 1998, and Virtanen & Knip 2003). Mother's intake of nitrite at the time of pregnancy is positively related to the risk of T1D (Virtanen et al. 1994).

#### *Energy, birth weight and height and growth*

Both maternal malnutrition and overnutrition are associated with subsequent T1D in the offspring (Jovanovic 2004). Surveys on the impact of birth weight and length on the risk of T1D remain controversial. Low birth weight and short birth length were related to a decreased risk of T1D in a pooled case-control analysis from seven different European centres (Dahlquist et al. 1999), but many case-control settings have reported similar birth size indicators in cases and control infants (reviewed by Virtanen & Knip 2003).

Increased early growth has been associated with increased disease risk in various European populations (Johansson et al. 1994, Hyppönen et al. 1999, Bruining 2000, Hyppönen et al. 2000, EURODIAB Substudy 2 Study Group 2002). The role of infant feeding in this association, if any, remains unclear. Greater weight gain related to greater intake of energy has been observed in formula-fed babies compared with breastfed infants from 3 months of age (Heinig et al. 1993). A

high frequency of intake of complementary foods rich in carbohydrates was a risk factor in 0 – 4 year old children developing T1D in a Swedish case-control study (Dahlquist et al. 1990). It could be concluded that the growth pattern possibly increasing the risk for T1D is the combination of relatively low birth weight and subsequent rapid growth during childhood.

### *Cow's milk and breastfeeding*

Cow's milk represents the first foreign protein source to which the overwhelming majority of infants are exposed. In comparison to breast milk, the protein concentration of cow's milk is higher mostly due to its greater casein content (Martin et al. 1991). The main whey protein component is  $\beta$ -lactoglobulin which is not an endogenous component of human milk. This evokes the highest rate of positive oral challenges (Hughes 1998). Experiments in animals have demonstrated the deleterious effect of cow's milk proteins on pancreatic  $\beta$ -cells (reviewed by Åkerblom & Knip 1998). Elimination of intact cow milk proteins from the diet has significantly reduced the incidence of T1D in the spontaneously diabetic BioBreeding (BB) rat, with the elimination being most effective when it occurs during the pre-weaning period (Martin et al. 1991). Ecologic findings on higher per capita consumption of cow's milk in countries with high T1D incidence support the hypotheses linking T1D to exposure to cow's milk, but none of the findings is based on cow's milk consumption by infants (Scott 1990, Dahl-Jørgensen et al. 1991, Muntoni et al. 2000, Patterson et al. 2001).

The first case-control data postulating that insufficient breastfeeding of genetically susceptible newborn infants may lead to  $\beta$ -cell infection and T1D later in life was published in 1984 (Borch-Johnsen et al. 1984). The Finnish case-control study of Virtanen et al. (1991) was the first human study to suggest a relationship between an early introduction of complementary cow's milk feeding and an increased risk of T1D, independently of the duration of breastfeeding. The importance of early exposure to cow's milk and a short duration of breastfeeding as nutritional risk determinants of T1D have further been studied in numerous case-control settings (reviewed by Åkerblom & Knip

1998, and Virtanen & Knip 2003), which have been the basis for two meta-analyses. Despite the rather similar odds ratios in the meta-analyses based on 13 (Gerstein 1994) and 17 case-control studies (Norris & Scott 1996), the conclusions were different. Gerstein concluded that early cow's milk exposure may be an important determinant of subsequent T1D and may increase the risk by approximately 1.5 times, while Norris and Scott summarized that the weak association between infant diet and risk of T1D may be attributable to methodological sources. Norris and Scott (1996) claimed that differences in the participation rates of cases and controls as well as long-term maternal recall may have biased the results of the analysed studies.

The putative effect of infant feeding on the development of T1D associated autoantibodies has been studied in four birth cohort series: the German (Hummel et al. 2000, Ziegler et al. 2003) and the Australian BABYDIAB (Couper et al. 1999) studies, the North-American DAISY Study in Colorado (Norris et al. 1996), and the Finnish DIPP Study (Kimpimäki et al. 2001a). The preliminary findings of these studies detected no association of breastfeeding or age at introduction of complementary milk feeding with emergence of up to 3 autoantibodies. However, the statistical power in all of these studies was low due to the small number of seroconverters. In the Finnish DIPP Study (Kupila et al. 2001), which was the only one evaluating the positivity for IA-2A or all four autoantibodies at the same time, short-duration exclusive breastfeeding and the early introduction of supplementary milk feeding were related to an increased risk of both of these outcomes (Kimpimäki et al. 2001a). The findings from the pilot study of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) suggest that the emergence of T1D associated autoantibodies can be prevented or delayed by avoidance of cow's milk proteins over the first 6 or 8 months of life (Åkerblom et al. 2005). This breakthrough evidence in humans of the possibility to manipulate spontaneous beta cell autoimmunity by dietary intervention in infancy was, despite the limited power of the study, the basis for the larger well-powered TRIGR -study. The current international

TRIGR -study is one of the largest pediatric trials in the world involving 40 study centres in North America, Europe and Australia (<http://www.trigr.org>).

Several studies suggest that patients with T1D have an enhanced humoral and cellular immunity to a series of dietary proteins as reviewed by Åkerblom & Knip (1998). High levels of antibodies to cow's milk proteins have been associated with increased risk of T1D independently of early formula feeding (e.g. Savilahti et al. 1993, Saukkonen et al. 1994). It is believed that a high titre of cow's milk antibodies is an epiphenomenon of an insult and immunization caused by cow's milk proteins, which in some genetically susceptible individuals may lead to T1D after a long but variable time interval.

Vaarala et al. (1998) proposed an interesting hypothesis that the bovine insulin present in cow's milk may immunize formula-fed infants. This hypothesis was based on the observation that induced antibodies that cross-reacted with human insulin were at significantly lower titres among breastfed and hydrolyzed formula-fed infants compared to babies fed ordinary formula.

### *Cereals*

Wheat gluten and some other plant proteins have shown to be diabetogenic in animal models (e.g. Scott et al. 1997, Nikulina et al. 2004). The only slightly elevated T cell responses to gluten in newly diagnosed children did not support a major role of gluten in the pathogenesis of human T1D (Klemetti et al. 1998). In an ecologic analysis of Muntoni et al. (2000) per capita consumption of cereals was an inverse predictor of elevated T1D incidence. Based on the findings in two birth-cohort studies, there may be a window of exposure to cereals in infancy outside of which initial exposure increases islet autoimmunity risk in susceptible children (Norris et al. 2003, Ziegler et al. 2003). In the US DAISY birth cohort study (Norris et al. 2003), it was found that children initially exposed to cereals (wheat, rye, barley, oats, or rice) between ages 0 and 3 months and those who

were exposed at 7 months or older exhibited an increased hazard of islet autoimmunity compared with those who were exposed during the fourth through sixth months. Similarly, in the German BABYDIAB cohort (Ziegler et al. 2003), food supplementation with gluten-containing foods before the age of 3 months was associated with a significantly increased islet autoantibody risk. Children receiving gluten foods first at the age of 6 months did not suffer an increased risk for elevated islet autoantibodies.

### *Vitamins and minerals*

Evidence from animal experiments and human observational studies suggests that some dietary micronutrients, most promisingly vitamin D, may be able to protect against the development of T1D (reviewed by Hyppönen 2004). Only the possible beneficial role of vitamin D has been studied in both case-control and cohort settings. A European multicentre trial consistently showed a protective effect of vitamin D supplementation in infancy (The EURODIAB Substudy 2 Study Group 1999), whereas contradictory results were found in a small Norwegian case-control study (Stene et al. 2000). However, in the latter study, cod liver oil taken during pregnancy was associated with reduced risk of T1D. Recommended vitamin D supplementation was observed to be associated with a reduction in the risk of T1D in the North Finland Mother-Child cohort (Hyppönen et al. 2001), but the number of incident cases was quite small (<10). Nevertheless, the risk of T1D by age of 31 years was remarkably reduced (>80%) if vitamin D supplementation during the first year was regular in comparison to no supplementation, and a further 80% risk reduction was seen if the given dose had been at least on the level of the contemporary recommendation of 50 µg per day (5 times the current recommendation). In the US birth cohort study (DAISY), maternal intake of vitamin D from food was significantly associated with a decreased risk of islet autoimmunity appearance, whereas vitamin D intake from supplements, as well as intakes of omega-3, and omega-6 fatty acids from food during pregnancy were not associated with appearance of IAA in the offspring after an average of 4 years' follow-up (Fronczak et al. 2003). The difference in the mean daily vitamin D

intake from food between an affected (n=16) and an unaffected (n= 206) group was 2.1 µg (4.2 vs. 6.3 µg, respectively).

Beneficial effects of nicotinamide and vitamin E in diabetes development remain hypothetical (Hyppönen 2004). Despite plausible theoretical background evidence from animal experiments and supportive data from pilot studies, randomized, controlled trials using nicotinamide have not provided any evidence for any beneficial effects (reviewed by Hyppönen 2004). The possible beneficial effects of vitamin E are based on its action as an antioxidant. In addition to several animal experiments, the hypothesis of a protective effect of vitamin E against development of T1D was supported by the human findings of Knekt and colleagues (1999). A nested case-control study within a 21-year follow-up indicated that the serum alpha-tocopherol concentration at the baseline examination was inversely associated with clinical T1D occurring 4-14 years later. However, a major role for vitamin E in the development of T1D remains unlikely.

### **3. Aims of the present study**

The purpose of this thesis was to evaluate diet during pregnancy and infancy and to assess the relation between the early dietary exposures to the development of cow's milk antibodies and T1D associated autoantibodies in a cohort of Finnish infants with increased genetic susceptibility to T1D.

The specific objectives of this thesis were as follows:

1. to evaluate the validity and reproducibility of a self-administered food frequency questionnaire developed to measure food consumption and nutrient intake during pregnancy (I),
2. to evaluate diet and nutrient intake of Finnish pregnant women (I, II),
3. to assess whether the maternal consumption of milk and milk products can affect the development of cow's milk antibodies in infants (III),
4. to investigate infant feeding patterns; the length of breast feeding and the age of introduction of complementary foods, during the first two years (III, IV, V), and to study their effects on the development of  $\beta$ -cell autoimmunity (V).

## 4. Subjects and methods

### 4.1 Subjects and study design (Table 4)

Research questions of the present doctoral thesis are addressed in three different populations: pregnant women taking part in a validation study in the city of Oulu (I, II), newborn infants belonging to the intervention group of Trial to Reduce Insulin dependent diabetes mellitus in the Genetically at Risk (TRIGR) study, (III), and a cohort of infants in Diabetes Prediction and Prevention (DIPP) Nutrition study (IV, V), (Table 4).

**Table 4. Subjects and study design** <sup>(1, 2)</sup>

Paper	Design	Subjects	N	Dietary methods	Immunological variables
I	Methodological survey among random population	Pregnant women in their third trimester	Validation study n = 113, Reproducibility study n = 111	2 x 5-day food records, FFQ 2 x FFQ	-
II	Population-based random survey	Pregnant women in their third trimester	118	2 x 5-day food records	-
III	Randomized double-blind pilot trial (TRIGR)	Genetically susceptible children having first-degree relative/s affected by T1D and randomized to receive hydrolyzed infant formula	97	Mothers: FFQ during pregnancy and lactation Children: 2-day food record (6 mo), 3-day food records (12, 24 mo)	IgA and IgG antibodies to cow's milk formula, $\beta$ -lactoglobulin, bovine serum albumin, and $\alpha$ -casein
IV	Population-based prospective birth cohort study (DIPP Nutrition)	Genetically susceptible children	429	follow-up record of introducing new foods (0-24 mo)	-
V	Population-based prospective birth cohort study (DIPP Nutrition)	Genetically susceptible children	3565	follow-up record of introducing new foods (0-24 mo) and structured dietary questionnaire (3,6,12,24 mo)	T1D associated autoantibodies: ICA, IAA, GADA, and IA-2A

<sup>(1)</sup> All of the parents have given their written informed consent, and the ethical committees of all participating hospitals have approved the study protocols.

<sup>(2)</sup> FFQ = food frequency questionnaire, TRIGR = Trial to Reduce Insulin dependent diabetes mellitus in the Genetically at Risk - study, DIPP = Type 1 Diabetes Prediction and Prevention -study, IgA = immunoglobulin A, IgG = immunoglobulin G, ICA = classic islet cell antibodies, IAA = insulin autoantibodies, GADA = Autoantibodies to the 65-kD isoform of glutamic acid decarboxylase, IA-2A = Autoantibodies to the protein tyrosine phosphatase-related IA-2 molecule, and T1D = type 1 diabetes

#### **4.1.1 Validation study (I and II)**

All of the pregnant women in their third trimester from 13 maternity clinics in the city of Oulu between the August 1995 and the April 1996 were invited to participate in either the reproducibility or the validation study. Of the 123 women invited to take part in the reproducibility study, five women refused to participate, five women did not return both of the questionnaires, and two were excluded because of poorly completed questionnaires. Of the 141 women invited to participate in the validation study, 11 women refused to take part, 14 did not return all questionnaires and/or food records, and three were excluded because of incomplete food records. Therefore, the final sample for the reproducibility study (I) comprised 111 women (90% of those invited) while there were 113 in the validation study (I) (80% of those invited). The research questions in study II were assessed in the framework of the validation study. The final series comprised 118 (83% of those invited) women having complete dietary records (11 refused to take part and 12 did not return both food records).

#### **4.1.2 Trial to Reduce Insulin dependent diabetes mellitus in the Genetically at Risk (TRIGR) study (III)**

The families of 521 newborn infants having at least one member (mother, father or sibling) affected by T1D were invited by letter to participate in the TRIGR pilot trial between April 1995 and November 1997 from 16 hospitals in Finland (Åkerblom et al. 2005). Altogether 471 (90% of those invited) were screened, and 234 (45% of those invited) were identified as having HLA-DQB1 genotype [DQB1\*02/\*0302, \*0302/x (x ≠ \*02, \*0301 or \*0602,\*0603) or 02/y (y ≠ 0302, \*0301, or \*0602/\*0603)], conferring increased risk for T1D. The families of these infants were asked to continue in the intervention study. Immediately after birth, the infants were randomized to receive, after exclusive breastfeeding in a doubled-blinded manner, either the extensively hydrolysed casein-based formula (Nutramigen®, Mead Johnson, Evansville, IN, USA) or a whey-adapted cow's milk based formula (Mead Johnson) as the first milk product introduced into the infant's diet. The dietary intervention lasted at least until the infant reached the age of 6 months or at most

until the age of 8 months, in those cases where the child had not received the formula for at least 2 months by the age of 6 months.

The subjects in study III belong to the intervention group (hydrolysed infant formula) of the TRIGR pilot trial. The final number of 97 infants (87% of those randomized to the intervention arm) comprised those for whom data of nutrition and cow's milk antibodies on at least one occasion were available.

#### 4.1.3 The Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition Study (IV and V)

The research questions in studies IV and V were assessed in the cohort of Diabetes Prediction and Prevention (DIPP) Study, which is a multidisciplinary prospective population-based prevention



study (Kupila et al. 2001). All the newborn infants from the catchment areas of three university hospitals: Turku, Oulu, and Tampere, in Finland are screened for genetic susceptibility for T1D from cord blood samples. All parents of these children have given their written informed consent for genetic screening. The high-risk children (HLA-DQB1\*0302/02 heterozygous and those positive to DQB1\*0302/x; x stands for homozygosity or neutral alleles) are

followed for diet, growth, viral infections, and T1D associated autoantibodies (ICA, IAA, antibodies to GAD65 and IA-2) at 3 to 12 months intervals. The families are offered the possibility to take part in a randomised double-blinded intervention trial with intra-nasal insulin when their child tests repeatedly positive for at least two autoantibodies.

The DIPP Nutrition study was performed in the framework of the DIPP Study. It was started on the 2<sup>nd</sup> of September, 1996 in Oulu, and on the 20<sup>th</sup> of October, 1997 in Tampere. The catchment area of the University Hospital of Oulu includes 42 municipalities and that of Tampere 34



municipalities. The subjects in the study IV comprise 429 infants (64% of those invited) who were recruited in the follow-up between September 1996 and December 1997, and who completed the “age at introduction of new foods” -form by the age of 2 years. The series in study V comprises the children born at the latest on the 30<sup>th</sup> of June, 2001 in Oulu and Tampere University Hospitals. The data were available from 3565 children (81% of the children invited), of whom 93.5% were followed up to 6 months, 81.9% up to 1 year, and 68.3% up to 2 years of age.

## **4.2 Dietary methods, (Table 4)**

### **4.2.1 Food records (I, II, III)**

In studies I and II (validation study), the data of diet were collected by means of two 5-day food records comprising 4 week days and one weekend day. The first record was kept during 29-32 weeks and the second one during 33-36 weeks of gestation. The time interval between the two records had to be at least one week. Everything consumed (including vitamin and mineral supplements) during the recording period was instructed to be recorded, this being facilitated by provision of a booklet with 126 portion pictures of common food items and mixed dishes (Haapa et al. 1985).

In study III, the diet of the offspring was assessed by means of a 2-day food record at the age of 6 months and by a 3-day food record at the ages of 1 and 2 years. The additional data about possible deviations from the advised diet and age at introduction of new foods were also inquired. Personal and written instructions on the record keeping were given in advance in all studies (I, II and III), and the records were checked by a nutritionist after each recording period.

### **4.2.2 Food frequency questionnaire, FFQ (I and III)**

The FFQ of 276 food items originally developed for measuring the dietary intake of 29,000 healthy middle-aged Finnish men participating in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study (Pietinen et al. 1988a; 1988b), and further modified to contain 110 food items for studying the diet of Finnish women participating in the Kuopio Breast Cancer Study (Männistö et al. 1996)

was used as the basis for the FFQ which was evaluated and used in studies I and III. Our FFQ was specifically designed to reflect the diet of Finnish women by using the information of the food consumption of women in childbearing age in the Finrisk Study (Kleemola et al. 1994). The ‘recipe’ behind each food-item was weighted by the frequencies of consumption in the food group considered (e.g., the recipe for breakfast cereals consists of rice crispies 33%, corn flakes 23%, cereals with added sugar 22%, and Weetabix 22%). Open frequency categories were used in increasing order: less than once per month, how many times per month, week, or day. The serving sizes were based on commonly used portions identified in earlier Finnish dietary studies (Leino 1984, Haapa et al. 1985) and for some foods, natural units (e.g. for eggs and beverages) were used.

The FFQ was designed to assess the whole diet over a period of 1 month aiming to rank individuals according to their food consumption and nutrient intake. The FFQ comprised 181 food items and mixed dishes, grouped under subheadings: “milk products“, “potato, rice and pasta as a side dish“, “cereals“, “fat on bread/spreads“, “fruits and berries“, “vegetables“, “salad dressings“, “warm main courses“, “fish and eggs“, “beverages“, and “desserts, sweets and snacks“. There were additional empty lines for each food group for recording foods not listed in the FFQ. Moreover, questions were asked about where the meals were usually taken (at home, in a cafeteria/restaurant), and the type of fat used in food cooking, baking and salad dressings. In the further analysis, the women’s personal choices for fat used in cooking were taken into account. Butter was used in cooking in all recipes for those subjects who generally ate their meals at home and who used butter in cooking. For other subjects, the recipe fat was determined to be butter, margarine, and oil in equal proportions, assuming that people who eat lunch at work do not have detailed knowledge of the type of fat used in cooking.

The participants in the reproducibility study (I) completed the FFQ twice, at an interval of 1 month, at the beginning and at the end of the 8<sup>th</sup> month of pregnancy. The participants in the validation

study (I) completed the FFQ 1 month before the start of the maternity leave (at the beginning of the 8<sup>th</sup> month of pregnancy) and 1 month after delivery.

In study III, mothers completed the FFQ after delivery covering retrospectively the maternal diet during the eighth month of pregnancy. If the mother was breastfeeding when the child was 3 months old, her diet during the preceding month was assessed using the FFQ. In the further analysis the cow's milk protein intake of the mother was grouped according to the type of processing into three groups: "Raw milk products" (regular milk, cream and ice cream), "Cheese" (all hard cheeses), and "Sour milk products" (sour milk, cultured milk and yogurt). In both studies (I and III) the completion of the FFQ was well guided in advance and returned FFQs were checked by a nutritionist.

#### **4.2.3 Follow-up record of introducing new foods (IV and V)**

Information on infant feeding patterns in studies IV and V was collected with the "age at introduction of new foods" form. During the first 2 years of life, parents were asked to mark down on the dietary follow-up form the age of the infant when exclusive and total breastfeeding were stopped and the age when the infant started to receive various complementary foods. The form included the following food groups: fruit and berries; potato; carrot; spinach or beetroot; turnip or swede; cabbages; lettuce; wheat; barley; oats; rye; maize, rice, millet or buckwheat; pork; beef; chicken; other meats; sausage; fish; egg; sour milk products; foods containing cow's milk; cow's milk or ice cream; and soy products. A blank entry for food items was taken to indicate that such foods were not consumed before the age of 2 years or before the time when the family dropped out of the study. The dietary form was completed at home and it was checked at every visit and the information was transferred to the dietary form when the family visited the clinic.

In study IV, the following food groups were used in the analysis of food consumption: fruit and berries; root vegetables (potato and carrot); vegetables high in nitrate (spinach, beetroot, swede and turnip);

gluten-containing cereals (wheat, barley, rye, oat); other cereals (rice, maize, buckwheat, millet); meat and meat products; sausage; fish and fish products; egg; and dairy products. Accordingly, dietary variables were constructed of the age when the first complementary food was given.

In the analysis of study V, the following food groupings were used: 1) fruit and berries; 2) root vegetables: potato, carrot, turnip, swede; 3) wheat, barley, rye and oats; 4) other cereals: maize, rice, millet or buckwheat; 5) cabbages (cauliflower, broccoli, kale, and Chinese, red and turnip cabbage); 6) milk products (cow's milk based infant formulas, sour milk products, milk, other milk products, foods containing milk); 7) cow's milk based infant formulas (excluding hydrolyzed and soy formulas); 8) fish; 9) meat (pork, beef, chicken, other meat); and 10) sausage. In addition, wheat, rye, barley and oats were each analyzed separately.

#### **4.2.4 Structured dietary questionnaire (V)**

Age specific (3, 6, 12, and 24 month) questionnaires were used to obtain information on infant feeding in study V. In the 3-month dietary questionnaire, the feeding at the delivery hospital was assessed in detail: whether the child was breastfed, had received banked breast milk, was exposed to infant formula and if so to which formula, and which of these was/were the main type of feeding during the delivery hospital stay. The duration of breastfeeding and the age at introduction and brand names of all infant formulas which the child had received as well as the age at introduction of other cow's milk and sour milk products (such as yoghurt, ice cream) and foods and drinks containing cow's milk were inquired for in all questionnaires. The brand name of the infant formula was recorded and the formulas were later classified as regular cow's milk based, special (hydrolyzed) or soy based. At each time point all the food items the infant had so far received were carefully recorded in the questionnaire.

#### **4.2.5 Breastfeeding**

In all studies exclusive breastfeeding was defined as the period during which the child received, in addition to breast milk, only water and/or vitamin/mineral supplements (mainly vitamin D starting 2 weeks after delivery). Neither infant formula nor other complementary foods were allowed. In study V, also the information on feeding in the maternity hospital could be taken into account when grouping infants according to the type of breastfeeding. Total breastfeeding was defined as the period during which the child received breast milk.

#### **4.2.6 Converting food records and questionnaires to food and nutrient intakes, (Figure 1)**

The food consumption data were analyzed using a software program developed at the National Public Health Institute (2005). For food consumption and nutrient intake (studies I – III), an estimate of an average daily intake was calculated by means of food records. Accordingly, the data on nutrient intake was analyzed as by food groups. A software program linking the food frequency data with the nutrient software program was developed in the National Public Health Institute (I and III). The program can accommodate each woman's personal fat choices (in cooking, baking and salad dressings) which were inquired in the questionnaire. FFQ-data was converted into a food record –type of data, which present an average values per day. To calculate the intakes of selected nutrients from dietary supplements (II) an Excel database was constructed for the present study based on information from the National Food Administration, the National Public Health Institute, and manufacturers. Nutrient intake from supplements was as well calculated as an average daily intake (a sum of nutrient divided by the number of recording days).

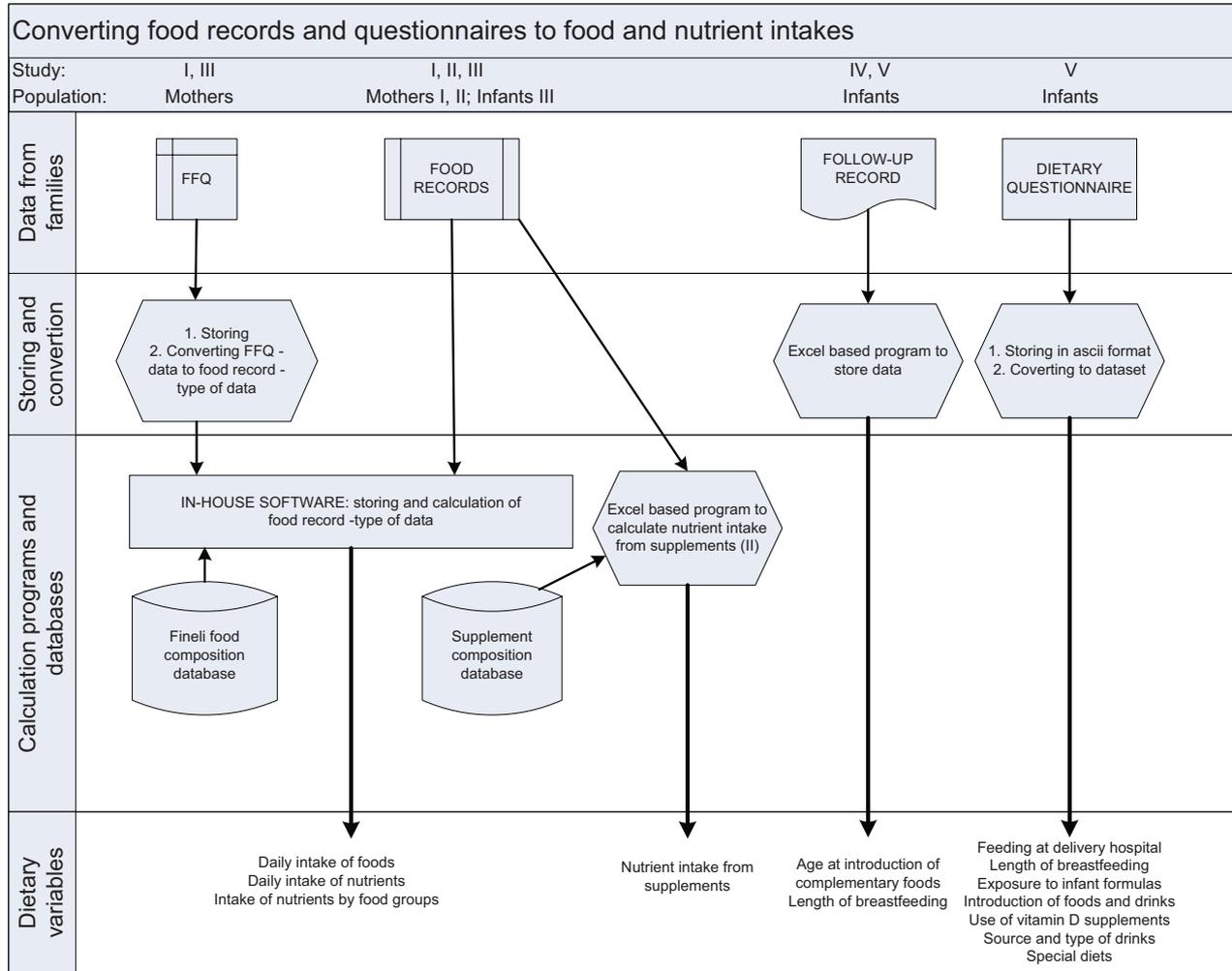


Figure 1. Converting food records and questionnaires to food and nutrient intakes, (FFQ = food frequency questionnaire)

The food composition database of the National Public Health Institute, called Fineli, (National Public Health Institute 2005) is continuously updated and is the most comprehensive one in Finland. The analytical nutrient values in the database are mostly based in Finnish studies. In addition, complementary data is obtained from the Finnish food industry and international food composition tables. The database currently includes over 3 000 individual food items and mixed dishes, 284 nutrient factors, and more than 400 supplements. The system allows for the creation or modification of specific recipes and personal recipes were used whenever possible. Standard recipes in the database are based on the current Finnish cookery books. As part of the DIPP Nutrition Study, the product information and nutrient content of infant formulas and baby foods have been stored and are continuously updated into Fineli.

#### **4.3 Sociodemographic and perinatal characteristics**

From the structured questionnaire completed at 3 months after delivery, the following information was obtained: parents' age, basic and vocational education, occupation, the number of siblings, the type of day care, and the home province (I, II, IV, and V). Information on duration of gestation, maternal weight development and smoking during pregnancy, mode of delivery, birth weight and height was obtained from the Medical Birth Registries of the Oulu and Tampere University Hospitals (II, IV, and V).

#### **4.4 Genetic methods**

HLA-DQB1 alleles in studies III, IV, and V were analyzed using a large-scale assay procedure that was developed for rapid screening purposes. The method utilizes time-resolved fluorometry to detect the hybridization of lanthanide-labeled allele-specific oligonucleotide probes with an amplified gene product as earlier described (Ilonen et al. 1996). In brief, a part of the second exon of the HLA-DQB1 gene was amplified using a primer pair with a biotinylated 3' primer. The biotinylated PCR products were then transferred to streptavidin-coated microtitration plates, denatured and hybridized with sequence-specific probes labeled with lanthanide chelates: europium

(Eu), terbium (Tb) or samarium (Sm). Two hybridization mixtures were used, one containing probes hybridizing with DQB1\*0602 and \*0603, DQB1\*0603 and \*0604 and a consensus sequence and the other containing probes specific to the DQB1\*02, \*0301 and \*0302 alleles. After appropriate incubations and washings, the specific hybridization products were detected using three-color time-resolved fluorescence of the lanthanide chelates.

#### **4.5 Immunological methods**

Blood samples were obtained from the infants at birth (cord blood) and at the ages of 3, 6, 9, 12, 18, and 24 months (III). The maternal blood samples were taken on an average 2 days after the delivery. Antibodies of IgA and IgG isotypes to whole cow's milk formula, beta-lactoglobulin (BLG), bovine serum albumin (BSA), and alpha-casein (CAS) were measured using modifications of the original enzyme-linked immunosorbent assay (ELISA) techniques as previously described (Savilahti et al. 1993, Saukkonen et al. 1994, Vaarala et al. 1995). Microtitre plates (MaxiSorp®, Nunc A/S, Roskilde, Denmark) were coated with one of the following antigens: adapted liquid cow's milk formula (Tutteli®, Valio Ltd, Helsinki, Finland); BLG (L-0130, Sigma Pharmaceuticals, St. Louis, MO, USA); BSA (2 µg/ml, grade V, Sigma), and CAS (C 6780, Sigma). Wells were blocked either with 0.5% sheep serum (for anti-cow's milk and BLG assays) or with 1% gelatin in PBS pH 7.4 (for BSA and CAS assays). Serum samples were diluted in the blocking buffers. Triplicate dilutions were used for dilutions of standard serum, duplicates for the assayed sera. Alkaline-phosphatase-conjugated affinity purified rabbit F(ab')<sub>2</sub> anti-human IgG, IgA or IgM antisera (Dako A/S, Glostrup, Denmark), and p-nitrophenyl-phosphate substrate, 1 mg/ml in TRIS buffer, (Sigma Fast p-nitro phenyl Phosphate Tablest sets N-2770, Sigma) were added. The reaction was followed and stopped when the absorbance of the highest standard was above 2 by addition of 100 µl 1 M NaOH. The end point measure of OD<sub>405</sub> nm was obtained in a semi-automatic multiwell photometer (Titertek Multiscan®, Elflab Inc., Helsinki, Finland). The mean value of two absorbancies for wells coated with blocking solution was subtracted from the mean value for the two absorbancies in antigen-coated wells. Results were subjected to point-to-point analysis in a

computerized photometer using 2-fold serial dilutions of a high titer standard serum as reference. The sample dilutions had to fall within the linear part of the standard curve, and antibody levels were expressed as percentages of the standard. Antibody titers of IgA isotype in umbilical cord blood were not included in the statistical analyses as IgA is not transported through the placenta to the fetus during pregnancy and the titers detected in a few infants were considered to be due to laboratory mistakes or contamination of the umbilical cord blood sample with maternal blood.

In the DIPP-Study (IV and V) ICA are used as the primary screening tool for  $\beta$ -cell autoimmunity in children with increased genetic risk of T1D (Kupila et al. 2001). Only children testing positive for ICA were analyzed for IAA, GADA and IA-2A (all preceding and subsequent samples). ICA were quantified by a standard indirect immunofluorescence method on sections of frozen human pancreas from a blood group O donor (Bottazzo et al. 1974). The end-point dilution titer of positive samples was recorded and the results expressed in Juvenile Diabetes Foundation (JDF) units. The detection limit was 2.5 JDF units. All samples initially positive for ICA were retested to confirm positivity. The sensitivity of the ICA assay in the used laboratory was 100% and the specificity 98% in the most relevant standardization workshop round. Serum levels of IAA were quantified with a microassay (Ronkainen et al. 2001) modified from that described by Williams et al. (1997). The IAA values representing the specific binding were expressed in relative units based on a standard curve run on each plate using the MultiCalc™ software program (PerkinElmer Life Sciences Wallac, Turku, Finland). A subject was considered to be positive for IAA when the specific binding exceeded 1.55 relative units (the 99<sup>th</sup> percentile in 371 non-diabetic Finnish subjects). The disease sensitivity of the IAA microassay was 44% and its specificity 98% in the CDC-sponsored Diabetes Autoantibody Standardization Program (DASP) Workshop 2002.

GAD antibodies were measured with a radiobinding assay as described (Savola et al. 1998a). The results were expressed in relative units based on a standard curve constructed from a dilution of

positive and negative samples. The cut-off limit for antibody positivity (5.35 relative units) was set at the 99<sup>th</sup> percentile in 373 non-diabetic Finnish children and adolescents. The sensitivity of the GAD antibody assay was 82% and its specificity 98% in the 2002 DASP Workshop. IA-2 antibodies were quantified with a radiobinding assay as described (Savola et al. 1998b). Antibody values were expressed in relative units based on a standard curve, as for GAD antibodies. The limit for antibody positivity was set at 0.429 relative units, which represents the 99<sup>th</sup> percentile in 374 non-diabetic Finnish children and adolescents. The sensitivity of this assay was 62% and its specificity 100% in the 2002 DASP Workshop. Samples with IAA, GAD, or IA-2 antibody levels between the 97.5<sup>th</sup> and 99.5<sup>th</sup> percentiles were reanalyzed to confirm the antibody status. Transplacentally transferred autoantibodies (Hämäläinen et al. 2000) were excluded from the analyses for the appearance of  $\beta$ -cell autoimmunity. In the analysis of study V, data on T1D associated autoantibodies until September 30, 2004 were used.

When data on T1D associated autoantibodies until September 30, 2004 were used, among the 3565 children with genetic risk for T1D, 237 (6.6%) were at least twice positive for ICA and 101 (2.8%) repeatedly positive for ICA plus at least one other antibody (of them 83 tested at least twice as being positive for IAA, 66 for GADA, and 49 for IA-2A) during the median follow-up time of 4 years (range 0.2 to 8.1 years) since birth. Altogether 42 children (1.2%) had progressed to clinical T1D at a median age of 3.2 years (range 1.0-6.4 years). We decided to include clinical T1D in the autoantibody endpoints due to three persistently seronegative children among those progressing to T1D. This resulted in 111 children (3.1% of all children) being positive for the ICA plus at least one other antibody, and this was used as an endpoint.

#### **4.6 Statistical methods**

Means and standard deviations for food consumption and nutrient intake were calculated for data derived from the FFQs and food records. In addition, pooled protein intake from different milk

products during pregnancy and lactation was separately calculated in study III. Median and range were used to describe the duration of breastfeeding and age at introduction of complementary foods (IV). Several variables based on cow's milk exposure were created in study V: any type of cow's milk exposure, exposure to cow's milk-based infant formulas, and early exposure in the delivery hospital. Log-transformation of food consumption and nutrient intake ( $\log [x + 0.1]$ ) was done in studies I – III. Accordingly, antibody titres were log transformed in study III. Adjustment for total energy intake was performed using the residual method of Willett (1997), (I – III). Further statistical methods were as follows.

### *Study I*

Intraclass correlation was used to measure the reproducibility between the questionnaires in the reproducibility study. Pearson product-moment correlation and Spearman correlation were used to compare food and nutrient intake from FFQs with those from food records. Pearson product-moment and Spearman rank correlations were used to compare food and nutrient intake from FFQs with those from food records in the validation study. The correction of the observed correlations for the attenuating effect of random within-person error can be written as

$$r_t = r_o \sqrt{(1 + \lambda_x/n_x)}$$

where  $\lambda_x$  is the ratio of the within- and between-person variances for x, and  $n_x$  is the number of replicates per person for the x variable (Willett 1998, Margetts & Nelson 1991). For study II,  $n = 10$  representing each recording day. Fisher's normalizing transformation

$$z = \frac{1}{2} \log_e [(1 + r)/(1 - r)] \text{ with } s \bullet e(z) = 1/(n-3)^{1/2}$$

was used to calculate 95% confidence intervals for the Pearson correlation coefficient  $r$ . The degree of misclassification across categories between the FFQ and the dietary records was examined by dividing food consumption and nutrient intake into quintiles based on both methods. The proportions of correctly categorized subjects in the same or adjacent quintiles were calculated.

### *Study II*

The t-test was used for testing the significance of differences in food and nutrient intake by education group and smoking, and analysis of variance by BMI-group and age group. Multiple linear regression was used to take account of the independent effects of age and length of education, and logistic regression to test the association between the use of supplements and sociodemographic variables.

### *Study III*

Spearman correlation coefficients and Wilcoxon signed rank test were used to study associations between maternal diet and maternal antibody titers, and between antibody titers in children and mothers. Partial correlation coefficients were calculated between dietary variables and cow's milk antibody titres. Logistic regression analysis was used to test the association between antibody titers in children (highest tertile = 1, others = 0) and maternal protein milk intake from different milk products (Hosmer & Lemeshow 1989). Wald statistics were examined for each variable included in the model.

### *Study IV*

Sociodemographic differences in feeding patterns were analyzed with the non-parametric Mann-Whitney and Kruskal-Wallis tests, and with the  $\chi^2$ -tests among categorized dietary variables. In the logistic regression models, dietary variables were categorized according to the previous Finnish nutrition recommendations for infants and young children (Hasunen et al. 1997), and the independent contribution and the corresponding odds ratio of all sociodemographic factors were estimated.

### *Study V*

Logistic regression analysis was used to study background factors associated with age at introduction of selected foods. A piece-wise exponential survival model with constant hazard in the intervals 0-1, 1-2 and  $\geq 3$  years was used to accommodate interval censored and most likely sibling dependent outcomes. Random effects for family were introduced to accommodate familial

dependence and these were assumed to follow a normal distribution. Time-dependent exposures were allowed to influence the risk in a given observational interval only if the exposure could reasonably be expected to affect the subject for at least half of the length of the interval. The models were fitted using maximum likelihood in SAS PROC NLMIXED, with standard errors of estimates derived from the observed information matrix. The proportionality of the hazards was tested by adding interaction terms of the exposure variables with time interval, to the models.

Statistical analyses were performed using SAS (SAS Institute, Inc: Cary, NC) procedures (studies I, II, and V) and the SPSS 7.5 (IV), 10 (III), and 12 (V) statistical packages for Windows (SPSS Inc., Chicago, IL, USA).

## 5. Results

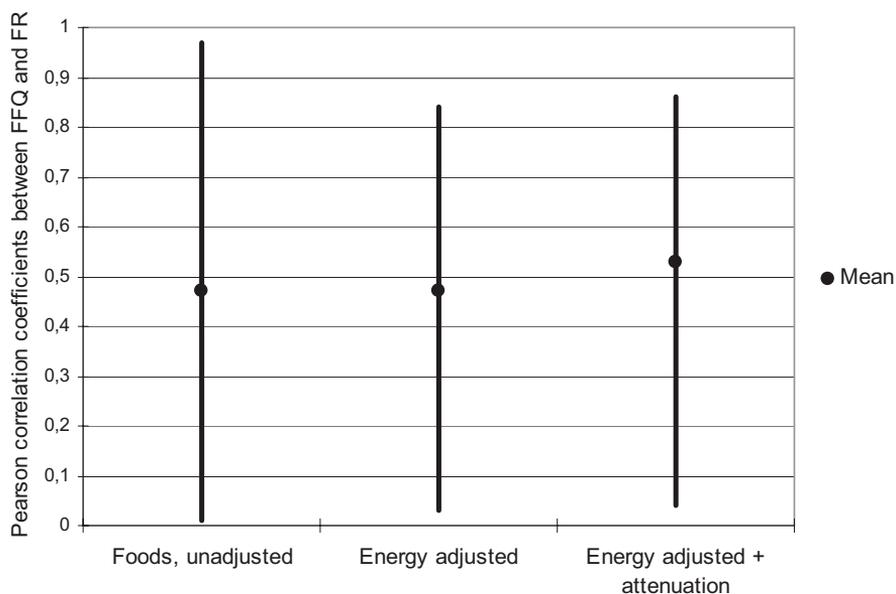
### 5.1 Validity of dietary methods (I, II, and III)

#### *Reproducibility of FFQ*

The mean intake of all foods and nutrients, except for high-fat milk, buttermilk, and soft margarine, were generally higher when estimated using the second FFQ than the first. The greatest differences ( $> | \pm 25 | \%$ ) between the first and the second FFQ among foods were found for high-fat milk, soft drinks, vegetables, ice cream, and fish, and among nutrients for ethanol. The intraclass correlation coefficients between the two FFQs ranged from 0.44 (ice cream) to 0.91 (coffee) for foods. The correlation coefficients were highest for those items consumed daily, such as coffee (0.91), low-fat milk (0.85), and butter (0.81), and lowest for occasionally eaten foods like ice cream (0.44), oils (0.54), and low-fat spreads (0.55). The intraclass correlation coefficients for nutrients ranged from 0.42 for ethanol to 0.72 for sucrose, riboflavin, and calcium. The average of all correlation coefficients for foods and nutrients was 0.65.

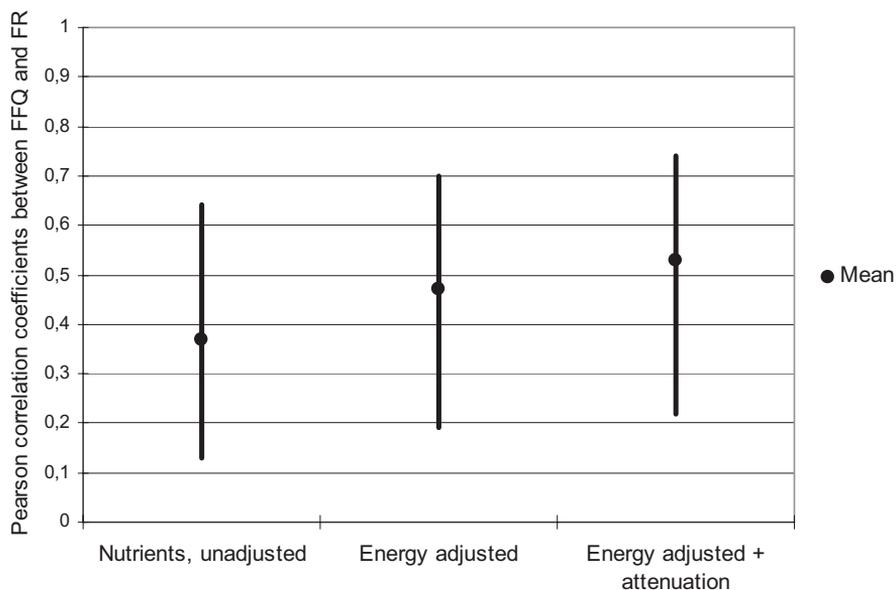
#### *Validity of FFQ*

The intake of foods and nutrients were higher as determined by FFQ than the intake assessed using food records; the estimates of FFQs for foods were approximately 129% (range from 83% for soft drinks to 188% for pork), and for nutrients 136% (range from 100% for ethanol to 169% for thiamine) of the values based on the means of 10-day food records. The unadjusted Pearson correlation coefficients for foods ranged from 0.01 for high-fat milk to 0.97 for coffee (Figure 2). The correlations for foods were stronger than the corresponding coefficients for nutrients (mean 0.47 vs. 0.37), and tended to be stronger for foods consumed at higher frequencies. Adjustment for energy led to the most conspicuous changes in the intake of butter, sausages, and pork, and the correction for attenuation for the consumption of eggs, potatoes, sausages, and berries. The adjusted and corrected correlation coefficients then ranged from 0.04 (high-fat milk) to 0.86 (low-fat milk).



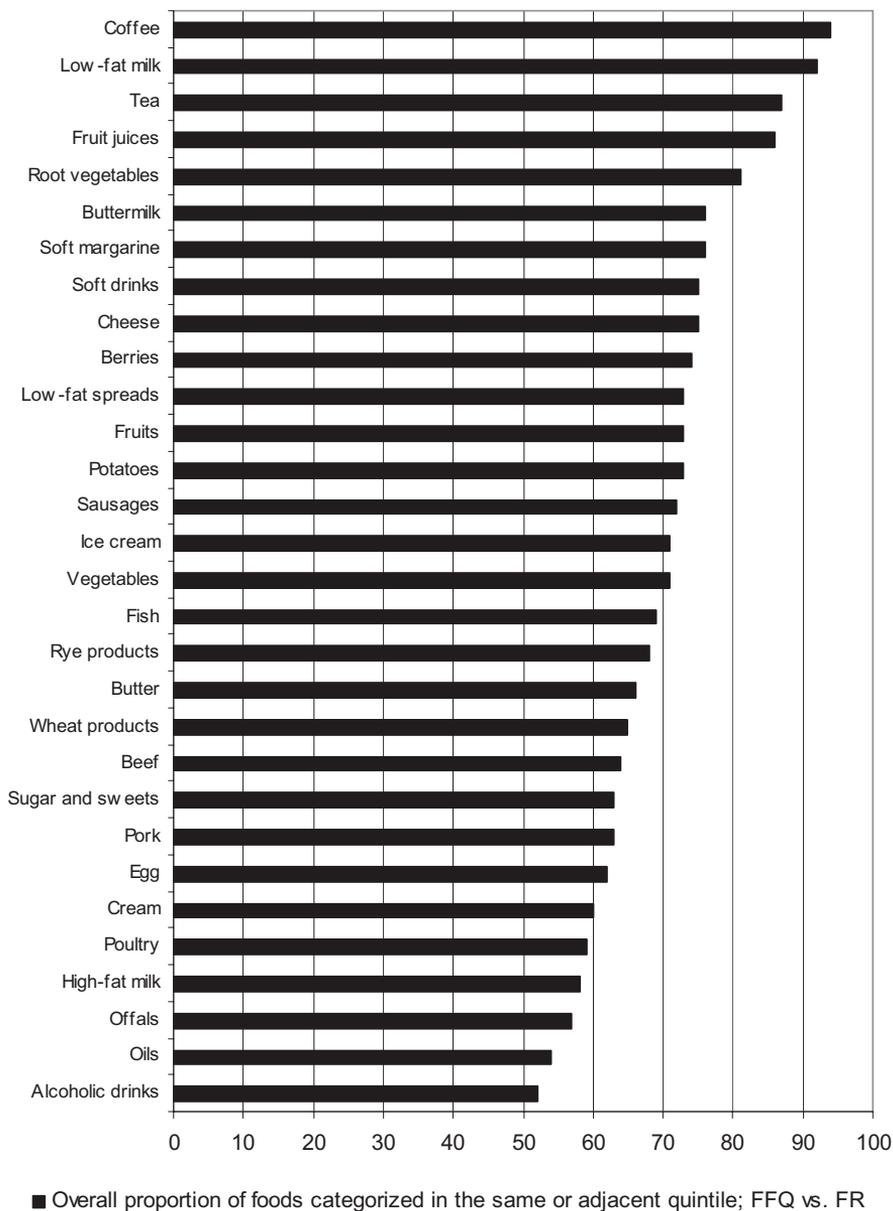
**Figure 2. Pearson correlation coefficients between food intake measured by FFQ and FR; mean and range (includes 30 foods, see Figure 4)**

The energy-adjusted Pearson correlation coefficients for nutrients (Figure 3) varied from 0.19 (vitamin E) to 0.70 (thiamine). When taking the effect of attenuation into account, the coefficients ranged between 0.22 (vitamin E) and 0.74 (thiamine). According to the improvement in the correlation coefficient, when attenuation was taken into account, the subjects had the greatest within-person variation with respect to their intakes of total triglycerides, vitamin D, mercury, nitrite, copper, and vitamin B<sub>12</sub>.

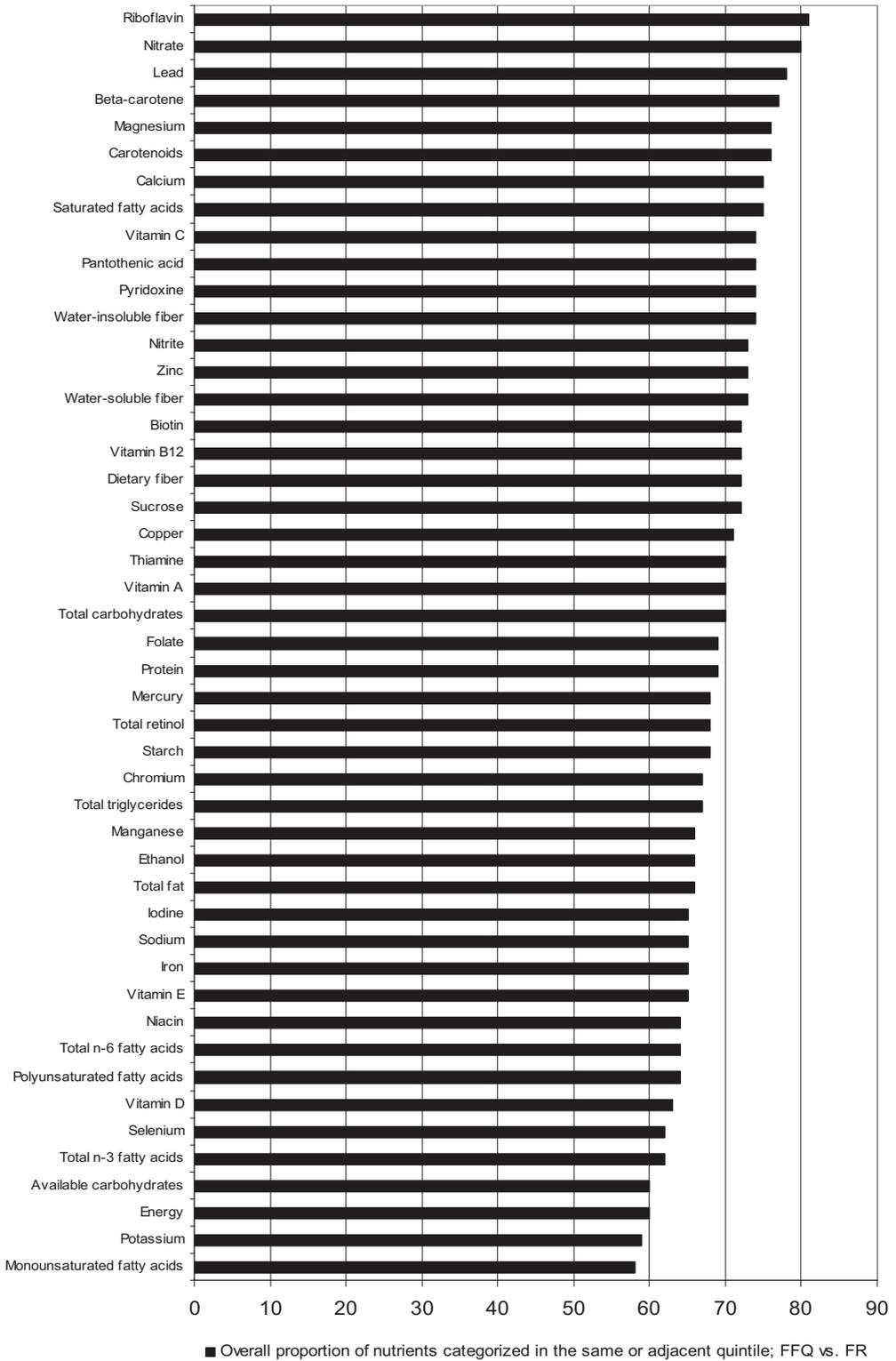


**Figure 3. Pearson correlation coefficients between nutrient intake measured by FFQ and FR; mean and range (includes 47 nutrients, see Figure 5)**

An average of 70% (52-94%) of the women were classified by both methods into the same or adjacent quintiles according to their food intake, and an average of 69% (58-81%) according to nutrient intakes (Figures 4 and 5). The greatest misclassifications (> 15% in the highest quintile on FFQs and in the lowest quintile on food records) in food groups were found for high-fat milk (23%), and offals (18%), and for thiamine (18%) in the nutrients. On average, only 6% of foods, and only 5% of nutrients were grossly misclassified into extreme quintiles.



**Figure 4. Overall proportion (%) of foods categorized in the same or adjacent quintile in cross classification of FFQ and FR**

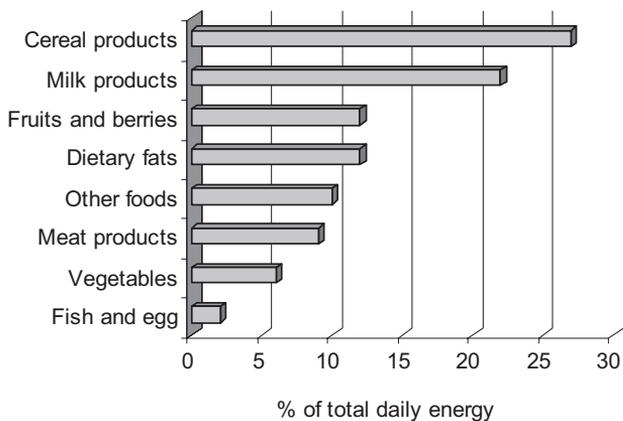


**Figure 5. Overall proportion (%) of nutrients categorized in the same or adjacent quintile in cross classification of FFQ and FR**

## 5.2 Dietary intake during pregnancy (I, II, and III)

### *Food consumption*

The main sources of energy in the diet of pregnant women were cereal products and milk products (Figure 6). The common use of cheese and foods rich in sugar (e.g. sweets, juices, yoghurt) increased the intake of saturated fat and sugar. The consumption of fish and poultry was low. More than one-fourth of the women (28%) ate liver or liver products despite to the general recommendation not to use liver dishes during pregnancy.



**Figure 6. The main sources of energy (% of total daily energy) in the diet of the pregnant women in study II**

The composition of the diet varied in relation to maternal age, education and smoking (Table 5), with the education having the greatest impact.

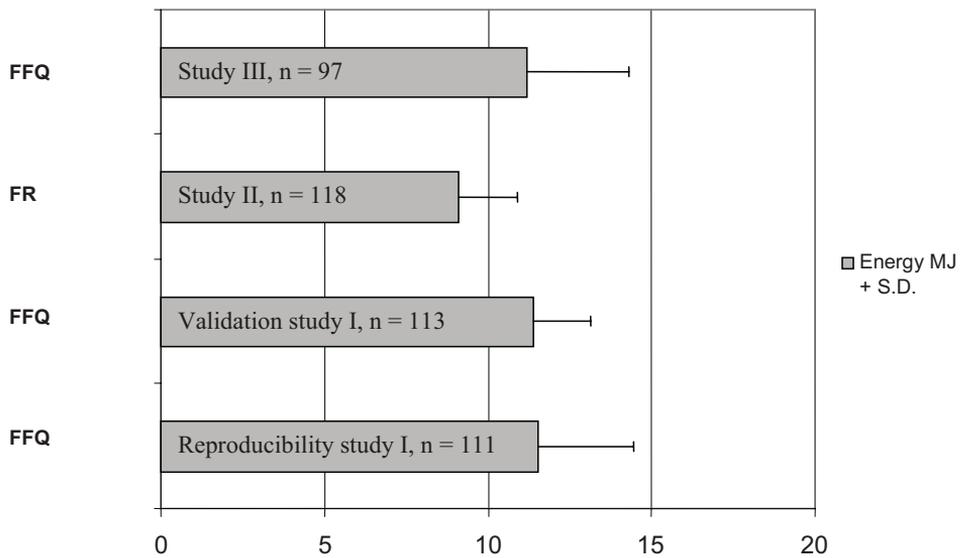
**Table 5. The associations of maternal age, education, and smoking with the diet during pregnancy; statistically significant ( $p \leq 0.05$ ) results from study II<sup>(1)</sup>**

	Age ↑	Education ↑	Smokers
<i>Intake of foods:</i>			
Sugar	↓		
Pork	↓		
Offals	↑	↓	
Sausages		↓	
Vegetables		↑	
Fruits		↑	
Fruit juices		↑	
Berries	↑		
Butter	↑		
Tea		↑	
Coffee		↓	↑
<i>Intake of nutrients:</i>			
Total energy		↑	
Total carbohydrate		↑	
Sucrose		↑	
Lactose		↑	
Dietary fiber	↑		
Vitamin C		↑	
Calcium		↓	
Iron	↑		
Magnesium	↑		
Ethanol			↑

<sup>(1)</sup> ↑ means that marked sociodemographic variable has a positive association and ↓ means an inverse association

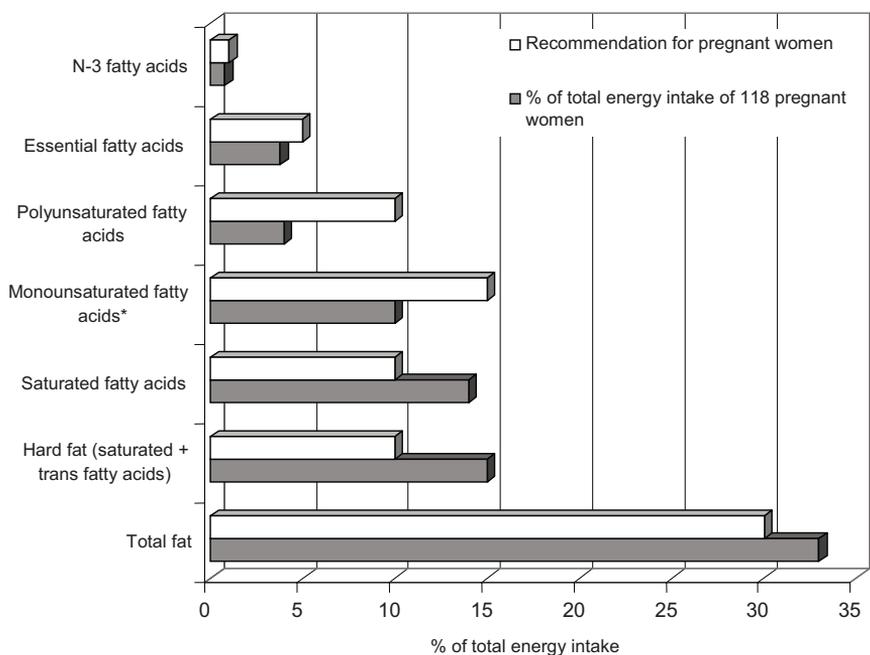
### Energy intake and nutrients

The obtained total energy intake during pregnancy was strongly dependent on the dietary assessment method used; the mean intake was about 2 MJ higher when estimated using FFQ in studies I and III than using the food record in study II (Figure 7). On average 15% of the total energy was supplied by protein, 33% by fats, 52% by carbohydrates, and 12% by sucrose (II).



**Figure 7. The mean intake of total energy (MJ + SD) during the eighth month of pregnancy by different dietary assessment methods in studies I – III, (FFQ = food frequency questionnaire, FR = food record)**

Compared to the Finnish nutrition recommendations (Valtion ravitsemusneuvottelukunta 2005), the intake of dietary fiber was low (19 g vs. 25-35 g/d) and the intake of sucrose high (12 en% vs.  $\leq 10$  en%). Longer educated women tended to have a higher intake of most carbohydrates (Table 5). Saturated fatty acids contributed about half of the total fat intake (Figure 8). The diet of those pregnant women not eating fish during the recording period ( $n = 17$ ) contained significantly less eicosapentaenoic acid, docosahexaenoic acid, and vitamin D ( $p < 0.001$  for all), compared to the diet of women consuming fish ( $n = 101$ ).



**Figure 8. Intake of dietary fat (en%) by pregnant women (II)**

(\*Recommended monounsaturated fatty acid intake ranges between 10 – 15 en%)

The intakes of vitamins and minerals met or exceeded the recommended allowances, except for vitamin D, folate, and iron (Table 6). The recommended daily intake of thiamine seemed also difficult to meet. The main dietary source of vitamin D was fish (36% of total vitamin D intake), and main sources of folate were vegetables and cereal products. The mean intake of vitamin A was higher among the women eating liver or liver products than women who did not use these products (1930 µg vs. 965 µg,  $p < 0.001$ ). Young and less educated pregnant women tended to encounter the greatest difficulties in meeting the dietary recommendations (Table 5).

**Table 6. Dietary intake of selected nutrients in comparison to nutrition recommendations for pregnant women (II)**

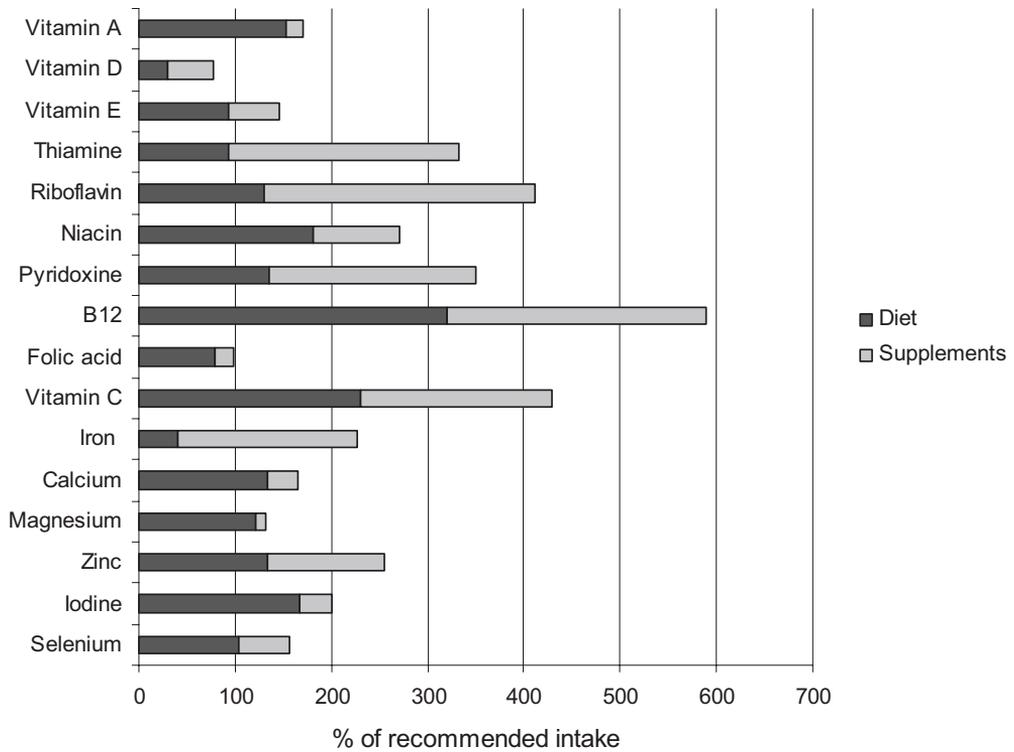
Nutrient	I quartile	Median	III quartile	Recommendations for pregnant women <sup>(1)</sup>
Vitamin A, µg	697	979	1433	800
Vitamin D, µg	2.2	2.9	4.5	10
Vitamin E, µg	8.2	9.5	11.1	10
Thiamine, mg	1.1	1.3	1.5	1.5
Riboflavin, mg	1.7	2.1	2.5	1.6
Niacin, mg	23	29	35	17
Pyridoxine, mg	1.6	2.0	2.2	1.5
Folate, µg	262	305	352	400 <sup>(2)</sup>
Vitamin B12, µg	4.6	5.9	7.9	2.0
Vitamin C, mg	100	143	194	85
Calcium, mg	1101	1342	1636	900
Iron, mg	9.6	11.4	12.9	<sup>(3)</sup>
Magnesium, mg	296	333	394	280
Zinc, mg	10	12	14	9
Iodine, µg	251	289	339	175
Selenium, µg	47	57	66	55

<sup>(1)</sup> Valtion ravitsemusneuvottelukunta 2005, Nordic Nutrition Recommendations 2004.

<sup>(2)</sup> In the new Finnish nutrition recommendations (Valtion ravitsemusneuvottelukunta 2005) the recommended daily intake for folate during pregnancy is 400 µg vs. 500 µg in the new Nordic nutrition recommendations (2004)

<sup>(3)</sup> Iron balance during pregnancy requires iron stores approx. 500 mg. The physiological need for iron during the later part of pregnancy cannot be supplied solely via the diet.

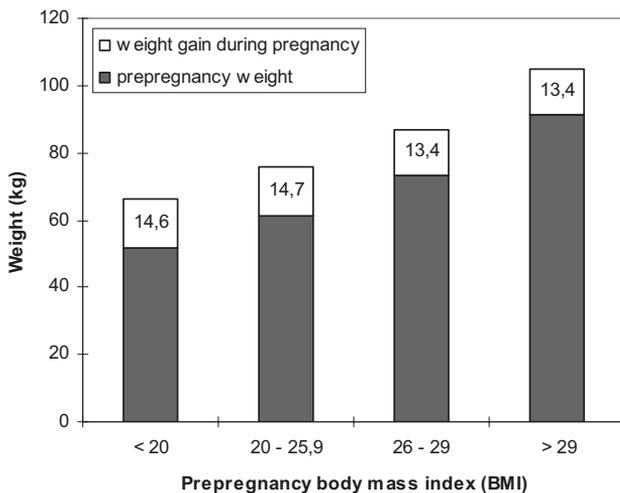
Altogether 70% of the pregnant women used dietary supplements during the recording period with iron supplements being the most frequently used products (48% of women). With the exception of vitamin D, iron, vitamin E, and thiamine, supplement users had an adequate intake of nutrients from their diet (Figure 9).



**Figure 9. Nutrient intake (% of recommended intake) from diet and supplements among supplement users (II)**

### Weight gain during pregnancy

The average weight gain during pregnancy was 14.0 kg (SD 4.6). The rate of weight gain differed between groups based on the prepregnancy body mass index (BMI), (Figure 10). The average weight gain of the women in the two highest BMI categories was 1 kg less compared to the women in the two lowest BMI categories (13.4 kg vs. 14.6 kg,  $p < 0.05$ ).

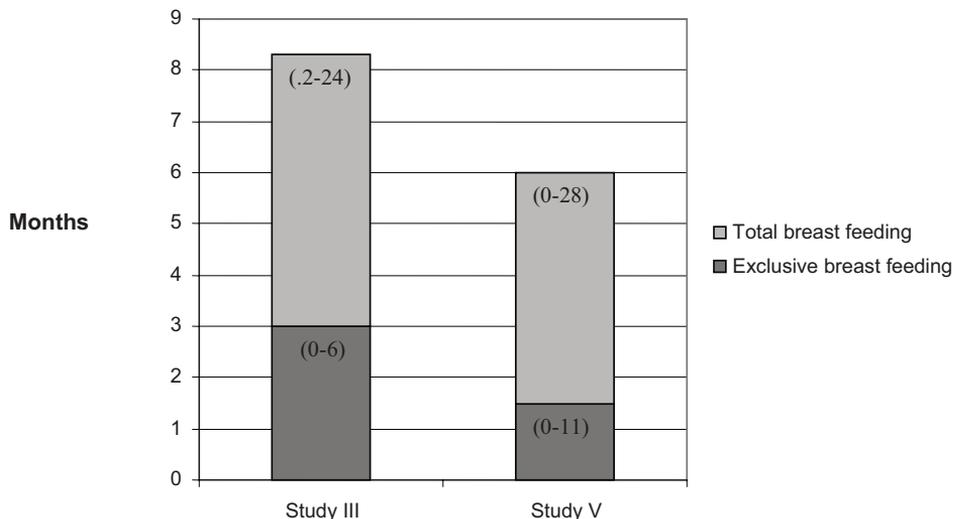


**Figure 10. Weight gain in women during pregnancy according to BMI category (II)**

### 5.3 Infant feeding patterns (III, IV, V)

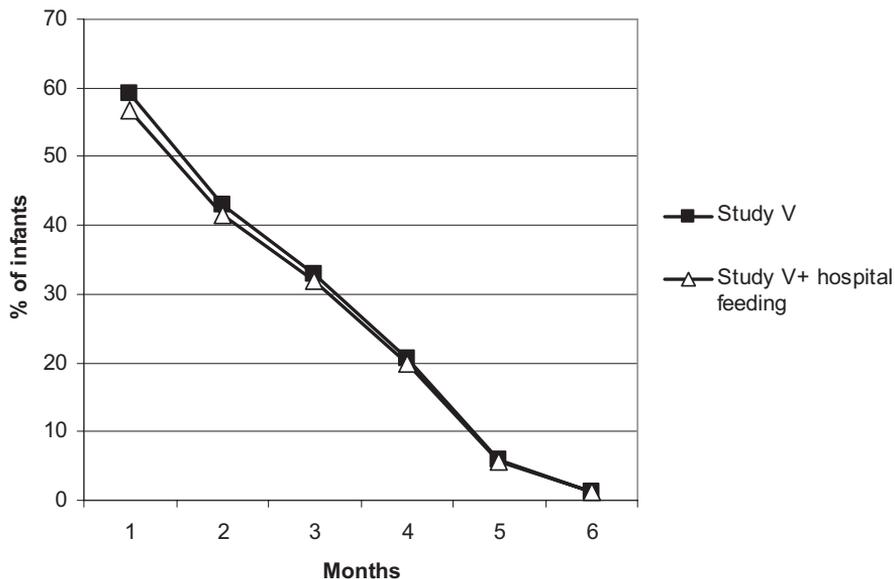
#### *Breastfeeding*

Both exclusive and total breastfeeding were on average one month longer in study III than in study V, which is most likely due to a different study design (Figure 11).



**Figure 11. Median duration (months, range in parenthesis) of exclusive and total breastfeeding in studies III and V. The participants were born 1995-1997 and 1996-2001, respectively.**

Altogether 71% of women in study V could recall their child being fed supplementary milk (donated breast milk or infant formula) at the maternity ward during the first days after delivery, and 9% of all women reported that the given milk was cow's milk based infant formula. Additional information about hospital feeding did not markedly shorten the observed median durations for breastfeeding; median duration of exclusive breastfeeding being 1.4 months (range 0 – 11) when taking into account vs. 1.5 months (range 0 – 11) not taking into account hospital feeding in calculations (Figure 12).



**Figure 12. Exclusive breastfeeding in study V. Feeding at the maternity ward is taken into account in the latter model.**

On average every second baby was still receiving breast milk at the age of six months, but the proportion of the exclusively breastfed was close to zero (Figure 12, Table 7). Some form of breastfeeding continued till the end of the first year on average in 15% of children (Table 7).

**Table 7. Children receiving breast milk in different age groups in study V, (% of all children in the same age group)**

Age months	≥ 1	≥ 2	≥ 3	≥ 4	≥ 5	≥ 6	≥ 7	≥ 8	≥ 9	≥ 10	≥ 11	≥ 12
<b>Breastfed children (% of all)</b>	84	77	70	64	57	51	45	38	32	25	20	15

Several sociodemographic variables were associated with the duration of breastfeeding (Table 8); parental education, and the age of the mother were positively and infant’s male gender negatively associated with the duration of breastfeeding. Accordingly infant’s ponderal index at birth was inversely associated with the duration of total breastfeeding (OR 0.32, 95% CI 0.14 – 0.73, p = 0.007).

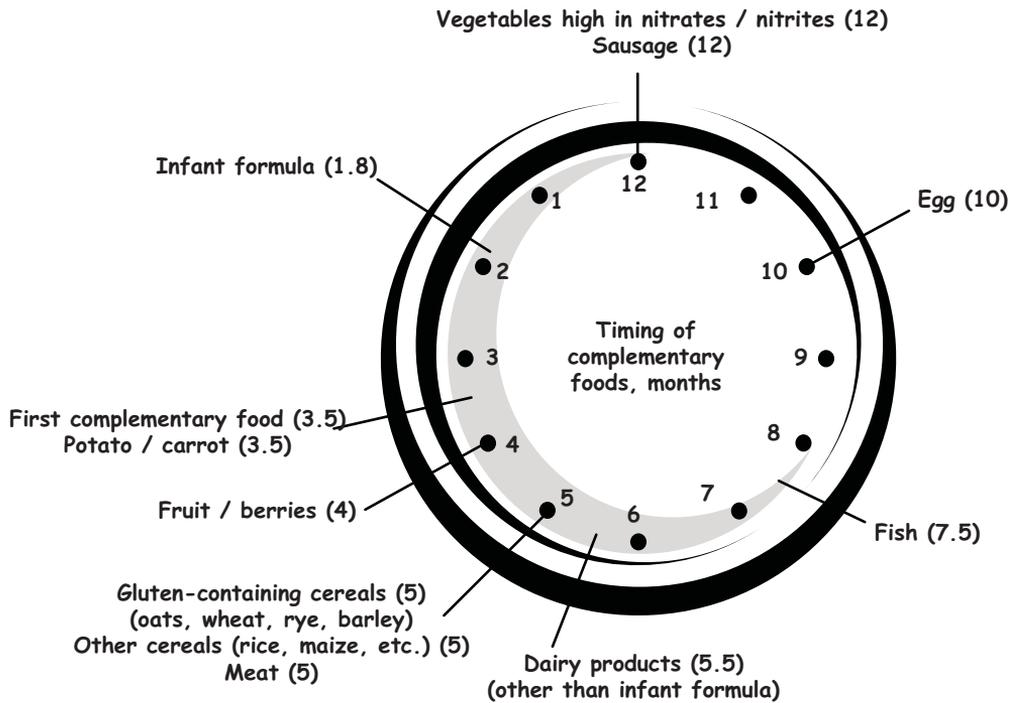
**Table 8. The association of selected sociodemographic factors with infants' diet; based on statistically significant odds ( $p \leq 0.05$ ) in study IV<sup>(1)</sup>**

	Mother		Infant	
	Age ↑	Education ↑	Number of siblings ↑	Male gender
<i>Duration of breastfeeding</i>				
- exclusive breastfeeding	↑	parental ↑		↓
- total breastfeeding	↑	parental ↑		↓
<i>Age at introduction of</i>				
- formula	↑	parental ↑		↓
- first complementary food	↑	parental ↑		↓
- vegetables high in nitrate	↑		one sibling vs. others ↓	
- meat and meat products	↑			
- sausage	↑		↓	

<sup>(1)</sup> ↑ means that marked sociodemographic variable has a positive association and ↓ means an inverse association

#### *Introduction of complementary foods*

The first complementary foods given after/in parallel with breast milk were infant formula (in 61% of children in study V), carrots and potatoes (16%), fruits and berries (6%), with the two latter groups being started at the same time in some infants (9%). Infant formula was introduced in study IV at a median of 1.8 months (range 0 – 25) and in study V at 1.6 (range 0 – 18) months of age, and the first complementary food in both groups at a median of 3.5 months of age (range 1 – 6 in study IV, and 0.7 – 8 in study V), (Figure 13). The median age at the introduction of dairy products (other than infant formula and infant food containing hydrolysed milk protein) was 5.5 months (range 2.5 - >24) in study IV. With the inclusion of infant formula and infant food containing hydrolysed milk protein, the median age at first cow's milk exposure in study V was 1.8 months (range 0 – 23.5). Timing of the introduction of most complementary foods was associated with the duration of breastfeeding; the association with age at the introduction of cow's milk products being particularly clear (correlation with the duration of exclusive breastfeeding 0.93, and total breastfeeding 0.57 in study V). Several sociodemographic variables were associated with the infants' diet: maternal age, parental education, and infant gender having the strongest influence (Table 8).



**Figure 13. Timing of the introduction of complementary foods in study V (median age at introduction, months)**

The associations between sociodemographic factors and age at introduction of selected foods were also studied in study V. Maternal age and education exhibited a positive and infant's male gender and maternal smoking an inverse association with age at introduction of fruits and berries and root vegetables. Maternal education and smoking were also positively associated with age at introduction of rye. The number of siblings was positively associated with age at introduction of root vegetables and rye. Accordingly caesarean section and Oulu as the area of birth were associated with early introduction of rye; root vegetables; and fruits & berries and root vegetables, respectively. In over-simplistic terms: being a female child of well educated parents with the mother being a non-smoker and 30 years or older seems to endow a Finnish infant with the best odds for being fed according to recommendations.

## 5.4 The associations between maternal diet and development of cow's milk antibodies in infant (III)

### *Maternal milk protein intake during pregnancy and lactation*

The average intakes of various milk products were slightly higher during pregnancy than lactation. Raw milk products represented the dominant source of milk protein in the maternal diet. Milk protein intake was rather similar during pregnancy and lactation. Spearman correlation coefficients for energy-adjusted protein intake during pregnancy and lactation were 0.56 ( $p < 0.001$ ) for all milk products, 0.70 ( $p < 0.001$ ) for raw milk products, and 0.66 ( $p < 0.001$ ) for cheese. Milk protein intake from raw milk products correlated inversely with milk protein intake from cheese ( $r = -0.25$ ,  $p = 0.017$  and  $r = -0.23$ ,  $p = 0.027$  during pregnancy and lactation, respectively).

### *Cow's milk protein antibodies in infants*

The proportions of children with detectable cow's milk antibodies of IgA class increased by age, whereas those of IgG class had a U-shaped form as shown in Figures 1 and 2 in the article III. At the age of 1 year, the duration of breastfeeding was inversely ( $r = -0.45$ ,  $p < 0.001$  and  $r = -0.54$ ,  $p < 0.001$ ), and the infant's current milk protein intake directly ( $r = 0.23$ ,  $p = 0.04$  and  $r = 0.31$ ,  $p = 0.004$ ) related to both the IgA and IgG BSA antibody titers, respectively. A clear association was noted between the detectable antibodies in the maternal circulation and the detectable levels of the same antibodies in cord blood (except for IgA antibodies to BLG, all p-values in Wilcoxon signed ranks test  $< 0.01$ ). The median levels of IgG class antibodies were higher in maternal samples than in the corresponding samples in cord blood, with only the median level of IgG antibodies to BLG being higher in cord blood. Maternal titers were differently related to antibody titers in the offspring in the two antibody classes, as might be expected. Stronger correlations were recorded for IgG antibody titers at early time points, at birth in particular, whereas the correlations between antibodies of the IgA class were stronger later.

Relatively few relationships were detected between the maternal consumption of different types of milk products and the antibody response to specific cow's milk proteins in the offspring. Maternal diet during pregnancy was related to IgA BLG antibody titers; milk protein intake from cheese was inversely related to IgA BLG antibody titers at the age of 3 (OR 0.16, 95% CI 0.03 – 0.81) and 6 months (OR 0.33, 95% CI 0.11 – 0.97). A similar inverse association was recorded for IgA CAS antibody titers at the age of 3 months (OR 0.34, 95% CI 0.12 – 0.95). Maternal diet during lactation was more clearly related to IgA CAS antibody titers. The maternal milk protein intake from raw milk products was associated with IgA CAS antibody titers at 6 months of age (OR 1.92, 95% CI 1.01 – 3.64), whereas an inverse relationship was seen at the age of 2 years (OR 0.44, 95% CI 0.21 – 0.92), similarly to that detected for IgG BSA antibodies (OR 0.46, 95% CI 0.21 – 0.97). Maternal milk protein intake from cheese was inversely related to IgA CAS antibody titers at 3 (OR 0.18, 95% CI 0.06 – 0.58) and 6 months of age (OR 0.37, 95% CI 0.17 – 0.81), and to decreased IgG CAS antibody titers at 6 months of age (OR 0.40, 95% CI 0.19 – 0.87).

### **5.5 Relation between dietary factors and appearance of autoantibodies (V)**

Among the 3565 children in study V, 111 children (3.1% of all children) tested positive for the ICA plus at least one other antibody until September 30, 2004, which was used as an endpoint. Early age at introduction of fruit and berries was related to greater risk of reaching the endpoint (Table 9). Accordingly, the age at introduction of root vegetables in the mid-tertile was related to increased risk of seroconversion to positivity for the endpoint. No evidence of interaction between HLA-DQB1 risk genotypes and age at introduction of fruits and berries or roots was seen in the development of the endpoint. The duration of total and exclusive breastfeeding and all of the studied measures of cow's milk exposure were not associated with the autoantibody endpoints. Using either the combined variable or the specific cereals, no statistically significant associations were observed with the endpoint of ICA plus at least one other autoantibody.

**Table 9. Hazard ratio (HR) and 95% CI adjusted for genetic risk for repeated positivity for type 1 diabetes associated autoantibodies related to age at introduction of foods<sup>(1)</sup>. Separate models were used for each dietary variable**

<b>Age</b>		
<b>Months</b>	<i>ICA plus at least one other autoantibody</i>	
	<b>N of positive (total N)</b>	<b>HR (95% CI)</b>
<i>Total breastfeeding</i>		
		ns <sup>(2)</sup>
- 1. tertile: 0-4	28 (1106)	0.96 (0.59-1.57)
- 2. tertile: 4.01-8.5	32 (1000)	1.09 (0.67-1.76)
- 3. tertile: >8.5	37 (1078)	1
<i>Exclusive breastfeeding</i>		
		ns <sup>(2)</sup>
- 1. tertile: <1	38 (1322)	0.87 (0.56-1.35)
- 2. tertile: 1-2.99	24 (934)	0.82 (0.49-1.35)
- 3. tertile: ≥3	42 (1174)	1
<i>Cow's milk</i>		
		ns <sup>(2)</sup>
- 1. tertile: < 1	37 (1196)	0.94 (0.61-1.47)
- 2. tertile: 1- 3.99	24 (1036)	0.76 (0.46-1.25)
- 3. tertile: >4	42 (1185)	1
<i>Fruits and berries</i>		
		p=0.026
- 1. tertile: < 3.5	33 (1001)	<b>2.02 (1.03-3.95)<sup>(3)</sup></b>
- 2. tertile: 3.5-4	47 (1330)	<b>1.97 (1.06-3.64)<sup>(3)</sup></b>
- 3. tertile: >4	21 (955)	1
<i>Roots</i>		
		p=0.014
- 1. tertile: <3	15 (669)	1.04 (0.57-1.90)
- 2. tertile: 3-3.99	49 (1198)	<b>1.82 (1.19-2.79)<sup>(3)</sup></b>
- 3. tertile: ≥4	38 (1431)	1
<i>Wheat, barley, rye or oats</i>		
		p=0.035
- 1. tertile: < 5	10 (571)	0.76 (0.37-1.54)
- 2. tertile: 5-5.49	55 (1471)	1.53 (0.99-2.37)
- 3. tertile: ≥ 5.5	36 (1192)	1
<i>Other cereals</i>		
		ns <sup>(2)</sup>
- 1. tertile: < 4.5	28 (961)	0.98 (0.61-1.59)
- 2. tertile: 4.5-5	32 (1042)	0.91 (0.57-1.45)
- 3. tertile: > 5	41 (1239)	1

<sup>(1)</sup>A piece-wise exponential survival model was used in the analysis. ICA were used as a primary screening tool. All samples of the children testing at least once positive for ICA were analyzed for IAA, GADA and IA-2A. Type 1 diabetes was included in the endpoints. For the endpoint based on repeated positivity for at least ICA plus one other autoantibody the trend across the tertiles was significant (p<0.05) for combined cereals (wheat, rye, oats, barley), rye alone, fruit and berries, but not for roots

<sup>(2)</sup>Loglikelihood ratio test was used to test whether the model with and without the food variables differed. Ns: p≥0.05

<sup>(3)</sup> P<0.05

When bringing age at introduction of fruits and berries and roots into the same model with socio-demographic and perinatal factors, both food variables retained their significance. When both food variables and the sociodemographic and perinatal factors were included in the same model, the association of fruits and berries with ICA plus at least one other autoantibody was of borderline significance ( $p=0.056$ ). The type of the first supplementary food, whether supplementary milk, roots, or fruits and berries, was unrelated to the endpoint (data not shown).

## 6. Discussion

### 6.1 Science and conclusions are only as good as our tools and their proper application; Validity of the food frequency questionnaire

The validity and reproducibility of our 181-item FFQ, which was designed to effectively study the putative effects of the maternal diet during pregnancy on the development of T1D, were found to be reasonably good (II). Similarly to most other validation studies among different populations, the FFQ overestimated food consumption and intake of nutrients (Willett 1998). However, more importantly, the quintile notation was acceptable for most of the foods, nutrients and other dietary factors.

Dietary measurement error has two consequences relevant to epidemiologic studies: first, a proportion of subjects will be misclassified according to exposure, and second, the distribution of reported intakes will be wider than the distribution of the true intakes. The first of these two errors is more consequential and has to be considered before we can draw firm conclusions from our FFQ. The greatest misclassifications were found for rarely consumed foods, highlighting the fact that when the time between the behaviour and the report increases, respondents may rely less on episodic memory and more on generic memory. In terms of the nutrients, the greatest misclassifications were found for different fatty acids, which needs to be taken into account when assessing the results from the studies using our FFQ. In order to improve the classification of dietary fats, the grouping and brand names of spreads in our FFQ are continuously checked and updated.

There are three recommended validity estimates for nutrients of interest in epidemiological studies: Spearman correlation coefficients should be above 0.5, more than 50% of subjects should be correctly classified and less than 10% of subjects should be grossly misclassified into thirds (Masson et al. 2003). When observing the validity estimates for dietary variables of special interest (Table 10) in the light of the abovementioned recommendations, the validity of our FFQ to assess intakes of vitamin D and E could partly be criticised. However, the quintile notation was acceptable for both of these vitamins. Since February 2003, Finnish milk products have been supplemented with vitamin D and,

therefore, measuring dietary intake of vitamin D has become clearer. The ability of our FFQ to measure the most frequently used milk products was found to be good (Table 10).

**Table 10. Validity estimates for dietary variables of special interest in studies on the putative effects of the maternal diet during pregnancy on the development of T1D; study I**

	<b>Spearman correlation coefficient between FFQ and food record (energy adjusted values + correction for attenuation)</b>	<b>Subjects grossly misclassified (%)</b>	<b>Overall proportion categorized in the same or adjacent quintile of food record (%)</b>
Low-fat milk	0.86	0	92
Cheese	0.61	5	75
Wheat products	0.52	5	65
Rye products	0.59	5	68
Sausage	0.72	0	72
Protein	0.55	5	69
Vitamin D	0.44	9	63
Vitamin E	0.22	5	65
Niacin	0.60	5	64
Vitamin C	0.65	5	74
Zinc	0.45	5	73
Nitrate	0.63	5	80
Nitrite	0.79	0	73

There is a paucity of validation studies of FFQs among pregnant women. To our knowledge, only eight validation studies of FFQs involving pregnant women have been published between 1989 and 2004 (Suitor et al. 1989, Greeley et al. 1992, Forsythe & Gage 1994, Brown et al. 1996, Robinson et al. 1996, Wei et al. 1999, De Vriese et al. 2001, Fawzi et al. 2004). They are difficult to compare with our study and with each other since they cover various phases of pregnancy and differ in the reference method, length of the FFQ, and statistical methods used. Four of the eight studies used from one to three 24-hour recalls as the reference method (Suitor et al. 1989, Greeley et al. 1992, Forsythe & Gage 1994, Wei et al. 1999), from four to seven food record days were used in three studies (Brown et al. 1996, Robinson et al. 1996, De Vriese et al. 2001), and the concentrations of long chain n-3 fatty acids,  $\alpha$ -linolenic acid, trans fatty acids, carotenoids and  $\gamma$ -tocopherol analyzed

in pooled blood samples were measured in the final study (Fawzi et al. 2004). Two studies were conducted in European populations (Robinson et al. 1996, De Vriese et al. 2001) and the rest in North America (including African American, Caribbean and Caucasian descendants), two of which focused on low-income pregnant women (Suitor et al. 1989, Wei et al. 1999). Our validation study could be considered as a more comprehensive study than the others in terms of the number of dietary variables, the use of several statistical methods, and the evaluation of validity estimates. The strengths of our FFQ are its possibility to provide information on the intake of a wide selection of nutrients and foods as well as nitrates, nitrites and some contaminants. All other validation studies have focused only on the intakes of nutrients (max 25 in the study of Wei et al. 1999), except for one (Robinson et al. 1996) in which the contribution of selected food groups to energy and energy-yielding nutrients was also assessed. The strength of our FFQ is also that the information of the most frequently used foods among the Finnish women of childbearing age (so. the target group of the FFQ) was used as a baseline tool to create food lists and recipes. Our FFQ makes it possible to take personal choices for fats into account. The additional questions about the use of dietary supplements mean that it is possible to include these compounds into calculations, although their validity was not assessed here.

Our study provided important information about a target group whose special characteristics as FFQ users have not been adequately evaluated. When one compares validity studies between non-pregnant and pregnant women, it seems that the tendency to over-report food consumption increases during pregnancy (e.g. Männistö et al. 1996 vs. study I). In the study among US low-income pregnant women, overestimation of food use was believed to occur in up to 20% of the subjects (Suitor et al. 1989). The poor correlation between instruments may be partly explained by appetite fluctuations and nausea, which could influence the long-term diet reports (Wirfält 1998). However, nausea is reported to peak around 8 to 12 weeks of gestation (Jewell & Young 2003), and therefore it should not markedly have influenced our measurements which were made during the third

trimester of gestation. Future research should develop and validate FFQs linking the data on most frequently used dietary supplements to the data on food intake. As revealed in our studies (II), there is widespread use of dietary supplements during pregnancy and, therefore, ranking the subjects according to nutrient intake exclusively from diet could lead to serious misclassification.

## **6.2 Focusing on quality rather than quantity when building basis for a new life; Diet during pregnancy**

The results of the dietary survey among pregnant women (II) emphasized that special attention should be paid to the type of fat as well as the amount of carbohydrates. An increase in the consumption of whole grain cereals, vegetables, and fish, and a decrease in the consumption of foods rich in sugar and saturated fatty acids should be the strategy adopted for improving the intake of critical nutrients: essential fatty acids, dietary fiber, vitamin D, folate, and thiamine. This is also a strategy for reducing the risk of overweight. Supplementation is needed to ensure an overall adequate intake of iron and vitamin D, and, in some reference groups, also folate. Dietary guidance at maternity clinics needs to be focused on young and less educated women as well as on smokers.

The total intake of energy increases during pregnancy (vs. non-pregnant women aged 25-34 years in Finravinto study; Finravinto 1997-tutkimuksen työryhmä 1998). The increasing energy content comes in most cases from increasing consumption of foods rich in sugar and fatty milk products and, therefore, it does not correlate positively with increasing wholesomeness of the diet. The critical vitamins and minerals in the present study were the same as in most of the western dietary surveys which have examined pregnant women during the last decade (Table 11). Overall, surveys using FFQ as the dietary assessment method have not found as many critical nutrients as surveys using food records (Table 11). This is most likely due to the tendency of FFQs to overestimate nutrient intake, and therefore, the nutritional guidelines should not rely only on information obtained from FFQs. Some of the estimated differences in food consumption according to the

length of education might reflect a tendency of well educated women to report their food intake, particularly the consumption of fruits and vegetables, in a socially desirable way.

The use of dietary supplements has become more common over time, and pregnancy seems to be an additional motivation to increase their use. According to the two most recent studies among Finnish pregnant women (study II and unpublished data of Arkkola et al.), the use of supplements does not target those nutrients with the highest risk of insufficient intake. In the present study II, only 31% of the women had taken vitamin D supplements. In the light of that result, the milk fortification, which started in February 2003, will be able to provide an important source of vitamin D to supplement the diet of Finnish pregnant women. The folate intake of Finnish women is not adequate for those who want to become pregnant (Alfthan et al. 2003); a finding which is common in many other countries as well (Table 11). The stricter implementation of recommendations on supplements before and during pregnancy might not be sufficient to reach the risk groups, young and less educated women. If future research confirms the hazards (known and potential) of an inadequate intake of dietary folate, the food fortification will surely need to be considered. It may also be debated if the same dietary recommendations are sufficient for such a heterogeneous group as Finnish pregnant women; specially focused additional recommendations could benefit some sub-groups.

**Table 11. Dietary surveys on pregnant diet in developed countries published in 1995 – 2005,** (FR = food record, FFQ = food frequency questionnaire)

<b>Year, country</b>	<b>Subjects</b>	<b>Trimester</b>	<b>Dietary methods</b>	<b>Nutrients not meeting recommendations</b>	<b>Impact of sociodemographic factors</b>	<b>Reference</b>
1995, Norway	38 Pakistani immigrants and 38 Norwegians	II	FFQ	Vitamin D	Place of origin; Pakistanis had lower dietary intake and total intake (incl supplements) of vitamin D	Henriksen et al. 1995
1997, Hungary	70 women	I, II, and III	FFQ + FR	Thiamine, riboflavin, pyridoxine, niacin, calcium, iron and zinc.	-	Antal et al. 1997
1998, UK	11 923 residents in the south-west of England.	III	FFQ	Energy, iron, magnesium, potassium and folate.	Income; the quality of diet in pregnancy falls with increasing difficulty in affording food.	Rogers & Emmett 1998, Rogers et al. 1998
1998, Finland	118 women in the north of Finland	III	2 x 5-day FR	Vitamin D, folate, iron, and thiamine.	Young, less educated and smokers have the biggest risk of not meeting the dietary recommendations.	Erkkola et al. 1998
2000, Ohio;USA	59 adolescents and 97 adults participating in a randomized clinical calcium trial	II and III	2 x 7-day FR	Energy, iron, zinc, calcium, magnesium, folate, and vitamins D and E.	-	Giddens et al. 2000
2001, Switzerland	381 women	II and III	Questionnaire on supplement use		-	Hess et al. 2001
2002, North-Carolina;USA	2063 women participating in the Pregnancy, Infection, and Nutrition (PIN) Study	II	FFQ	Folate and iron	Women >30 years old, >350% of poverty, nulliparous and high school graduates had higher overall Diet Quality Index for Pregnancy scores.	Bodnar & Siega-Riz 2002; Siega-Riz et al. 2002
2003, Florida;USA	63 low risk women	I, II, and III	9 x 3-day FR	Iron, magnesium, zinc, vitamin B6, selenium, and vitamin C.	-	Turner et al. 2003

### **6.3 Goals of infant feeding have not been achieved**

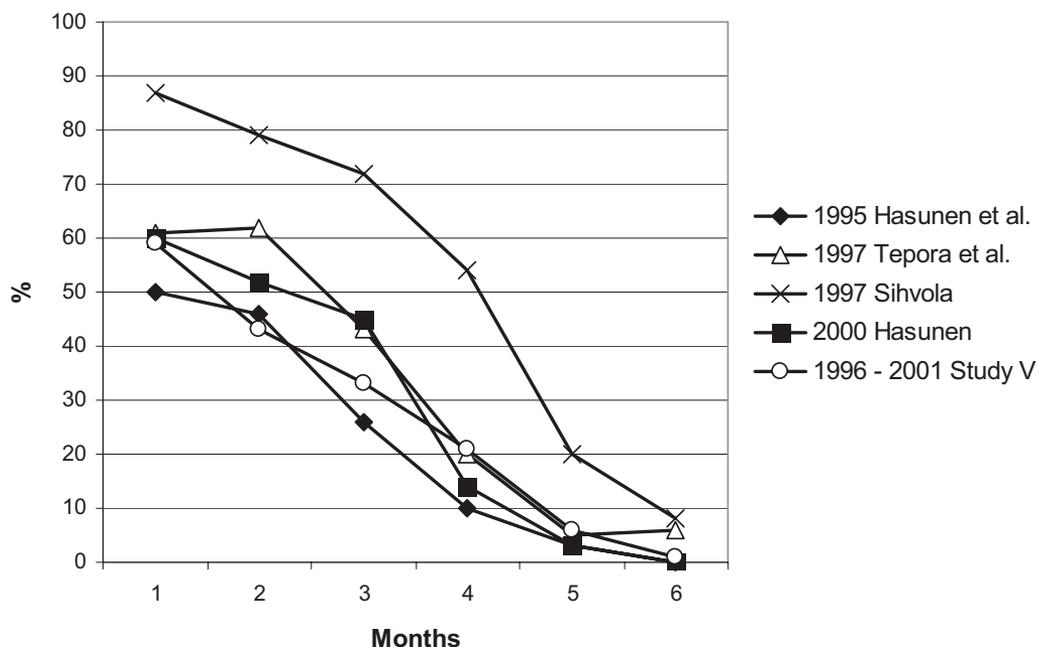
The goals for infant feeding have not yet been achieved in Finland (IV, V). The median duration of exclusive breastfeeding is more than 4 months shorter than the recommendation (1.5 vs. 6 months), (WHO 2003, Hasunen et al. 2004), and that of total breastfeeding half a year less than the Finnish recommendation (6 vs. 12 months), (Hasunen et al. 2004). Only 37% of the infants were introduced to complementary foods no earlier than at the age of 4 months, and thus only this percentage adhered to the national recommendation (Hasunen et al. 2004). The weaning process seems to be strongly influenced by sociodemographic characteristics; with high parental education, older maternal age and infant's female gender predicting later introduction of complementary foods.

The new method developed for studying the age at introduction of complementary foods among DIPP-infants seemed to function well. The age at introduction of foods as an exposure is easier to measure and is not so easily biased as is encountered for example with quantitative measurements of foods. However, the time windows for exposure of most food groups are narrow in Finland, and it could make the analyses less sensitive for detecting relations between the time at first exposure and the endpoint variables. The questionnaire on the age at introduction of complementary foods does not require high literal skills or burden parents to the same extent as other diet recording methods.

Our findings on the duration of exclusive breastfeeding are in line with the results from other Finnish studies completed between 1995 and 2000 (Figure 14; Hasunen et al. 1996, Tepora et al. 1999, Sihvola 2001, Hasunen 2002). The studies of Hasunen (1996; 2002) represent data from national surveys, whilst others come from local surveys (Tepora et al. 1999, Sihvola 2001). The breastfeeding trends based on studies IV and V, show that a subcohort (12%) of a larger cohort (IV) gave us a very similar figure on the duration of breastfeeding as obtained with a larger cohort (V). The figure based on the regional study of Sihvola (2001) differs from the others and demonstrates

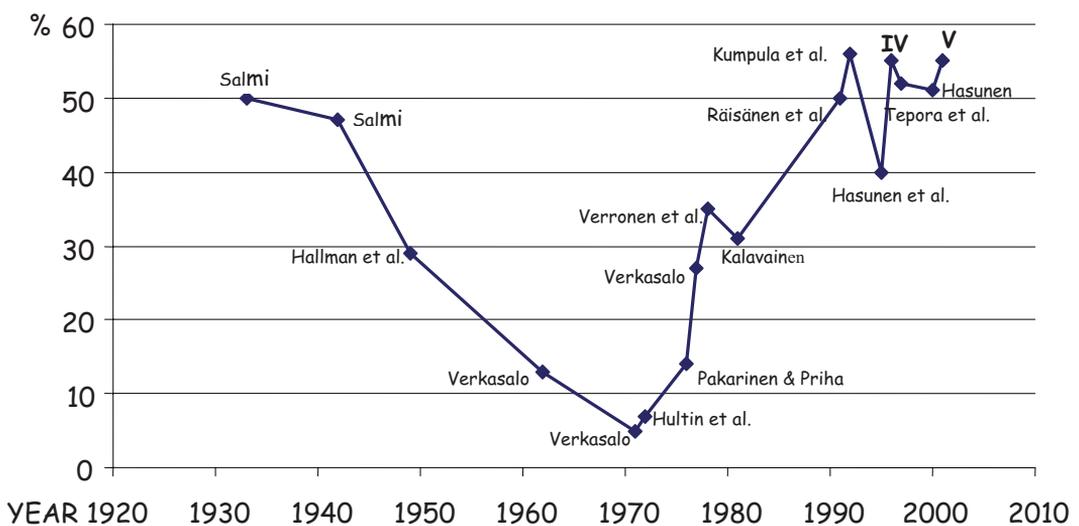
that consistent education and motivating professional support provided by maternal clinics can have a positive impact.

The impact of hospital feeding on the duration of exclusive breastfeeding was smaller than expected. The proportion of infants requiring supplementary milk (donated breast milk or infant formula) at the maternity ward in our study was markedly smaller (71% vs. 87%) than the proportion reported in a large prospective study (Saarinen et al. 1999) which was conducted in the Helsinki region about 2 years before the beginning of our study. However, the Finnish infants are rarely introduced to infant formula in the maternity ward in comparison to other countries. For example: more than 80% of infants received infant formula during their first days in the hospital in Denmark (Michaelsen 1997), whilst only 9% of mothers in our study could recall formula being given to their offspring (V).



**Figure 14. Exclusive breastfeeding in Finnish studies 1995 – 2001 (% of all infants)**

When one reviews Finnish breastfeeding studies during the last 70 years, it can be appreciated that the set goal of 6 months' exclusive breastfeeding has been difficult to achieve even in the form of total breastfeeding for most of the mothers (Figure 15). After the catastrophic decline in the 1970's attributable to the improved availability of alternative feeding methods (infant formulas became available in Finland in 1974), increasing migration to urban areas, and greater employment of women, the breastfeeding trend has been positive until the 1990s.



**Figure 15. Proportion of children breastfed for at least 6 months in Finnish studies from 1933 to 2001<sup>(1, 2)</sup>.**

<sup>(1)</sup> If there is more than one result from the same year, the one with the highest number of subjects or not being an intervention study has been chosen.

<sup>(2)</sup> References: Salmi 1944, Hallman et al. 1952, Hultin et al. 1977, Verkasalo 1980, Verronen et al. 1981, Kalavainen 1984, Pakarinen & Priha 1984, Kumpula et al. 1994, Hasunen et al. 1996, Räisänen et al. 1998, Tepora et al. 1999, Hasunen 2002)

The age at introduction of complementary foods is strongly associated with the duration of breastfeeding as shown in our studies and supported by results from earlier studies (e.g. Dubois et al. 2003, Lande et al. 2003). The new recommendation to start introducing complementary foods at the earliest of 4 months of age was set in Finland after the country joined the European Union (Sosiaali- ja terveystieteiden ministeriö 1994). According to nationwide surveys in 1995 and 2000 (Hasunen

et al. 1996, Hasunen 2002) and the present studies, Finnish mothers have succeeded in delaying the average age of starting complementary feeding from the previous peak age of 3 months towards to a new peak of 4 months. This clear milestone for the first introduction of complementary foods is something of a Finnish phenomenon; the variation around the age at starting complementary feeding is wider in other European countries (Savage et al. 1998, Euro-Growth Study; Freeman et al. 2000, Lande et al. 2003). For most of the infants, cow's milk protein is the first protein introduced when beginning the use of infant formula. After the first six months, infants are surprisingly often given cow's milk in the form of regular milk products even though unmodified cow's milk is believed to be unsuitable for infants until 12 months of age. The long tradition favouring the use of milk products in Finland might partly explain their early introduction. Early introduction of cow's milk has been associated with the risk of T1D (Virtanen & Knip 2003) and some allergic diseases (Chandra 2002), although not consistently. However, no associations were found between all of the measures of cow's milk exposure used and the autoantibody endpoints in the present study (V).

Socioeconomic differences in the diets of Finns start to develop already in pregnancy and the trend seems to continue in infancy and thereafter; the youngest and less well educated groups exhibit a greater risk of not following dietary advice. This is not only a Finnish phenomenon; similar findings have been reported in several studies among pregnant women and infants in other Western countries (Michaelsen 1997, Savage et al. 1998, North et al. 2000, Hörnell et al. 2001, Yngve and Sjöström 2001b, Dubois et al. 2003, Lande et al. 2003, Vingraite et al. 2004). A poor diet in pregnancy, commonly correlating with smoking, leads to a greater risk of low birthweight babies, shorter pregnancy, and possibly predisposes the infant later to diseases such as coronary heart disease and diabetes as he/she enters adulthood. During infancy, the children born to less educated and young women have a greater risk of being breastfed for a shorter time and introduced to complementary food earlier than recommended (Tepora et al. 1999, IV, V). This may increase the risk for some

diseases (e.g. T1D, allergies) and perhaps can influence the neurocognitive development (Anderson et al. 1999, Chandra 2002, Ivarsson et al. 2002, Jain et al. 2002, Owen et al. 2002; 2003; 2005, Virtanen & Knip 2003). The potential to achieve an enormous health gain through improved diet in pregnancy and infancy is unquestionable and, therefore, it should be encouraged at all levels of our health care system. It is a challenge in Finland, as well as in most Western countries, to develop strategies to improve the diet of families having young and less educated parents. Based on the present study and some earlier reports (Räsänen & Ylönen 1992, Simell et al. 2000, Hasunen 2002) gender differences in infant feeding do exist in Finland and their possible consequences require further investigation.

Finland has long been recognized for its excellent maternal and child health care and the high quality of the educational system. There seems to be a dark cloud obscuring the light behind these outstanding statistics; young and less educated families are not able, or in some cases not willing, to seek the full benefit of the maternal and child health care system. Issues related to the recommended diet during infancy and beyond should be included in the basic school curriculum. There is a good channel that could be used in Finland; all girls and boys in the 7<sup>th</sup> grade have home economics lessons for one year. The introduction of issues related to breastfeeding and weaning at that stage could particularly target and benefit prospective young families. Increasing the awareness of boys is important, as support from the male partner has been shown to be the most important factor for initiating breastfeeding (Baranowski et al. 1983).

There is an urgent need in Europe to develop a valid, comparable surveillance system for infant feeding which uses common definitions and methodology. Because of this, it is difficult to compare and assess the results from different studies in this area. Overall the situation in Scandinavia appears better than that in the Southern European countries; more than half of the infants have been at least partially breastfed for at least 6 months in Denmark, Sweden, Norway, and Finland, a goal which is

seldom achieved in other European countries (Michaelsen 1997, Freeman et al. 2000, Yngve and Sjöström 2001a, Lande et al. 2003, IV and V). The global goal of exclusive breastfeeding for the first 6 months followed by partial breastfeeding until the end of the second year and for longer, defined by WHO and UNICEF (UNICEF 1999, WHO 2003) does seem to be unachievable in Finland as well as in most other Western societies. It has recently been questioned in Finland, if the unachievable recommendation of 6 months' exclusive breastfeeding without any clear scientific evidence for its benefits in developed countries will decrease the overall willingness of mothers and health care personnel to follow nutritional recommendations in infancy (Savilahti 2005). However, trends point towards higher prevalence and duration of breastfeeding though there are some exceptions (Yngve and Sjöström 2001a).

#### **6.4 New perspectives on antibody responses; Do milk products differ in their effects on cow's milk antibody responses?**

In the unique intervention setting, few relationships were detected between the maternal consumption of different types of milk products and the antibody response to specific cow's milk proteins in the offspring (III). An inverse association was found between maternal intake of cheese during pregnancy and lactation and the humoral immune response to cow's milk proteins in the offspring. Accordingly, a positive relation between maternal intake of raw milk proteins during lactation and the antibody response to cow's milk proteins in infants at 6 months was converted to an inverse relationship at 2 years of age. Thus, this study introduced a novel perspective into research on cow's milk antibodies in infants and young children.

Children belonging to the intervention group in TRIGR were not exposed to other cow's milk proteins except breast milk during the first 6 months of life, which provided us with an excellent basis to study the impact of maternal cow's milk consumption on the humoral immune response to cow's milk proteins in the offspring. To our knowledge, there are no comparable studies in the field of cow's milk antibodies. There are no other studies in which milk products have been separated

according to their type of processing, which we subdivided into high casein group (cheese) vs. all milk proteins (raw milk products). The maternal diet during lactation was more strongly associated with cow's milk antibody titers in the offspring than with the diet during pregnancy. High levels of cheese consumption were inversely associated with antibody titers until 6 months of age, whereas a high consumption of raw milk products appeared to decrease the antibody titers later. Maternal protein intake from raw milk products was inversely related to protein intake from cheese. These findings might imply that raw milk products are the true effectors, and the impact of maternal cheese intake on the antibody titers in infants is mediated via the intake of raw milk products.

Our study confirmed the connection between IgG antibodies in the maternal circulation and those found in cord blood (Sarvas et al. 1993, Malek et al. 1996). Finnish women seem to increase their consumption of milk products during pregnancy and lactation (I - III vs. Kleemola et al. 1994 and Männistö et al. 2003). Maternal protein intake from raw milk products and cheese tended to inhibit the humoral immune responses to cow's milk proteins in the offspring; cheese during the first 6 months and raw milk later, around 2 years of age (III). This raises the issue of whether cow's milk proteins transferred through placenta and/or breast milk could induce tolerance to cow's milk proteins in the offspring. The rather narrow range in the consumption of different milk products among mothers, probably partly caused by a flat-slope syndrome (large portions of food are underestimated and smaller portions are overestimated), could have made the analyses less sensitive for detecting any such effects. The increased levels of cow's milk antibodies in subjects with T1D could be an epiphenomenon of an insult and immunization caused by cow's milk proteins, which in some genetically susceptible individuals may lead to T1D after a long but variable time interval (Åkerblom et al. 1996). However, it is definitely premature to issue dietary advice on the consumption of milk products by pregnant and lactating women in an attempt to limit the development of T1D even in high risk offspring. The evidence that T1D associated autoantibodies may start to emerge early in infancy (Ziegler et al. 1999, Kimpimäki et al. 2001b), and that some

intrauterine environmental factors having links to the diet are related to the risk of T1D emphasize the importance of studying early dietary risk predictors.

### **6.5 New findings observed in the largest T1D focused birth cohort study challenges future studies**

Large sample size and a long follow-up are needed in studies examining potential nutritional factors in the etiology of T1D due most likely to the lengthy period of time between exposure and outcome, the imprecise quantification of exposure measurements and important covariates, and the complex intercorrelation of dietary exposures. In the largest prospective cohort series so far reported, an early age ( $\leq 4$  months) for the introduction of fruit and berries and introduction of roots between 3 to 4 months of age were related to the risk of advanced  $\beta$ -cell autoimmunity (V). The numbers of autoantibody endpoints were markedly larger than in other birth cohort studies (Table 12), but still slightly too small to provide precise risk estimates.

The collection of dietary data before the development of the autoantibody endpoint excluded the possibility of differential bias in the selection of subjects and recall bias in the reporting dietary habits. It could also be questioned if our questionnaires allow families to specify foods given to their infants adequately enough to be able to identify potential interesting aspects in the etiology of T1D. This is a basic question in long-term dietary studies in which all the dietary factors of interest are not known at the starting point. The major limitation of our study is that only information on the age at introduction of new foods, but not on the amounts of foods consumed could be studied. Whether age at introduction of foods is related to later food consumption patterns is not known. The surprising finding on fruits and berries, foods that are considered healthy, as potential nutritional risk factors in the etiology of T1D is the result on a comprehensive cohort study in a well-defined study population with a high participation rate. It needs to be taken into consideration in future studies on the etiology of T1D.

The steep increase in the incidence of T1D most likely reflects changes in lifestyle and environmental factors. Dietary exposures measured in study V can act at least partly as proxies for other life style characteristics, and therefore it would be necessary to try to identify broader behavioural patterns behind the early introduction of fruits, berries and roots, which were related to emergence of early  $\beta$ -cell autoimmunity in our study. Sociodemographic characteristics associated with early introduction of these foods were young maternal age, less extensive education, maternal smoking, male gender of infant, small number of siblings, caesarean section and Oulu as the area of birth. There are inter-relationships between some of these characteristics, since the education of young people is in many cases still continuing and the number of children (i.e. family size) will increase. The slight male predominance in the incidence of T1D among white children (Karvonen et al. 2000) and the recently reported male majority with signs of humoral  $\beta$ -cell autoimmunity (Williams et al. 2002) provided us with an interesting perspective when examining gender differences in infant feeding. Given that boys are breastfed for a shorter time and introduced to complementary food earlier than girls, one could propose the hypothesis that there is a relation between earlier exposure to foreign proteins in boys and possibly increased weight gain induced by complementary feeding, and the greater incidence of T1D. According to case-control findings, rapid weight gain in infancy and early exposure to cow's milk are both independent risk predictors of T1D (Hyppönen et al. 1999). These hypotheses, however, need to be confirmed in further investigations. Among the other characteristics associated with early introduction of fruits and berries and roots in our study, younger maternal age and maternal smoking during pregnancy have been linked to reduced risk of T1D in earlier studies (Blom et al. 1989, Dahlquist & Källén 1992, Patterson et al. 1994, Dahlquist et al. 1999, McKinney et al. 1997, Stene et al. 2004). The impact of education has been controversial (e.g. Virtanen et al. 1998, Sipetic et al. 2004, Sepa et al. 2005).

If early consumption of fruits, berries and/or roots itself has a real relationship with the risk for early and/or more advanced  $\beta$ -cell autoimmunity, we need to focus on the nature of the food

components. All of the food components are not necessarily nutrients (e.g. they may contain natural or synthetic toxins) and therefore complete food composition data may not be available. The hidden cause could also be another item used in some industrial food processing. For example; rice starch is commonly used in the manufacture of fruit and berry purees for children. There is increasing evidence that gut-associated lymphoid tissue is involved in the development of T1D and since the immune defence mechanisms of the infant gut mature with age (Vaarala 1999), the putative dietary regulation of autoimmunity may well depend on age. There is an urgent need in Finland for further studies to elucidate if the frequent use of manufactured baby foods and their “hidden constituents” have any impact on the developing gut and immune system of infants. It may also be questioned if it would be better to serve fruits and berries, as well as other foods, as such, instead of in the form of the processed pulp present in ready-to-eat manufactured baby foods.

There are altogether four birth cohort studies which have focused on nutrition in the etiology of T1D (Table 12). BABYDIAB studies (Couper et al. 1999, Hummel et al. 2000, Ziegler et al. 2003) have focused mainly on breastfeeding patterns, while a wider selection of maternal and infant dietary variables was included in DAISY (Norris et al. 1996; 2003, Fronczak et al. 2003) and DIPP studies (Kimpimäki et al. 2001a, Kupila et al. 2001). In the DAISY and BABYDIAB studies positivity already for a single autoantibody specificity was used as an endpoint in the reported results so far, even though that represents, in most cases, harmless nonprogressive  $\beta$ -cell autoimmunity. Our study differs from the others in that we used as an endpoint repeated positivity for ICA plus at least one other autoantibody, which reflects more advanced and stable beta-cell autoimmunity than positivity for only one autoantibody. Unlike the statistical analyses used in previous comparable studies (inadequately described in some of these studies), we have accommodated between-sibling dependence through a frailty model, and in addition to interval censoring of the seroconversion times in the model, we have accounted for the time-dependent nature of the principal explanatory

variables. Accordingly, as in the present study (V), several sociodemographic variables have been taken into account as possible confounding factors only in the DAISY Study.

**Table 12. Birth cohort studies on nutrition in the etiology of pretype 1 diabetes <sup>(1)</sup>**

<b>Study and the location</b>	<b>BABYDIAB Germany</b>	<b>BABYDIAB Australia</b>	<b>DAISY Colorado, US</b>	<b>DIPP Nutrition Finland</b>
The year of study start	1989	1993	1994	1996
Setting	Newborns of parents with T1D.	Newborns with first-degree relative of T1D	Newborns with an increased genetic risk of T1D or first-degree relatives of T1D individuals	Newborns with an increased genetic risk of T1D
Blood samples	At birth, age 9 months, 2, 5, and 8 years.	Taken at six months intervals.	At ages 9, 15, and 24 months and yearly thereafter.	Taken at 3 to 12 months intervals.
Endpoint used	At least one of IAA, GADA, IA-2A, ICA	One of IAA, GADA, IA-2A, one repeatedly	At least one of IAA, GADA, IA-2A	Repeated positivity for ICA and at least one of IAA, GADA, IA-2A
Dietary methods; pregnant and infant diet	Prospective questionnaire (the length of breastfeeding)	Home diary	Age at introduction of new foods interview form	Lactation: FFQ Children: A follow-up record on introducing new foods, age specific structured questionnaires (3, 6, 12, 24 mo).

<sup>(1)</sup>References:

German BABYDIAB: Hummel et al. 2000, Ziegler et al. 2003

Australian BABYDIAB: Couper et al. 1999

North American DAISY: Norris et al. 1996, Fronczak et al. 2003, Norris et al. 2003

Finnish DIPP: Kimpimäki et al. 2001a, Kupila et al. 2001, (V)

Previous cohort studies of children with increased genetic risk have reported associations between age at introduction of cereals and the development of an early and potentially reversible beta-cell autoimmunity, i.e. positivity for at least one autoantibody (Norris et al. 2003, Ziegler et al. 2003).

However, we did not observe a significant association between age at introduction of cereals and

the development of ICA plus at least one other autoantibody. The findings of the birth cohort studies showed no association between breastfeeding or age at introduction of complementary milk feeding and the emergence of up to three autoantibodies. In our earlier study among DIPP- infants, short-term exclusive breastfeeding and the early introduction of complementary milk feeding were related to an increased risk of developing all four autoantibodies and IA-2A (Kimpimäki et al. 2001a). This inconsistency could be a result of the different study design (cohort vs. case-control), or a chance finding. The cohort study could be considered as being more comprehensive because of its higher number of endpoints, extensive statistical methods, and more accurate dietary data collection. It should be noted, however, that the prospective studies done so far have been underpowered for the detection of such low risk ratios (about 1.5) as have been observed in case-control studies. However, it is noteworthy that in the present study when one includes only those children with a first-degree relative with diabetes, then a positive association of borderline significance was observed between early introduction of cow's milk and the increased risk of developing ICA (V).

#### **6.6 The inferences drawn cannot be stronger than the weakest part of the study; Evaluating selected issues in studies I – V**

The distribution of subjects in studies I and II by age and education were comparable to Finnish pregnant women in general (Meriläinen et al. 1995). However, the dietary habits of women living in Oulu region might differ from women in other areas in Finland (Nummela et al. 2000). According to the Findiet 2002 study, the use of fresh vegetables is lower in the Oulu province compared to other areas (Männistö et al. 2003). Nonetheless, we consider the subjects in studies I and II to represent rather well Finnish pregnant women in general. In studies III, IV and V, the children were genetically susceptible index children. In addition subjects in study III needed to have first-degree relative/s affected by T1D. The cohort of infants in studies IV and V is expected to be

representative of the population of Finnish infants, whereas the subjects in study III could more clearly be seen as representatives of the children having a major risk for T1D.

A complicated and cumbersome dietary method might have increased the number of dropouts in some of our present studies (e.g. in the validation study). However, the participation rates in the present validation study are high in comparison to other validation studies done in pregnant women; the percentages in other studies having ranged from 45 to 60% (vs. 80% in our study) of those invited (Brown et al. 1996, Wei et al. 1999, Fawzi et al. 2004). The validity of the cohort study is related to the completeness of the follow-up (Freudenheim 1999). People willing to participate in a cohort study may be more interested in health and health practices than the average individual and therefore may have different dietary practices from the general population. A highly selected study population (i.e. those still remaining at the end of long-term studies) could lead to minimized dietary exposure contrasts, which can decrease the informativeness of the epidemiologic studies. The follow-up rates in four birth cohort studies on nutrition in the etiology of pretype 1 diabetes (Table 12) are difficult to compare because of deficient and incoherent information provided in the published articles (Norris et al. 1996, Couper et al. 1999, Hummel et al. 2000, Kimpimäki et al. 2001a, Kupila et al. 2001, Fronczak et al. 2003, Norris et al. 2003, Ziegler et al. 2003). Scientific journals should develop comparable methods for reporting follow up rates and include them in the requirements for submitted manuscripts. It is almost impossible to make direct numerical comparisons of participation rates between the DIPP Nutrition study cohort and other birth cohorts. It is mentioned in the article of Hummel et al. (2000) that 82% of recruited children in German BABYDIAB Study participated in the 2-year follow-up visit (vs. 68.3% followed up to 2 years in the present study). However, it is difficult to obtain a clear picture of the baseline participating rates in the German BABYDIAB Study (Hummel et al. 2000, Ziegler et al. 2003). The DAISY Study (Norris et al. 1996; 2003, Fronczak et al. 2003) and the Australian BAYDIAB Study (Couper et al. 1999) report only median ages at follow up.

In the DIPP Nutrition study there is a risk that the knowledge of increased genetic risk would affect food choice. The impact of possible health behaviour changes on the diet of the DIPP-infants, if any, may be strongest at the very start of follow-up time, immediately after obtaining information about increased risk (Bennett Johnson & Tercyak 1995). This is usually the period of full breastfeeding or/and formula feeding. The health behaviour changes might favour breastfeeding, which in accordance to the National nutrition recommendations for infants (Hasunen et al. 2004). However, the length of exclusive and total breastfeeding among DIPP-children was very similar to that of Finnish infants in general (Hasunen 2002). All the nutritional guidance given to families was based on the National nutrition recommendations for infants (Hasunen et al. 2004) and was kept as neutral as possible.

## **7. Conclusions; Implications on public health and future perspectives**

The validity and reproducibility of our food frequency questionnaire were found to be reasonably good to be used as a dietary instrument, which could be administered after delivery to effectively study the putative effects of the maternal diet during pregnancy on the development of T1D. This validity study represents an internationally important addition to the methodology of studies on diet during pregnancy. Pregnancy seems to be an incitement for dietary over reporting. Since it is possible that the extensive FFQ used to assess diet is also subject to over reporting, the observed absolute intakes should be viewed with caution. In long-term cohort studies, the FFQ should be continuously updated due to new foods becoming available over the duration of the study and changes in dietary patterns. Improving the grouping of those foods and nutrients subjected to the greatest misclassification should be considered. A new validation study will be required for every revised version.

A balanced diet will meet the increased nutrient requirements during pregnancy, with the exception for some critical nutrients: vitamin D, folate, and iron. The quality of diet during pregnancy should be improved by increasing of the consumption of whole grain cereals, vegetables, and fish, and decreasing of the consumption of foods rich in sugar and saturated fat. Supplementation is needed to ensure an overall adequate intake of iron and vitamin D. The use of dietary supplements increases during pregnancy, but their use tends to be focused on the wrong nutrients. Milk fortification provides an important additional source of vitamin D into the diet of Finnish pregnant women. Future challenges include ensuring an adequate folate intake among women of childbearing age and during pregnancy, levelling the dietary inequalities existing between pregnant women with different sociodemographic characteristics, and preventing the trend to greater pregnancy weight gain in Finland and the prevalence of obesity accelerated by pregnancy. There have been few dietary surveys conducted in Finnish pregnant women and these should be done regularly so that we are able to detect new and emerging problems, and to ensure that the advice is up-to-date. The need

for consistent assessment is also emphasized by the complex relationship between maternal nutritional and birth outcomes and possible lifelong consequences. Promoting the health and nutrition of women of reproductive age could be considered as one way to circumscribe health problems in future generations.

The duration of breastfeeding in Finland is considerably shorter than recommended. Compliance is relatively poor with the current recommendations on the timing of the introduction of first complementary food and dairy products. Feeding infant formulas in the maternity wards does not compete with breastfeeding. The diet during infancy is strongly influenced by the extent of parental education, maternal age, and infant's gender. The potential for achieving marked health gains through improved diet in pregnancy and infancy should be encouraged at every level of our health care system as well as in basic school education to prevent the development of even wider sociodemographic dietary differences. Gender differences in infant feeding and their possible health consequences should be further investigated. The dispersed local data on infant feeding in Finland should be pooled to create national databases and linked to an international follow-up. This would require the establishment of nationally and internationally comparable surveillance systems, which would have common definitions and would use similar methodology. It is recommended that this should be started at the national level.

We introduced a new approach to the research into cow's milk antibodies in infants and young children. Few associations were established between maternal cow's milk protein intake during pregnancy and lactation and cow's milk protein antibody levels in the offspring. The milk and milk products taken by the mother seem to slightly differ in their impact on the emerging cow's milk antibody response in the offspring. Protein intake from raw milk products and cheese tend to slightly inhibit the humoral immune response to cow's milk proteins in the offspring; i.e. cheese during the first 6 months and raw milk later, close to 2 years of age, the impact being stronger

during lactation than during pregnancy. Further studies are needed to confirm whether cow's milk proteins transferred through the placenta and breast milk could induce tolerance to cow's milk proteins in the offspring, and to identify the possible pathogenic role of increased levels of cow's milk antibodies in subjects with T1D.

T1D is the second most important public health concern in Finnish children after allergies and asthma and its prevalence in Finland is the highest in the world. Nutrition may play a major role in the development of T1D. In the largest prospective cohort series so far reported, an early age at introduction of fruit and berries was related to the risk of advanced  $\beta$ -cell autoimmunity. The finding is a totally new discovery. The next step is to assess whether the finding can be replicated and if so, whether these foods are proxies of other life style characteristics, or whether there is a true causal relationship. The study design should be repeated when a larger number of autoantibody endpoints becomes available. Although more studies are needed to confirm the present findings, the conclusion can be drawn that the risk of pre-type 1 diabetes and T1D could be reduced by following the current national guidelines on age at introduction of complementary foods. In future studies, data on infant feeding should be linked to data on maternal nutrient intake during pregnancy as well as with data on other environmental factors (e.g. viral infections) to identify possible interactions among these parameters. Given the relatively low prevalence and the fact that the disease is most likely multifactorial, the long-term diet is of interest when studying diet-based relations of T1D. The DIPP study is emerging as the most comprehensive birth cohort study so far and its potential for providing answers to open questions in the etiology of T1D continues to expand. Despite the fact that findings from well-conducted cohort studies carry considerable weight in the epidemiologic literature, no single study in itself can be definitive. Multiple studies taken together and new innovative designs, models and methodologies are needed for future research settings.

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