Early Life Nutrition and Gastrointestinal and Allergic Outcomes: The Generation R Study

JESSICA KIEFTE - DE JONG

Early life nutrition and gastrointestinal and allergic outcomes

The Generation R Study

Jessica Kiefte-de Jong

ACKNOWLEDGEMENTS

The general design of the Generation R Study was made possible by financial support from the Erasmus Medical Center Rotterdam, the Erasmus University Rotterdam, the Netherlands Organization for Health Research and Development (ZonMW), the Netherlands Organisation for Scientific Research (NWO), the Ministry of Health, Welfare and Sport, and the Ministry of Youth and Families, the Netherlands. Grant support was provided for the studies described in this thesis by Europe Container Terminals (ECT).

Financial support for this dissertation was kindly provided by:
Erasmus University Rotterdam.
Nederlandse Coeliakie Vereniging
J.E. Jurriaanse Stichting, Rotterdam.
Astmafonds
Danone Research – Center for Specialised Nutrition
Yakult Nederland B.V.
Nutricia Nederland B.V.
Nestlé Nutrition
GE Healthcare
Nutri-akt B.V.
Stichting Astmabestrijding

ISBN: 978-94-6169-244-3

Cover design: Marieke Klaasse, MillerDesign, Rotterdam, the Netherlands.
Lay-out and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands.

Early Life Nutrition and Gastrointestinal and Allergic Outcomes

The Generation R Study

Voeding in het vroege leven en gastrointestinale en allergische uitkomsten: Het Generation R Onderzoek

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 12 september 2012 om 15.30 uur.

door

Jessica Christina Kiefte – de Jong

geboren te Den Helder

ERASMUS UNIVERSITEIT ROTTERDAM

PROMOTIECOMMISSIE

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Paranimfen	Thamar Post Lianne Kerkhoven

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Chapter 3

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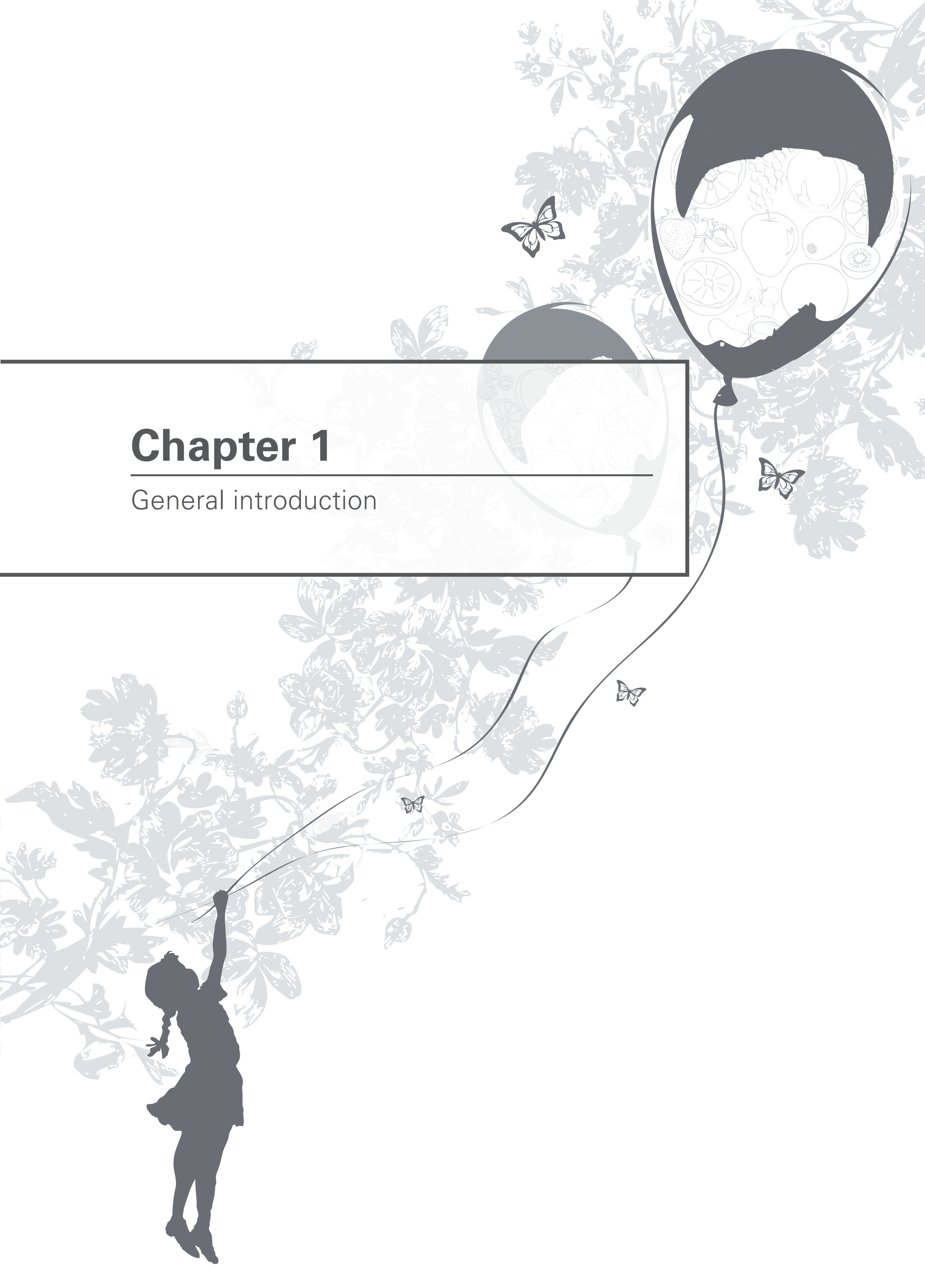
Chapter 1

General introduction



EARLY LIFE NUTRITION

A large number of epidemiological studies indicate that there is an association between early life nutrition, poor fetal and early postnatal growth and the development of diseases [1-2].

Adequate nutrition throughout life is fundamental from infancy until adulthood. The first year of an infant's life is a time of rapid transition from breast-feeding or formula-feeding to a varied diet from nearly all food groups being consumed on a daily basis by the majority of children. The World Health Organization (WHO) has recommended to breastfeed exclusively for at least 6 months [3]. Several studies have convincingly shown that breast-feeding is the most eminent type of feeding in order to prevent infections in childhood [4]. Studies also suggest, that prolonged breast-feeding may reduce the risk of obesity and other chronic disease such as diabetes mellitus and cardiovascular disease later in life [4]. After the age of 6 months, introduction of complementary feeding in addition to breast-feeding or formula-feeding is essential for both developmental and nutritional needs[3].

The optimal age of the introduction of complementary feeding is still a hot topic and is debated widely. As stated by the WHO "Complementary feeding should be *timely*, meaning that all infants should start receiving foods in addition to breast milk from 6 months onwards. It should be *adequate*, meaning that the complementary foods should be given in specific amounts, frequency, and consistency and offer a variety of foods to cover the nutritional needs of the growing child while maintaining breast-feeding" [5]. This decision to introduce complementary feeding after the age of 6 months was based on a Cochrane review updated in 2006 and republished in 2009, which concluded that exclusive breast-feeding for 6 months (i.e. breast-feeding without any other solids, water or milk) reduces the risk of infectious morbidity and does not increase the risk of observable growth deficits. No benefits of introducing complementary foods between 4 and 6 months were demonstrated, with the exception of improved iron status in developing countries [6]. However, because of minimal scientific evidence on the appropriate timing of complementary feeding from developed countries, several advisory boards in developed countries adopted different recommendations for the introduction of solid foods. Since 2008, the European Society of Paediatric Gastroenterology, Hepatology and Nutrition recommends that the introduction of complementary food, including solid food should be given after 4 months and before 6.5 months [7]. In the Netherlands, on the other hand, the nutritional board advises the introduction of solid food after 6 months of life, according to the WHO recommendations [5] unless there are indications that earlier introduction of solids (i.e. between 4 and 6 months) are needed [8].

However, several studies suggested recently that delaying complementary feeding until the age of 6 months can be beneficial [9]. For example, some studies showed that delaying complementary feeding is associated with a reduced risk of overweight [10-11]. Others claim that especially postponing complementary foods until 6 months might increase the risk of allergic disease and celiac disease since there may be a window of tolerance in introducing complementary feeding between 3 and 6 months for certain food allergens [12-13]. Therefore, further studies are needed to establish the optimal timing of complementary feeding in relation to health.

The transition from milk feeding to solid foods generally continues until the age of 12 months, and from 12 months onwards the variation in the consumption of food products is comparable to older children [14]. Studies suggest that altered feeding practices after this transition period may contribute to the rising incidence of diseases in children [15-16].

Although valuable knowledge has been gained with studies focused on single nutrients or food products, these may fail to account for the interactions between nutrients, and they do not take into consideration that some nutrients are interrelated [17]. Thus during the last decennium interest has shifted to the study of dietary patterns representing a broader picture of food and nutrient consumption and may therefore be more predictive in studies of various health- and disease outcomes [17]. The Mediterranean diet is one of dietary patterns that has been extensively studied. Adherence to this dietary pattern has shown to be associated with a lower prevalence of overweight, cardiovascular disease, and several types of cancer [18].

Although these associations between dietary patterns and diseases have been reviewed for adults[19], less is known about effects of dietary patterns of children at pre-school age. A review by Smithers et al showed that only two studies addressed dietary patterns before the age of 18 months [15]. In addition, the Norwegian Mother and Child Cohort Study were able to define an 'Unhealthy' dietary pattern at the infant's age of 18 months which was associated with maternal negative affectivity [20]. Similarly, in the Southampton Women's Survey a dietary pattern related to infant's guidelines (e.g. high intake of fruit, vegetables, and home-prepared foods) at the age of 12 months was defined and associated with better body composition at 4 years of age [21].

Taking this into consideration, specific knowledge about the determinants and health-effects of common dietary patterns in children below the age of 2 years are needed. This will improve our understanding regarding which aspects of health are most vulnerable to early–life diet and can also improve targeted dietary recommendations to parents of young children.

EPIDEMIOLOGY OF CELIAC DISEASE AND FUNCTIONAL BOWEL DISORDERS

Functional constipation is one of the major gastrointestinal symptoms presenting in children younger than 15 years of age [22] of which the prevalence is increasing since the early 90's[22]. In addition, 0.7–29.6% (Median 8.9%) of the pediatric population has functional constipation [23]. The onset of constipation is in about half of the children in the first year of life with the highest prevalence at pre-school age [23]. The term 'functional' refers to constipation of which no physical or organic cause is known. Different definitions of functional constipation are used in practice, but the so-called 'Rome- criteria' are generally considered as the major criteria to be used to define functional constipation in the population. The Rome II criteria have been developed in 1999 and attempted to provide a symptom-based definition of functional childhood constipation (table 1.1) [24]. Several environmental and social factors have suggested as being associated with childhood constipation, including transition from breast- to formula feeding, low physical activity levels, low social economic background, and obesity [23, 25]. Also, low intake of dietary fiber, fluid, fruit, and vegetables have been suggested to be associated with constipation but the effects of these dietary components are still inconsistent in very young children [23, 26].

Food hypersensitivity, such as cow's milk allergy, has been proposed in some studies as a potential cause of 'functional' constipation in childhood. Cow's milk allergy affects 0.1-5% of the children up to 2 years of age [27]. Several studies have suggested that cow's milk allergy may be a cause of constipation in children; but due to the late-onset allergic reaction on intestinal motility, it is frequently unrecognized [28-29]. Another cause of constipation that has gained interest is celiac disease (CD)[30]. CD is a lifelong disorder caused by intolerance for gluten characterized by villous atrophy of the small intestine. In the Netherlands, the prevalence of CD is though to be around 0.5-1%, but is highly unrecognized because of the broad spectrum of clinical features [31]. In addition, symptoms of CD vary from classical symptoms as diarrhoea and growth retardation; to less specific symptoms such as anemia, fatigue, osteoporosis and reproductive complications[31]. This phenomenon is also known as the 'iceberg of celiac disease'.

The etiology of celiac disease includes genetic causes, however infant feeding practices and infectious disease may also play a role [31]. The diagnosis of CD requires demonstration of villous atrophy with hyperplasia of the crypts in the small intestine,

Table 1.1: ROME II criteria for children (Rasquin 1999)

In infants, and pre-school children at least two weeks of:
· Scybalous, pebble-like, hard stools for a majority of stools; or
· Firm stools two or less times/week.
· And no objective evidence of an organic disease responsible for the symptoms.

but tests based on the detection of anti-tissuetransglutaminase (anti-tTG) is used as an initial screen for CD which is useful in epidemiological studies [32]. Although these tests have high specificity and sensitivity, it is still unclear what the consequences of intermediate anti-tTG or positive anti-tTG without villous atrophy are on later health.

EPIDEMIOLOGY OF ASTHMA-LIKE SYMPTOMS AND ATOPIC DERMATITIS

Asthma is a common chronic disease in the pediatric population with a prevalence around 10% in Europe [33]. In pre-school children, asthma-like symptoms are commonly defined as wheezing or shortness of breath [34]. The majority of all children have at least one episode of asthma-like symptoms such as wheezing, or shortness of breath in the first year of life [35]. However, it has been demonstrated earlier that only 11% of the children with asthma-like symptoms at pre-school age develop asthma later on [36]. In addition, most of the wheezing symptoms are transient and usually dissipate at school age [35]. Atopic dermatitis (AD) is associated with asthma and is characterized by itching, dryness and an increased sensitivity to allergens due to an altered skin barrier, which leads to skin inflammation [37]. Most of the children with AD will outgrow their disease. At school age, almost half of the children with previous AD will be in remission and approximately 20% will have persistent symptoms of AD [37]. It has been debated whether AD is truly linked to atopic disease. Children with AD may develop a typical sequence of asthma and (food) allergies at later ages. Some of these symptoms may persist for several years or may resolve with increasing age. This progression of various allergic symptoms is also considered as the 'atopic marsh' [38].

Despite genetic factors, the environment also plays a fundamental role in the development of asthma and atopic dermatitis. Factors proposed to influence the risk of asthma and allergic diseases include breast-feeding, crowding, maternal age, gender, exposure to smoking, and viral/bacterial infections [39]. There is considerable interest in potential links between diet and atopic disease. Breast milk has well established immunological activity and has been shown to protect against infectious disease [40], however beneficial effects of breast-feeding on AD and wheezing have shown to be inconclusive [41-42]. In addition, a cluster randomized trials showed no protective effect of breast-feeding on the development of asthma and allergy [43]. Studies examining the timing of introduction of solid foods to the infant diet also showed inconsistent results. Traditionally, it was thought that delaying the introduction of solid foods may reduce the risk of development of allergic disease by decreasing the dietary antigen load [44]. However, others suggest that early solid introduction may decrease the risk of asthma or allergic rhinitis by providing a window of opportunity to induce oral tolerance [9, 13]. This emphasizes that the relation between timing of introduction of solid foods and allergic outcomes needs further study.

In recent years, studies have reported associations between either maternal nutrient status or child nutrient status, and the development of asthma and atopic disease. Weak epidemiological evidence supports the association between intake of vitamin A, D, E, fruit, and vegetables, and the protection of asthma. A Mediterranean diet during pregnancy also seems to be protective for the development of asthma in childhood [45]. The topic of nutrient status and the association with AD remains controversial. There is weak evidence for vitamin A and C, which may have a benefit in preventing AD in a selected population whereas there is no evidence to support a role for vitamin A, C, E, and D in the prevention of AD in the general population [41]. Most studies focused on the nutrient status during pregnancy or in older children, but little is known about dietary habits in pre-school children and the development of asthma-like symtpoms and AD.

AIMS OF THE STUDY

With this thesis, we aimed to elucidate the following aspects concerning early life nutrition, allergic and gastrointestinal outcomes:

- **Infant nutrition:**
  - o Consequences of timing of complementary feeding
  - o Determinants of dietary patterns in toddlers

- **Gastrointestinal outcomes:**
  - o Consequences of celiac disease autoantibodies during pregnancy
  - o Nutritional and endocrinological determinants of functional constipation in childhood

- **Asthma-like symptoms and atopic dermatitis:**
  - o Nutritional determinants of asthma-like symptoms and atopic dermatitis during the pre- and postnatal phase.

THE GENERATION R STUDY

The objectives of this thesis have been explored within the framework of the Generation R Study [46]. The Generation R Study is a population-based prospective multi-ethnic cohort study from fetal life until young adulthood which has been designed to identify early environmental and genetic causes of normal and abnormal growth, development and health during fetal life, childhood and adulthood.[46]

Eligible participants were mothers who were residing in Rotterdam, the Netherlands at their delivery date. Enrolment was aimed in first trimester, but was allowed until birth of the child. All children were born between April 2002 and January 2006. Assessments during pregnancy were planned in each trimester and included physical examinations, blood and urine collection, fetal ultrasound examinations, and self-administered questionnaires. In the pre-school period, which refers to the period from birth to 4 years of age, information was collected by parental derived questionnaires at the ages of 2, 6, 12, 14, 24, 36, and 48 months and regular routine visits to the child health centres. More detailed assessments were conducted in a subgroup of Dutch children after visiting the research centre at 1.5, 6, 14, and 24 months. Measurements during these visits included, among other things, physical examinations, and body fluid specimen collection [46].

OUTLINE OF THIS THESIS

Subsequent to this general introduction, chapter 2 of this thesis describes the consequences of complementary feeding on growth in early life. It also describes determinants of the child's diet after the weaning and lactation period by using a dietary pattern approach. Chapter 3 refers to: the role of maternal celiac disease auto antibodies on fetal growth along with nutritional and endocrinological determinants of functional constipation, by assessing the timing of introduction of food allergens, dietary patterns and cortisol levels in the second year of the child's life. In chapter 4, the influence of nutritional exposure on the development of asthma-like symptoms or atopic dermatitis is reported in chronological order (exposure during pregnancy, in infancy and in early childhood). Finally, in chapter 5 an overview will be given on the conclusions and discussion points that have arisen from these studies. Also, recommendations and implications for future research will be highlighted in chapter 5. An overview of the studies described in this thesis is shown in table 1.2.

Table 1.2: Overview of studies reported in this thesis

Chapter	Study group	Population of analysis	Research question
2.1	Generation R cohort	*n*=3184	Timing of introduction of solids in first year of life and postnatal growth
2.2	Generation R cohort (Dutch only)	*n* =2420	Socioeconomic and lifestyle factors of dietary patterns of toddlers
3.1	Generation R (prenatally included)	*n* =7046	Celiac disease auto antibodies and fetal growth and birthweight
3.2	Generation R	*n* =4651	Timing of introduction of food allergens and breast-feeding duration in the first year of life and functional constipation in childhood
3.3	Generation R (Dutch only)	*n* =2420	Dietary patterns, overweight, and sedentary behavior of the child and the development of functional constipation in childhood
3.4	Generation R (Subsample)	*n* =483	Cortisol stress reactivity and cortisol diurnal rhythm and functional constipation and abdominal pain.
4.1	Generation R (prenatally included)	*n* =8742	Folic acid supplementation, plasma folate and vitamin B12 levels during pregnancy and asthma-like symptoms and atopic dermatitis in the offspring.
4.2	Generation R	*n* =6905	Timing of introduction of food allergens and asthma-like symptoms and atopic dermatitis in childhood
4.3	Generation R (Dutch only)	*n* =2173	Dietary patterns and asthma-like symptoms and respiratory infections in childhood.
4.4	Generation R	*n* =7210	Timing of introduction of fish and fish consumption in infancy and asthma-like symptoms in childhood.

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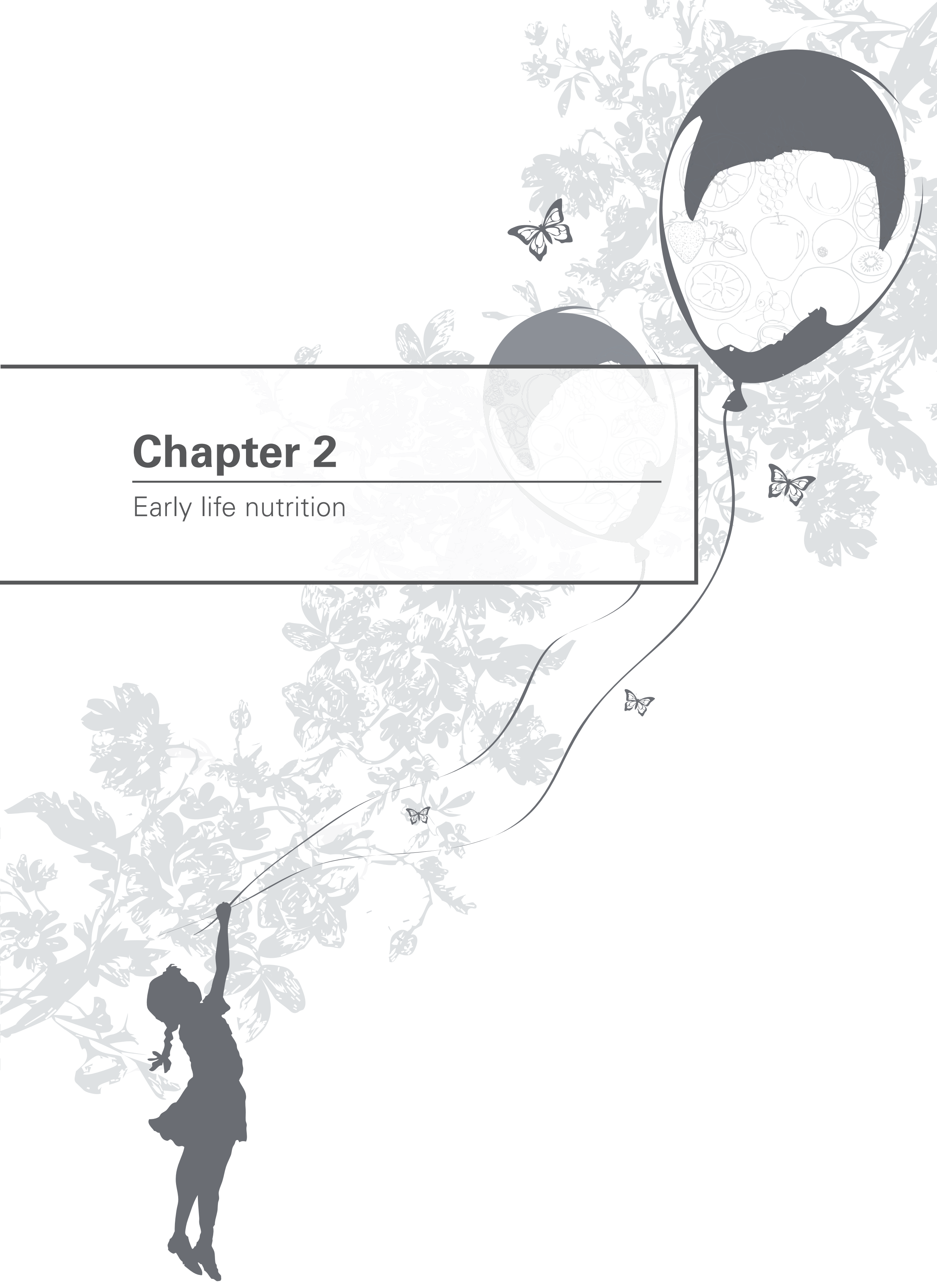
Chapter 2

Early life nutrition



Chapter 2.1

Weight change before, during and after introduction of solids

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Br J Nutr. 2012 Apr 5:1-6

ABSTRACT

We studied the association, and its direction, between the introduction of solids and weight-for-height (WFH) change between birth and 45 months. Pregnant women were asked to participate in a birth cohort during their first antenatal visit. Data from 3184 children were used. Timing of introduction of solids was reported by the mother from a questionnaire at 12 months postpartum, and categorized into very early (0-3 months), early (3-6 months) and timely (after 6 months) introduction of solids. Anthropometric data were collected during standardized child health center visits. WFH was converted into a z score. Repeated measurements analyses with splines positioned according to the moments of solid introduction were used to obtain estimates for WFH change before and after introduction of solids. Analyses were adjusted for educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding, history of food allergy, and infant's hospital admission. Before solids were introduced, weight gain was higher in children introduced to solids early (z=0.65, 95% CI: 0.34, 0.95) than in children introduced to solids very early (z=0.02, 95% CI: -0.03, 0.08) and timely (z=-0.04, 95% CI: -0.05, -0.03). Shortly after the introduction of solids, children introduced to solids very early and early showed a relative decrease in WFH. WFH change did not differ between solid introduction groups after 12 months, and at that time, weight change was as expected (i.e. z= 0). We therefore conclude that differences in WFH in childhood are not the result of early introduction to solids.

INTRODUCTION

Infant feeding (breast-feeding and complementary feeding) may be important for a healthy weight in later life. However, results on complementary feeding as a determinant of overweight are still conflicting: a recent review[1] concluded that there is not sufficient evidence for an association between timing of introducing solids and obesity. Since then, two papers were published that reported a clinically relevant association between timing of introduction of solids and childhood obesity.[2, 3] One proposed mechanism to explain the higher prevalence of obesity in children introduced early to solids is rapid infant weight gain after the introduction of solids. Results on net increase in energy intake in children introduced to solids are controversial, but intake of fatty and sugary foods have been reported to be higher at 12 months in children introduced to solids early.[4] However, reverse causality can be the cause: an alternative explanation is that infant weight gain precedes the introduction of solids. Indeed, an earlier study showed that one of the reasons for parents to introduce solids earlier than recommended is that their infant was big for their age.[5]

Most studies reporting the association between introduction of solids and infant weight gain defined infant weight gain as the difference in weight between two time points, and do not take into account that introduction of solids may be related to weight change before and after introduction of solids to the infant's diet, which is important to confirm or reject the proposed mechanism.

The aim of the present study was to study the association between very early (before 3 months), early (between 3 and 6 months) and timely (beyond 6 months) introduction of solids and weight change in infancy and early childhood. We hypothesize that infants that were introduced to solids very early and early were already heavier before introduction than infants who were introduced to solids after the age of 6 months.

METHODS

Study design and population

This study was embedded in The Generation R study, an observational cohort study that follows children from fetal life onwards.[6] The Generation R study was designed to identify early determinants of growth, development and health. Invitations to participate in the study were made to all pregnant mothers who had an expected delivery date between April 2002 and January 2006 and who lived in the study area (Rotterdam, the Netherlands) at time of delivery. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethical Committee at Erasmus MC, University Medical Center Rotterdam. Written informed consent was obtained from all subjects.

Of the 7295 mother-infant pairs who were followed from birth 5088 received the 12-months food questionnaire, because data collection on food started from 2003 onwards: 3643 (72%) mothers completed this questionnaire. Excluded were twins (n=82), children born before 37 weeks of gestation (n=158), and children with less than four measurements on weight and height (n=219). Data of 3184 children were analyzed.

Compared to those with missing information on the introduction of solids and those having less than four weight and height observations, mothers included in the present study were more often breast-feeding at 6 months (32.9% vs. 26.9%), higher educated (33.4% vs. 21.8%), more often had infants with a normal birth weight (85.0% vs. 81.8%), were more often native Dutch (67.7% vs. 44.2%), less often smoked during pregnancy (7.7% vs. 12.5%) (p<0.001 for all), and more often had a normal weight, defined as a body mass index (BMI) ≤ 25 kg/m^2 (75.9% vs. 72.0%) (p<0.01).

Measurements

Infant feeding

For this study, we were interested in the age of first introduction of any solids. At the child's age of 12 months, mothers reported in a questionnaire the age of the first introduction of the following foods:[7] dairy products, porridge, bread, biscuits, crackers, baby cookies, pasta, meat products, vegetarian meat substitutes, fish, shellfish, vegetables, fruit, peanuts and nuts. Answer categories included 'never given', 'between zero and three months', 'between three and six months', 'between six and nine months', and 'older than nine months', which was recoded into three categories: 'zero to three months', 'three to six months', and 'six months or later'. The latter group adheres best to the feeding recommendations of the WHO and is therefore used as the reference group.[8]

Anthropometrics

Length/height and weight were measured according to a standard schedule (one, two, three, four, six, 11, 14, 18, 24, 30, 36, and 45 months of age) and performed by well-trained staff at each visit to the child health center. Length was measured in supine position to the nearest millimeter until the age of 14 months using a neonatometer, after which height was measured in standing position by a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Hamburg, Germany). Weight change was defined as increase or decrease in weight-for-length/height (WFH) z score, which were calculated from a national reference using the Growth Analyzer program (Growth Analyzer version 3.5, 2007, Dutch Growth Research Foundation, Rotterdam, the Netherlands).[9]

Covariates

Potential confounders were maternal educational level, ethnicity, body mass index (BMI), smoking during pregnancy, gestational age, child's birth weight, breast-feeding, history of (any) food allergy in the infant's first year of life and hospital admission during the first year after birth. Mother's educational level and ethnicity were asked at enrollment. Mother's body mass index (BMI) was calculated from self-reported pre-pregnancy weight and measured height at intake. Smoking during pregnancy was self-reported in a prenatal questionnaire. Birth weight and gestational age were obtained from medical records. Mothers reported in a postal questionnaire that was sent at two months after birth whether they gave breast-feeding, formula feeding or a combination of both. History of (any) food allergy in the infant's first year of life and hospital admission during the first year after birth were asked at 12 months after birth, and were taken into account because if these events took place in the first months after birth, they could have confounded the associations.

Statistical analyses

Differences in characteristics between mothers introducing solids before three or six months were compared with those of mothers introducing solids after six months with the Chi^2 test (categorical variables) or by ANOVA (continuous variables).

A mixed linear model (proc mixed in SAS) was performed with WFH *z* score as outcome. Linear splines for WFH change by age were created, which are useful when estimates for weight change are expected to differ between time periods. The knots for the splines were positioned according to the moments of solid introduction (i.e. 0-3, 3-6 and after 6 months), and set after the start of the introduction of solids (mid-point of each category), the following 3 months after the start point for introduction, the following period until 12 months, and after 12 months (Table 2.1.1). The four periods obtained with the splines were put in the model as time variables, and the WFH development from birth to pre-school age was obtained with stratified analyses for each group of solid introduction.

Residuals of the spline model were normally distributed with a mean difference of 0.01 (95% CI: -1.91, 1.89) between actual and predicted values of wfh z-score, and residuals did not vary over time (Pearson's r=0.001, *p*=0.90) or for subgroups (i.e. breast-fed children, or children of low socio-economic status). Analyses were conducted with Statistical Package for Social Sciences for Windows version 17.0 (SPSS, Inc., Chicago, IL, USA) and Statistical Analysis Software package version 9.1 (SAS Institute, Cary, NC, USA).

Table 2.1.1: Time periods for weight change estimates

Categories of timing of introduction of solids	Birth until start of introduction of solids	Shortly after introduction of solids	After the introduction of solids	After 12 months of age
0-3 months	0 – 1.5 months	1.5 – 4.5 months	4.5 – 12 months	12 – 45 months
3-6 months	0 – 4.5 months	4.5 – 7.5 months	7.5 – 12 months	12 – 45 months
After 6 months	0 – 7.5 months	7.5 – 11.5 months	11.5 – 12 months	12 – 45 months

RESULTS

Thirty-eight percent of mothers introduced solids after the recommended age of six months. Relative to mothers that introduced solids before three or six months, mothers that introduced solids after six months were more often higher educated, native Dutch, non-smokers, breast-feeding for at least six months, and had more often an infant with a history of food allergy. Hospital admission and mother's weight were not significantly associated with timing of the introduction of solids. The number of children with either a low or high birth weight did not differ between groups, but mean birth weight of children that were introduced to solids before three months was slightly lower than children introduced to solids after three or six months (Table 2.1.2). Table 2.1.3 shows the growth pattern from birth to pre-school age for each group of solid introduction. Children that were introduced to solids very early (before three months) had a weight-for-height change as expected (i.e. WFH z score ≈ 0) before they were introduced to solids. This was followed by a relative decrease in WFH until 4.5 months, but after 4.5 months, they were growing as expected (i.e. WFH z score ≈ 0). Children that were introduced to solids early (between three and six months) had a high WFH gain before they were introduced to solids (z=0.65, 95% CI: 0.34, 0.95), but once introduced to solids, this was followed by a relative decrease in WFH (z=-0.13, 95% CI: -0.18, -0.08). After 7.5 months, they were growing as expected with WFH z score ≈ 0 (table 2.1.3, figure 2.1.1). Children introduced to solids according to the recommendation (after six months), had a small decrease in WFH before they were introduced to solids (z=-0.04, 95% CI: -0.05, -0.03), but after the introduction of solids, they followed a growth pattern as expected (i.e. WFH z score ≈ 0).

At 4.5 months, children in the very early and early introduction of solids groups, were on average 0.5 kg heavier than children introduced to solids timely (Supplementary material table S2.1.1 and Figure S2.1.1)

Because the exact timing of history of food allergy and hospitalization is unknown, these variables could be either confounders or mediators. We have therefore run the models without these variables (see supplementary material, table S2.1.2), but results did not change.

Figure 2.1.1 shows WFH z score development from birth to pre-school age, and is a graphical representation of Table 2.1.3. At the ending age (pre-school age), WFH z

Table 2.1.2: Characteristics of 3184 participants according to the timing of introduction of solids*

		Total (%)	Introduction of solids (%)			*p*-value
			0-3 months (*n*=171)	3-6 months (*n*=1808)	≥6 months (*n*=1205)	
Socio-demographic factors						
Educational level	Low	14.1	21.1	15.9	10.5	<0.001
	Mid-low	26.8	36.0	28.1	23.6	
	Mid-high	25.7	23.6	24.8	27.5	
	High	33.4	19.3	31.2	38.5	
Mother's ethnicity	Native Dutch	67.6	53.7	67.5	69.6	<0.001
	Other Western	11.5	12.3	10.1	13.3	
	Non-Western	20.9	34.0	22.3	17.0	
Parental characteristics						
Maternal smoking during pregnancy		7.8	13.8	9.5	4.3	<0.001
Mother's BMI (kg/m^2)	Normal (<25)	75.9	74.3	75.2	77.1	0.47
	Overweight (25-30)	16.9	17.1	16.8	16.8	
	Obese (>35)	7.2	8.6	7.9	6.0	
Perinatal characteristics						
Birth weight (grams)	Low (<2500)	1.4	1.2	1.2	1.7	0.41
	Normal (2500-4000)	81.9	86.5	82.0	81.0	
	High (>4000)	16.8	12.3	16.8	17.3	
Birth weight (grams)	Mean±SD	3521±497	3422±476	3528±494	3525±503	0.03
Postnatal characteristics						
Child was breastfed at 2 months of age		69.2	56.3	66.9	74.4	<0.001
Child was breastfed at 6 months of age		32.8	22.2	27.3	42.6	<0.001
History of food allergy		6.4	5.6	5.1	8.6	<0.001
Hospital admission in first year of life		6.1	7.0	5.4	7.0	0.20

*Missing data were: 99 (3.1%) for educational level, 58 (1.8%) for ethnicity, 532 (16.7%) for maternal smoking, 698 (21.9%) for mother's BMI, 3 (0.1%) for birth weight, 87 (2.7%) for breast-feeding at 2 months, 56 (1.8%) for breast-feeding at 6 months, 172 (5.4%) for history of allergy, 199 (6.3%) for hospital admission.

scores are 0.01 (95% CI: -0.18, 0.19) for children introduced to solids very early, 0.11 (95% CI: 0.05, 0.17) for children introduced to solids early, and 0.04 (95% CI: -0.03, 0.11) for children introduced to solids according to the recommendation.

DISCUSSION

This study shows that children introduced early, but not very early, to solids had a higher increase in WFH prior to the introduction of solids than children introduced timely to solids. Differences in weight change disappeared during the first year of life. At pre-school age, children introduced to solids early had a slightly higher WFH than children introduced to solids very early or timely.

Table 2.1.3: Weight-for-height change prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=104), early (n=1120) and timely (n=771)

Timing of introduction of solids	Change in weight-for-height *z* score and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 – 4.5 months	4.5 – 12 months	12 – 45 months
z score	0.02	-0.13	0.02	-0.01
95% CI	-0.03, 0.08	-0.23, -0.04	-0.03, 0.08	-0.01, 0.002
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12 – 45 months
z score	0.65	-0.13	0.005	-0.004
95% CI	0.34, 0.95	-0.18, -0.08	-0.01, 0.02	-0.01, -0.001
Timely (≥ 6 months)	<7.5 months	7.5 – 11.5 months	11.5 – 12 months	12 – 45 months
z score	-0.04	0.02	-0.05	-0.003
95% CI	-0.05, -0.03	-0.01, 0.05	-0.10, 0.002	-0.01, -0.001

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding, history of allergy, and hospital admission in the first year of life

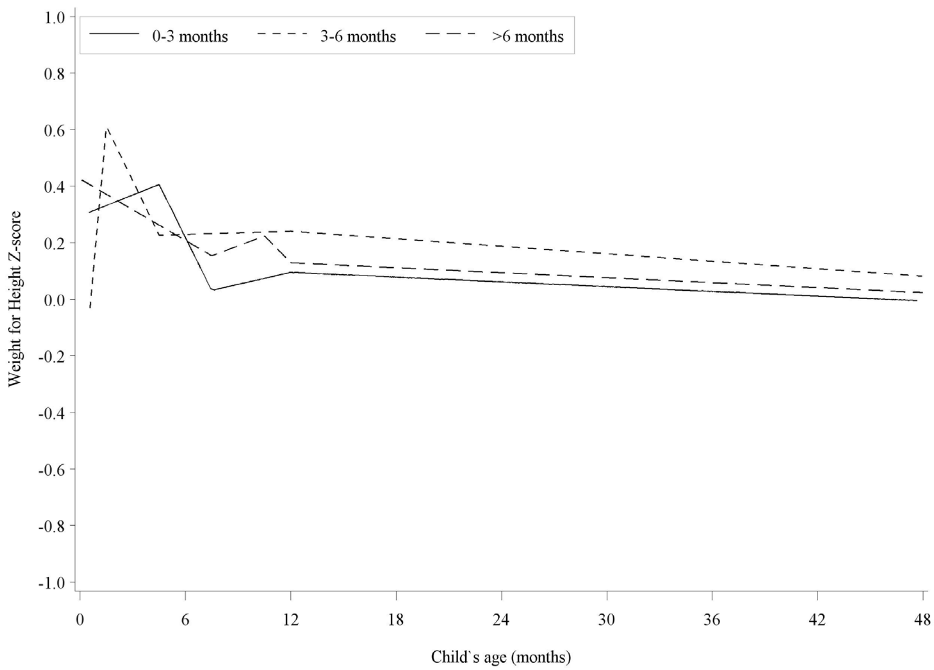

Figure 2.1.1: Estimated weight-for-height *z* score for each age group of introduction to solids (this figure is a graphic presentation of the data in Table 2.1.3).

Studying the association between early introduction of solids and weight gain is quite a methodological challenge. First, the association between early introduction of solids and weight gain may be subject to reverse causality: infants experiencing rapid weight gain may be earlier, or later, introduced to solids. Therefore, some studies adjusted the analyses for birth weight. However, this does not take rate of weight gain shortly after birth into account. Our results revealed no significant differences in birth weight *z* score among subgroups, but showed that infants that were introduced early to solids, were already heavier prior to introduction of solids. Second, the association between early introduction of solids and weight gain may be confounded by several factors. Although the associations were adjusted for the most important confounders, no detailed information was available on breast-feeding exclusivity, i.e. breast-feeding with no other fluids or solids at all. We also did not have detailed information on exact amount of formula feeding and solids intake, and therefore not on exact calorie and protein intake. This may have caused bias in any direction, but is most likely to affect weight change after the introduction of solids. However, this may not change our conclusion that children introduced to solids early were heavier before any solids were introduced. Timing of introduction of solids was based on the 12-months food frequency questionnaire. This could have induced some recall bias. However, there is no reason to assume that the recall differed according to weight change. Lastly, 72% of participants returned the 12-months food frequency questionnaire. Although this response rate is relatively high, mothers included in the analyses were general healthier and wealthier, which was also reflected in the mean *z*-scores being above 0. However, we think it is unlikely that this has led to selection bias as we assume the missing data being MAR (missing at random), which means that the missing data depend on covariates in the model only. Selection bias would for example occur when children of less healthy or wealthy families had more often missing data but were also introduced to solids earlier than recommended and had a higher WFH after solids introduction.

Despite the use of different cut-off points to define early introduction of solids, our findings that solid introduction is associated with weight during the first months of life is consistent with other studies. Baker et al. reported that infants introduced to solids before 4 months of age had a higher weight gain from birth to 1 year.[10] Baird et al. found that children introduced to solids after 6 months, were lighter and shorter than children introduced to solids before that age.[11] One study included several measurement points and used a similar cut-off point as in our study (at 3 months), making this study best comparable to ours. Infants receiving solids before 12 weeks were heavier at four, eight, 13, and 26 weeks of age, but not at 52 weeks or later.[12] There is also one trial that found no weight differences at 3, 6, and 12 months in children introduced to solids at 3-4 months of age compared to children introduced to solids at 6 months.[13] Wright et al found that babies who were heavier at 6 weeks and 3 months were weaned earlier, but that infant weight gain between birth and 6 weeks was a stronger predictor.[14] Also, Wright *et al* in their study asked mothers for their reasons starting introduction of solids, and 'my baby seems hungry' was a predictor of early weaning. Thus, our hypothesis that infant's are heavier prior to introduction of solids, making mother's believe it is necessary to introduce solids, fits well in our findings and the findings from the current literature.

After one year of age, we found no differences in weight change between children introduced to solids very early or early and children introduced to solids timely. However, as a result of differences in weight change during the first year of life, children introduced to solids early had a slightly higher WFH *z* score compared to children introduced to solids according to the recommendation. This result is consistent with a study describing that early introduction of solids before four months of age is not associated with weight change adjusted for height between birth and age 3.[15] However, associations between early introduction of solids (defined either as continuous variables or before the age of 4 months) and weight status after one year of age have been reported at several ages, ranging between 3 and 40 year old. [2, 3, 16, 17] Two of these studies did not find an association between introduction of solids before 4 months of age and weight in the same cohort at an earlier age.[16, 17] Huh et al. adjusted their analyses for weight gain between 0-4 months of age, and this hardly influenced the association between early introduction of solids and overweight at age 3.[2] They also reported that the association was only present in formula-fed children. We have stratified our analyses according to type of milk feeding to reveal possible effect modification, but our conclusions do not change (Supplementary Material, Tables S.2.1.3a-c) We hypothesize that early introduction of solids may therefore be associated with characteristics that determine later overweight. These characteristics may be related to lifestyle or may be related with biological programming. Grummer-Strawn for example found that children introduced to solids before the age of 4 months, were more likely to have a higher intake of fatty and sugary food at 1 year of age.[4]

Conclusion

In conclusion, although prior size may be related to introduction of solids, we have found no evidence that early introduction of solids increases WFH. Infant weight change may therefore not be one of the underlying causal mechanisms for the association between early introduction of solids and later overweight.

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SUPPLEMENTARY MATERIAL

Table S2.1.1: Absolute weight change (in kg per cm gain in length) prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=104), early (n=1120) and timely (n=771)

Timing of introduction of solids	Change in weight for height and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 - 4.5 months	4.5 – 12 months	12-45 months
weight change	0.85	0.27	0.29	0.09
95% CI	0.80 , 0.90	0.18 , 0.35	0.24 , 0.34	0.08 , 0.10
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12-45 months
weight change	1.74	0.70	0.31	0.09
95% CI	1.60 , 1.88	0.68 , 0.73	0.31 , 0.32	0.09 , 0.10
Timely (≥ 6 months)	<7.5 months	7.5 - 11.5 months	11.5 – 12 months	12-45 months
weight change	0.63	0.04	0.36	0.09
95% CI	0.62 , 0.65	0.02 , 0.07	0.31 , 0.41	0.09 , 0.10

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding, history of allergy, and hospital admission in the first year of life.

Table S2.1.2: Weight-for-height change prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=113), early (n=1217) and timely (n=809) – Associations with no adjustment for potential mediators (allergy and hospitalization)

Timing of introduction of solids	Change in weight-for-height *z* score and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 - 4.5 months	4.5 – 12 months	12-45 months
z score	0.01	-0.13	0.02	-0.01
95% CI	-0.05 , 0.07	-0.22 , -0.04	-0.03 , 0.08	-0.01 , 0.001
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12-45 months
z score	0.64	-0.13	0.004	-0.004
95% CI	0.35 , 0.95	-0.18 , -0.08	-0.01 , 0.02	-0.01 , -0.002
Timely (≥ 6 months)	<7.5 months	7.5 - 11.5 months	11.5 – 12 months	12-45 months
z score	-0.04	0.02	-0.06	-0.003
95% CI	-0.05 , -0.03	-0.005 , 0.05	-0.11 , -0.01	-0.01 , -0.001

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding

Table S2.1.3a: Weight-for-height change prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=26), early (n=428) and timely (n=408) – subgroup of children exclusively breastfed until 2 months

Timing of introduction of solids	Change in weight-for-height *z* score and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 - 4.5 months	4.5 – 12 months	12-45 months
z score	-0.16	-0.15	0.06	0.003
95% CI	-0.28 , -0.05	-0.32 , 0.01	-0.04 , 0.15	-0.01 , 0.02
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12-45 months
z score	0.73	-0.19	0.02	-0.003
95% CI	0.50 , 0.95	-0.23 , -0.16	0.01 , 0.03	-0.01 , 0.0001
Timely (≥ 6 months)	<7.5 months	7.5 - 11.5 months	11.5 – 12 months	12-45 months
z score	-0.06	0.03	-0.03	-0.003
95% CI	-0.08 , -0.05	-0.01 , 0.07	-0.10, 0.04	-0.007, 0.0004

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding

Table S2.1.3b: Weight-for-height change prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=29), early (n=300) and timely (n=163) – subgroup of children formula fed only at 2 months

Timing of introduction of solids	Change in weight-for-height *z* score and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 - 4.5 months	4.5 – 12 months	12-45 months
z score	0.08	-0.12	-0.07	-0.01
95% CI	-0.01 , 0.17	-0.28 , 0.03	-0.10 , 0.08	-0.02 , 0.003
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12-45 months
z score	0.73	-0.08	-0.005	-0.005
95% CI	0.49 , 0.96	-0.12 , -0.04	-0.01 , 0.005	-0.01 , -0.001
Timely (≥ 6 months)	<7.5 months	7.5 - 11.5 months	11.5 – 12 months	12-45 months
z score	-0.006	0.004	-0.06	-0.005
95% CI	-0.03 , 0.02	-0.05 , 0.06	-0.17 , 0.04	-0.01 , 0.001

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding

Table S2.1.3c: Weight-for-height change prior, shortly after, and after solid introduction, and after 12 months of age for children introduced to solids very early (n=49), early (n=392) and timely (n=200) – subgroup of children mixed feeding at 2 months

Timing of introduction of solids	Change in weight-for-height *z* score and 95% CI*			
	Before introduction	Shortly after introduction	After introduction	After 12 months of age
Very early (0-3 months)	<1.5 month	1.5 - 4.5 months	4.5 – 12 months	12-45 months
z score	0.08	-0.12	-0.07	-0.01
95% CI	-0.01 , 0.17	-0.28 , 0.03	-0.10 , 0.08	-0.02 , 0.003
Early (3-6 months)	< 4.5 months	4.5 - 7.5 months	7.5 – 12 months	12-45 months
z score	0.73	-0.08	-0.005	-0.005
95% CI	0.49 , 0.96	-0.12 , -0.04	-0.01 , 0.005	-0.01 , -0.001
Timely (≥ 6 months)	<7.5 months	7.5 - 11.5 months	11.5 – 12 months	12-45 months
z score	-0.006	0.004	-0.06	-0.005
95% CI	-0.03 , 0.02	-0.05 , 0.06	-0.17 , 0.04	-0.01 , 0.001

*Adjustments were made for mother's educational level, ethnicity, smoking during pregnancy, mother's BMI, breast-feeding

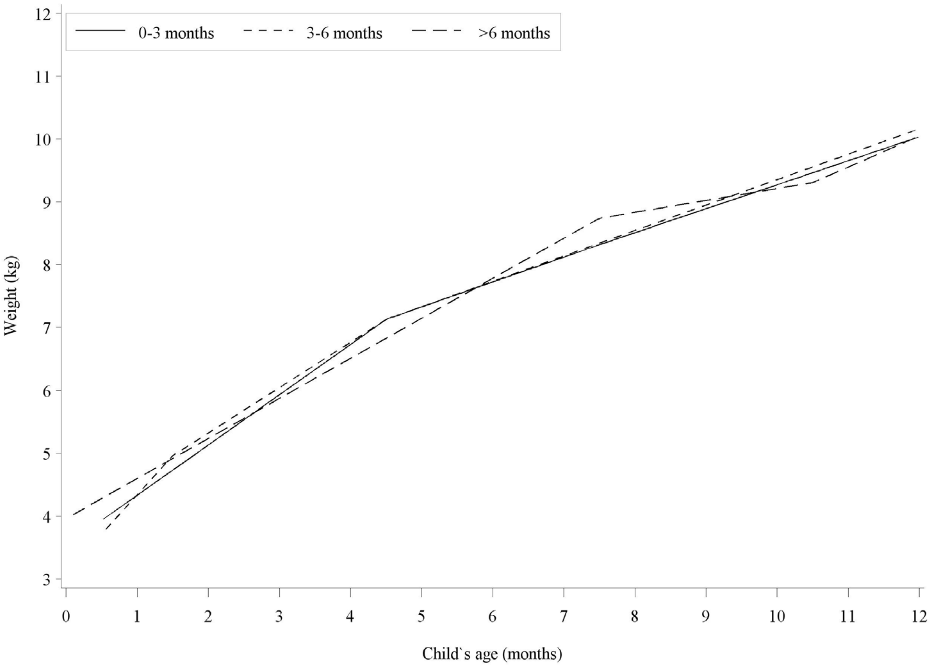

Figure S2.1.1: Estimated weight (in kg) until 12 months for each group of introduction to solids (n=3184). This figure is a graphical presentation of table S2.1.1, but for interpretation, weight is on the y-axis, and the analysis are adjustment for length/height.

Chapter 2.2

Socio-demographic and lifestyle determinants of a 'Western-like' and 'Health conscious' dietary pattern in toddlers

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Br J Nutr. 2012 Apr 5:1-11

ABSTRACT

Determinants of the child's diet shortly after weaning and lactation have been relatively understudied. The aim of this study was to identify common dietary patterns in toddlers and to explore parental and child indicators of these dietary patterns. The study was a population-based prospective birth-cohort study in Rotterdam, the Netherlands. Food consumption data of 2420 children aged 14 months was used. A 'Health conscious' dietary pattern characterized by pasta, fruits, vegetables, oils, legumes and fish, and a 'Western-like' dietary pattern characterized by snacks, other fats, confectionery and sugar-containing beverages were extracted from Principal Component Analysis.

Low paternal education, low household income, parental smoking, multiparity, maternal BMI, maternal carbohydrate intake, and watching television (TV) of the child were determinants of a 'Western-like' diet whereas parental age, dietary fiber intake during pregnancy, introduction of solids after 6 months, and female gender were inversely associated with a 'Western-like' diet of the child. Maternal co-morbidity, alcohol consumption during pregnancy, and female gender were inversely associated with a 'Health conscious' dietary pattern of the child while single parenthood, folic acid use, and dietary fiber intake during pregnancy were positively associated. All above associations were statistically significant.

In conclusion, both a 'Western-like' and 'Health conscious' diet can already be identified in toddlers. Particularly adherence to a 'Western-like' diet is associated with unfavorable lifestyle factors of the parents and child, and low socioeconomic background. These findings can form a basis for future epidemiological studies regarding dietary patterns and health outcomes in young children.

INTRODUCTION

Adequate nutrition is fundamental from infancy until adult life. However, the increase in the rates of chronic diseases such as obesity may suggest that children are not using an optimal diet since studies show that nutritional practice in early childhood have a role in the etiology of obesity[1].

There is evidence that many obesity-promoting behavior, including unhealthy eating habits that are learnt during childhood, track to adulthood[2]. The social environment is very important for young children to develop eating habits [3]. Not only the parent's lifestyle has been shown to play a significant role in the development of a healthy diet in children[4] but also parental education and financial background are associated with children's eating behavior. Similarly, it has been demonstrated that longer TV watching is linked to a higher consumption of salted and sugary snacks in children[6].

Particularly feeding patterns in the first year of life have been studied to a great extent but the primary objective of these studies was to assess socio-demographic determinants of breast- or bottle feeding[7] along with complementary feeding[8], whereas determinants of the child's diet shortly after the weaning and lactation period have been relatively understudied.

During the last decennium, an alternative approach within nutritional research has been developed by using dietary patterns analysis [9-10]. This approach takes into account that dietary components can be highly correlated with each other and represents a more comprehensive reflection of food consumption than assessing single nutrients or foods [9-10]. In addition, several studies have shown in adults that a 'Western' dietary pattern is associated with an increased risk of obesity [11-12] and metabolic disease[13], whereas 'Healthy' dietary patterns are associated with a lower all-cause mortality in adults [13]. So far, dietary patterns in toddlers have not had extensive study yet. However, to identify young children at potential risk for unhealthy eating behavior and in order to develop targeted strategies to improve adequate nutrition during infancy and childhood, knowledge about common dietary patterns in very young children and its determinants will be important. Dieticians and health care workers can provide targeted guidance to promote the development of healthy eating when taking into account the combination of different food items that young children eat. Also, to perform further studies on dietary patterns and various health outcomes in toddlers, knowledge about these determinants to elucidate potential confounders or mediators are necessary. For that reason the aim of this study was to assess whether meaningful dietary patterns may already be observed among toddlers and further identify parental and child determinants of adherence to these dietary patterns.

METHODS

Study population

This study was embedded in a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands and has been described in detail previously[14].

In total, 9778 mothers with a delivery date between April 2002 and January 2006 were enrolled in the study of which 7893 provided consent for follow-up. From 2003 onwards, data collection on nutritional intake at the age of 14 months was implemented in the study. In total, 5088 mothers received a food frequency questionnaire (FFQ) for their child at 14 months of age (figure 2.2.1). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving

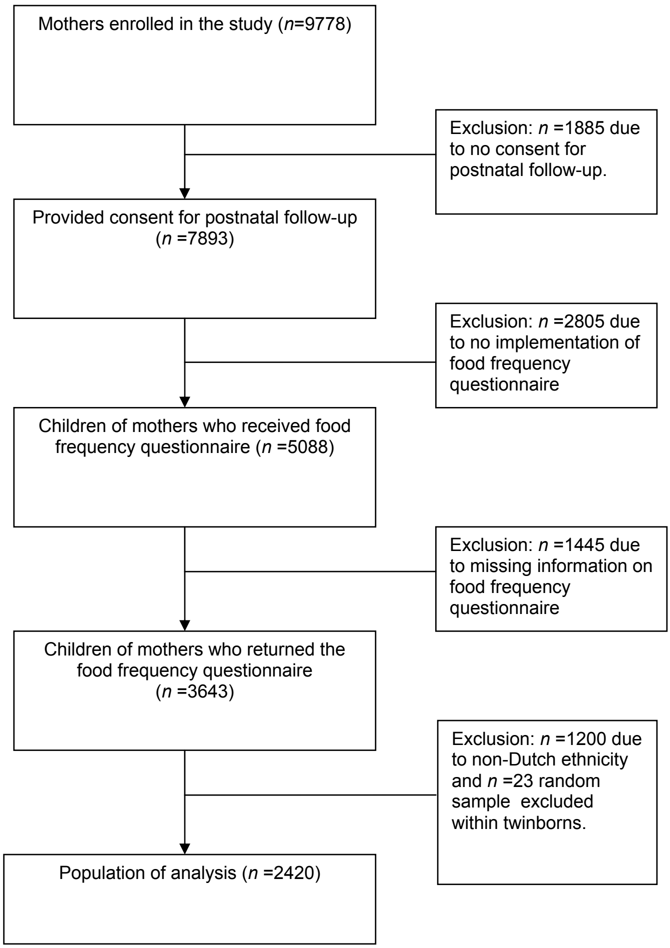

Figure 2.2.1: Flow chart of the study

human subjects were approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam, the Netherlands. Written informed consent was obtained from all subjects.

Dietary assessment of the children

Out of 5088 mothers who received the FFQ to assess the child's nutrition, 3643 (72%) filled in the FFQ and were eligible for analysis (figure 2.2.1). The FFQ was developed in cooperation with the division of Human Nutrition of Wageningen University, the Netherlands and based on an existing validated FFQ developed and described in detail previously [15]. This existing FFQ was modified by only comprising foods frequently consumed during the second year of life according to a National Dutch food consumption survey in 941 Dutch children aged 9 – 18 months [16]. In addition, only foods contributing ≥ 0.1% of the total consumption of energy, protein, fat, carbohydrates, and dietary fiber in the latter survey were incorporated in the FFQ. The final FFQ was validated against three-days 24h-recalls carried out by trained nutritionists in a representative sample of Dutch children aged 14 months living in Rotterdam, the Netherlands. This validation showed the following intra-class correlation coefficients for macronutrients: total energy: 0.4, total protein: 0.7, total fat: 0.4, carbohydrates: 0.4, and dietary fiber: 0.7. The final FFQ consisted of 211 food items and included questions on the frequency of consumption of these food items over the last month, the amount and type of the food item, and preparation methods. Portion sizes in grams a day were estimated using standardized household measures [17]. To calculate average daily nutritional values the Dutch food composition table 2006 was used [18].

A total energy intake of <300 or >3000 kcal/day was considered as implausible values for total energy intake. However sensitivity analyses showed no different results when excluding these values, therefore these children were kept in the final dietary pattern analyses.

Interquartile range of the child's age when the FFQ was filled out ranged from 12 – 14 months with a minimum age of 11.6 months and a maximum age of 33 months. Since sensitivity analyses showed similar results even when excluding outliers (age above the 99th percentile: 20 months of age), children older than 20 months were kept in the final analyses.

Dietary pattern analysis was restricted to children with a Dutch ethnicity (*n*=2420) since the definitions of dietary patterns can be race specific [19] and because this FFQ was only valid in this population. Ethnicity of the child was defined as follows: if both parents were born in the Netherlands, the ethnicity was defined as Dutch; if one of the parents was born in another country than the Netherlands, that country applied; if parents were born in different countries other than the Netherlands, the country of the mother applied[20].

Parental indicators

From obstetric records assessed in mid-wife practices and hospital registries data on maternal age, body mass index and parity at intake were available[14]. Total macronutrient intake during pregnancy was assessed at intake (median 13.5 weeks of gestation, range: 3.4) using a validated semi quantitative food frequency questionnaire of Klipstein-Grobusch et al[21]. Only the energy-providing nutrients: total fat and carbohydrate intake during pregnancy were used as predictors in this study since they are associated with both diet and fat mass in the offspring [22]. Adjustment for total energy intake was performed by a multivariate nutrient density method [23-24]. Other prenatal questionnaires completed by the mother included information on mother's educational level, household income, maternal smoking, maternal alcohol consumption, folic acid supplementation during pregnancy, marital status, co-morbidity (i.e. any history of or medical treatment for diabetes mellitus, hypertension or hypercholesterolemia). Folic acid exposure during early pregnancy was assessed by a questionnaire that included the following question: "Have you taken folic acid, either as a single supplement or as part of multivitamin supplement during the first trimester?" Partners of the mothers received one questionnaire in the prenatal phase which included information on paternal education, age, smoking, and any history or medical treatment for diabetes mellitus, hypertension or hypercholesterolemia.

Level of maternal and paternal education was defined as follows: I) low: no education, elementary or middle school or less than four years of high school, II) Mid: college, associate degree or Bachelor's degree, and III) high: Master's degree [25].

Child indicators

Gender of the child, birth weight and gestational age were available from obstetric records and hospital registries[14]. Timing of introduction of solids in the first year of life was retrospectively assessed by supplementary questions in addition to the FFQ at the age of 14 months. Parents were asked at what age they had first introduced solids in the child's diet in addition to breast-feeding or bottle-feeding which we coded into two categories: before the age of six months or six months and later according to the feeding recommendations of the WHO[26]. After the age of six months all children received complementary feeding. Breast-feeding duration was assessed according to five indicators: ever breast-feeding, cessation of breast-feeding and receiving any breast-feeding at the age of two, six and 12 months. Data on ever breast-feeding was collected from delivery reports and data on breast-feeding cessation or continuation was derived from postnatal questionnaires at two, six and 12 months. Accordingly, breast-feeding was categorized into the following three groups: I) Never breast-feeding, II) Any partial breast-feeding in first 4 months of life, and III) Any full breast-feeding in first 4 months of life. A definition of full breast-feeding was established on the basis of whether the child received breast-feeding without any other formula feeding, milk or solids. Partial breast-feeding indicated children receiving both breast-feeding and formula feeding and/or solids in this period.

The presence of cow's milk allergy was obtained by questionnaires at the age of six and 12 months in which parents were retrospectively asked whether their child had a history of doctor-attended cow's milk allergy. At the age of 12 months, questionnaire data was available on additional care giving of the child by au-pair or daycare. The duration of watching TV during week and weekend days was assessed by questionnaire in the second year of the child's life. According to the American Academy of Pediatrics, the answers categories were divided into <2 hours/day and ≥2 hours/day[27]. Height and weight were measured at the Community Child Health Centres at the age of 14 months. Height was measured in standing position by a Harpenden stadiometer (Holtain Limited, Crymych, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). Weight-for-age and height-for-age *z*-score was calculated from the national reference using the Growth Analyzer program (http://www.growthanalyser.org) [28].

Statistical analysis

All 211 food items from the FFQ data of all Dutch children (*n*=2420) were classified into 21 food groups on the basis of the Dutch food consumption survey among pre-school children[16] and nutrient content (Table S.2.2.1). Subsequently, we applied principal component analysis (PCA) on 21 food groups (assessed in grams a day) of the children to construct overall dietary patterns by explaining the largest proportion of variation in the food group intake [10]. To reduce correlation between the factors, the varimax method by maximizing the sum of the variance of the loading components was used [29]. To reduce bias as a result of multiple testing and to better identify meaningful dietary patterns, only the dietary patterns with an eigenvalue of ≥ 1.5 were extracted. This cut-off was on the basis of the screeplots which indicated a clear break after the second factor (ie. dietary pattern) with an eigenvalue of 1.7[30] (Table 2.2.1). These two dietary patterns accounted for 24.5% of the variability in food consumption within our study population. Accordingly, regression-based factor scores were extracted and used as adherence scores of these dietary patterns.

To assess the association between adherence to the dietary pattern and each of the potential parental and child indicators, we included all indicators simultaneously in a multivariate linear regression model. The addition of these potential indicators was predominantly on the basis of previous studies in toddlers and pre-school children [5, 22, 31-34]. Additionally, folic acid supplementation during pregnancy was added as a proxy for other health behaviours of the mother during pregnancy [35]. In order to improve model fit these analyses were followed by a backward stepwise elimination procedure retaining only the strongest predictors with $p=0.10$ as endpoint.

To diminish potential bias associated with attrition, missing values of subject characteristics (approximately 0.1% - 28%) were multiple imputed ($n=5$ imputed datasets). The multiple imputation was based on the correlation between each variable with missing values with the other subject characteristics as described previously in detail [36-37]. Briefly, the multiple imputation procedure begins with generating 5 copies of the original data set each with missing values replaced by values randomly generated from the predictive distribution on the basis of the correlation between each variable with missing values and the other subject characteristics. The second stage is that the main statistical analyses (i.e. regression analyses) are repeated in each of the 5 imputed data sets. The final effect sizes (beta's or regression coefficients) are estimated by taking the average effect size of the 5 imputed datasets. To calculate the 95% confidence interval, the combined standard errors from the 5 imputed datasets were calculated using Rubin's rules[37] taking into account the uncertainty associated with missing data. Analyses were repeated in the original data and after the multiple imputation procedure. Since we found similar results, the final results in our paper are presented as the pooled regression coefficients (ß) with its 95% Confidence Intervals (95%CI) after the multiple imputation procedure. A p-value <0.05 was considered as statistically significant.

RESULTS

The observed correlations of the food groups for the two constructed dietary patterns are presented in table 2.2.1. Component 1 represented a 'Health conscious' dietary pattern characterized of high intake of fruit, vegetables, legumes and fish. Component 2 represented a 'Western-like' dietary pattern comprising high intakes of savory and snacks, other fats, confectionery and sugar-containing beverages (Table 2.2.1). Adherence score ranged from -1.91 to 7.47 for the 'Health conscious' dietary pattern and from -2.09 to 8.43 for the 'Western-like' dietary pattern. Characteristics of the study population are shown in table 2.2.2.

Determinants of adherence to a 'Western-like' dietary pattern of the child

Multivariate associations between parental and child indicators and adherence to a 'Western-like' dietary pattern are presented in table 2.2.3.

In the multivariate model that included all parental and child indicators kept after the backward selection procedure, low paternal educational background ($p=0.01$), low household income ($p<0.01$), parental smoking ($p<0.01$), high maternal body mass index ($p<0.01$), high intake of carbohydrates ($p=0.01$, after adjustment for total energy intake),

Table 2.2.1: Characteristics of the 'Health conscious' and 'Western-like' dietary pattern in Dutch children aged 14 months (retaining factor loadings > 0.2 or <-0.2)

	Mean intake grams/day	Health conscious dietary pattern	Western-like dietary pattern
Food group		Factor loading	Factor loading
Refined bread and breakfast cereals	15	-	0.60
Whole bread and breakfast cereals	62	-	-
Starchy foods	23	0.62	-
Dairy	626	-	-
Fruit	162	0.32	-
Soy substitutes	4	-	-
Vegetables	52	0.74	-
Potatoes	34	0.61	-
Soups and sauces	9	-	0.23
Savory and snacks	4	-	0.60
Confectionery	28	-	0.72
Vegetable oils	1	0.50	-
Other fats	11	-	0.59
Fish	8	0.22	-
Shellfish	0.3	-	-
Meat	26	0.21	0.27
Eggs	2	-	-
Legumes	4	0.59	-
Sugar-containing beverages	198	-	0.59
Non-sugar containing beverages	56	-	-
Composite dishes	102	-	-
Eigen value**		3.4	1.7
Variance explained (%)		16.3	8.2

Nutrients		Pearson's correlation coefficient	Pearson's correlation coefficient
Energy (kcal)	1275 kcal	0.30*	0.50*
Proteins (en%)	13 en%	0.10*	-0.20*
Fat (en%)	28 en%	-0.10*	0.10*
Saturated fat (en%)	10 en%	-0.10*	0.11*
Monounsaturated fat (en%)	8 en%	-0.02	0.10*
Polyunsaturated fat (en%)	5 en%	0.10*	0.20*
Carbohydrates (en%)	59 en%	0.02	0.03
Mono- and disaccharides (en%)	35 en%	-0.02	0.13*
Polysaccharides (en%)	24 en%	0.23*	-0.13*
Dietary fiber (grams)	18 grams	-	-

PCA was used as an extraction method in which the factor score represent the relative contribution of that food group to the identified dietary pattern. **p*-value<0.05; **The Eigen value was used as indicator of the amount of variation explained by each dietary pattern.

Table 2.2.2: Parental and child characteristics of the study population (*n*= 2420)

		n	%
Mother			
Maternal educational background			
	Low	41	(2%)
	Mid	1703	(70%)
	High	677	(28%)
Household income per month			
	< 2000 euro	301	(12%)
	≥ 2000 euro	2119	(88%)
Marital status			
	Married/living together	2298	(95%)
	No partner	122	(5%)
Smoking during pregnancy		508	(21%)
Alcohol consumption during pregnancy		1420	(59%)
Maternal body mass index in kg/m2 (mean±SD)		24	±4
Folic acid supplementation in early pregnancy		2230	(92%)
Total energy intake during pregnancy in kcal/day (mean±SD)		2150	±488
Total protein during pregnancy in energy percentage/day (mean±SD)		15	±2
Total fat intake during pregnancy in energy percentage/day (mean±SD)		37	±5
Saturated fat intake during pregnancy in energy percentage/day (mean±SD)		13	±2
Monounsaturated fat intake during pregnancy in energy percentage/ day (mean±SD)		13	±2
Polyunsaturated fat intake during pregnancy in energy percentage/day (mean±SD)		7	±2
Total carbohydrate intake during pregnancy in energy percentage/day (mean±SD)		48	±6
Total dietary fiber intake during pregnancy in grams/MJ (mean±SD)		3	±1
Maternal age in years (mean±SD)		32	±4.2
Nulliparous		1498	(62%)
Maternal history of diabetes mellitus		128	(5%)
Maternal history of hypertension		172	(7%)
Maternal history of hypercholesterolemia		388	(16%)
Father			
Paternal educational background			
	Low	87	(4%)
	Mid	828	(34%)
	High	1505	(62%)
Paternal smoking		962	(40%)
Maternal body mass index in kg/m2 (mean±SD)		25	±3
Paternal age in years (mean±SD)		34	±5
Paternal history of diabetes mellitus		183	(8%)
Paternal history of hypertension		93	(4%)
Paternal history of hypercholesterolemia		166	(7%)
Child			
Age of food assessment in months (mean±SD)		14	±2

Table 2.2.2: Parental and child characteristics of the study population *Continued* (*n*= 2420)

	n	%
Male gender	1201	(50%)
Birthweight in grams (mean±SD)	3503	±570
Gestational age in weeks (mean±SD)	39.9	±1.7
Never breast-feeding	302	(13%)
Partial breast-feeding until 4 months of age	1439	(59%)
Full breast-feeding until 4 months of age	679	(28%)
Timing of introduction of solids ≤ 6 months of age	1628	(67%)
History of food allergy in first year of life	152	(6%)
Daycare of au-pair in first year of life >16 hrs/week	1640	(68%)
Weight for age *z*-score (mean±SD)	-0.1	±0.9
Height for age *z*-score (mean±SD)	-0.2	±0.9
Watching TV ≥ 2 hrs a day	335	(14%)

Table 2.2.3: Predictors of adherence to a 'Western-like' dietary pattern of children aged 14 months (*n*=2420)

	Multivariate regression (fully adjusted model) ß (95%CI)	*p*- value	Multivariate regression model after stepwise backward selection* ß (95%CI)	*p*- value
Maternal indicators				
Maternal educational background at intake				
Mid vs. High (reference)	0.05 (-0.06, 0.15)	0.38	-	
Low vs High (reference)	0.17 (-0.29, 0.64)	0.47	-	
Household Income at intake <2000 euro vs ≥2000 euro (reference)	0.21 (0.05, 0.38)	0.01	0.19 (0.07, 0.32)	<0.01
Marital status at intake Living alone vs Married/living together (reference)	-0.17 (-0.42,0.09)	0.21	-	
Maternal smoking during pregnancy Yes vs. No (reference)	0.16 (0.02, 0.29)	0.02	0.16 (0.04, 0.27)	<0.01
Maternal alcohol consumption during pregnancy Yes vs. No (reference)	-0.03 (-0.14, 0.07)	0.53	-	
Maternal Body Mass Index before pregnancy (kg/m2)	0.02 (0.01, 0.03)	0.01	0.02 (0.01,0.03)	<0.01
Energy intake during pregnancy (MJ/day)	-0.03 (-0.07, 0.001)	0.06	-0.02 (-0.05, 0.01)	0.10
Fat intake during pregnancy (energy percentage/day)	0.02 (-0.01, 0.05)	0.20	-	
Carbohydrate intake during pregnancy (energy percentage/day)	0.02 (-0.01, 0.05)	0.09	0.01 (0.002, 0.02)	0.01
Dietary fiber intake during pregnancy (grams/MJ/day)	-0.15 (-0.22, -0.08)	<0.01	-0.15 (-0.21, -0.09)	<0.01
Folic acid supplementation during pregnancy Yes vs No (reference)	-0.02 (-0.26, 0.23)	0.90	-	
Maternal age at intake (years)	-0.03 (-0.04,-0.01)	<0.01	-0.03 (-0.04, -0.01)	<0.01
Parity at intake (0-6)	0.22 (0.14,0.30)	<0.01	0.22 (0.16, 0.29)	<0.01

Table 2.2.3: Predictors of adherence to a 'Western-like' dietary pattern of children aged 14 months *Continued* (*n*=2420)

	Multivariate regression (fully adjusted model) ß (95%CI)	*p*- value	Multivariate regression model after stepwise backward selection* ß (95%CI)	*p*- value
Maternal history of diabetes, hypertension or hypercholesterolemia at intake Yes vs No (reference)	0.06 (-0.24, 0.35)	0.69	-	
paternal indicators				
Paternal educational background at intake				
Mid vs. High (reference)	0.11 (-0.01, 0.23)	0.07	0.16 (0.07, 0.25)	<0.01
Low vs High (reference)	0.23 (-0.11, 0.56)	0.18	0.41 (0.13, 0.70)	<0.01
Paternal Body Mass Index at intake (kg/m2)	-0.001 (-0.02, 0.01)	0.88	-	
Paternal history of diabetes, hypertension or hypercholesterolemia at intake Yes vs No (reference)	-0.06 (-0.27, 0.15)	0.57	-	
Paternal age at intake (years)	-0.01 (-0.02, 0.01)	0.29	-0.01 (-0.02, -0.003)	0.01
Paternal smoking at intake Yes vs No (reference)	0.17 (0.06, 0.27)	<0.01	0.12 (0.03, 0.21)	<0.01
Child indicators				
Age of food assessment (months)	0.12 (0.10, 0.14)	<0.01	0.10 (0.08,0.12)	<0.01
Gender female vs. male (reference)	-0.18 (-0.27, -0.08)	<0.01	-0.19 (-0.26, -0.11)	<0.01
Birth weight (SDS)	0.03 (-0.03, 0.08)	0.29	-	
Breast-feeding in first year of life				
Any partial breast-feeding in first 4 months vs Never breast-feeding (reference)	-0.08 (-0.23, 0.07)	0.32	-	
Any full breast-feeding in first 4 months vs Never breast-feeding (reference)	-0.11 (-0.28, 0.08)	0.23	-	
Timing of introduction of solids in first year of life ≥ 6 months vs < 6 months (reference)	-0.13 (-0.23, -0.03)	0.02	-0.14 (-0.22, -0.05)	<0.01
History of food allergy in first year of life Yes vs. no (reference)	-0.17 (-0.39, 0.06)	0.14	-0.16 (-0.33, 0.01)	0.07
Daycare or au-pair in first year of life > vs. ≤ 16 hrs/week (reference)	-0.06 (-0.18, 0.06)	0.33	-	
Weight for age at 14 months (*z*-score)	0.01 (-0.07, 0.08)	0.82	-	
Height for age at 14 months (*z*-score)	0.02 (-0.05, 0.09)	0.55	0.04 (-0.01, 0.08)	0.09
Watching TV in second year of life ≥2 hours vs. <2 hours per day (reference)	0.32 (0.16, 0.47)	<0.01	0.33 (0.20, 0.46)	<0.01

ß, regression coefficient indicating difference in factor score of a 'Western-like' diet compared to reference group or per unit of a continuous variable; CI, confidence interval.* After using the backward selection procedure with p=0.10 as endpoint.

and multiparity ($p<0.01$) were significantly associated with a higher adherence to a 'Western-like' dietary pattern of the child. High dietary fiber intake during pregnancy ($p<0.01$, after adjustment for total energy intake), and high parental age ($p\leq0.01$) were inversely associated with adherence to a 'Western-like' dietary pattern of the child.

Adherence to a 'Western-like' dietary pattern of the child was significantly associated with a higher age of food assessment ($p<0.01$), and more TV watching in the second year of the child's life ($p<0.01$). Children who where female ($p<0.01$), and children who received solids after the recommended age of six months ($p<0.01$) had a lower adherence score on a 'Western-like' dietary pattern (Table 2.2.3).

Determinants of adherence to a 'Health conscious' dietary pattern of the child

Predictors of adherence to a 'Health conscious' dietary pattern of the child are presented in table 2.2.4.

In multiple regression analyses, children of mothers who consumed alcohol consumption during pregnancy ($p=0.04$), and whose mothers had a history of co-morbidity ($p<0.05$), and children who were female ($p=0.03$), had a lower adherence score on a 'Health conscious' dietary pattern. Folic acid supplementation during pregnancy ($p=0.02$), high dietary fiber intake of the mother ($p<0.01$, after adjustment for total energy intake), and single parenthood ($p<0.01$) were positively associated with adherence to a 'Health conscious' dietary pattern of the child (table 2.2.4). Children who received any full breast-feeding in the first 4 months of life had a higher score on a 'Health conscious' dietary pattern ($p<0.05$, Table 2.2.4).

DISCUSSION

The results from this prospective cohort study among children aged 14 months have several points with potential health implications that need to be emphasized.

First, a 'Western-like' dietary pattern was already identifiable in the second year of the child's life. We found a 'Western-like' dietary pattern that was characterized by high consumption of refined grains, savory and snacks, confectionery, other fats and sugar-containing beverages. An increase in the consumption of sugar-containing beverages is considered to be related to poor diet quality[38]. Also, consumption of sugar-containing beverages has shown to be correlated with other unhealthy food choices such as savory, snacks and sweets [39]. From the Bogalusa Heart Study it is known that mean intake of sugar-containing beverages, snacks and sweets increases from childhood until adulthood with an overall decrease in diet quality over the years [40]. Indeed we found that the older the child was at moment of food assessment the more likely it adhered

Table 2.2.4: Predictors of adherence to a 'Health conscious'dietary pattern of children aged 14 months (*n*=2420)

	Multivariate regression (fully adjusted model) ß (95%CI)	*p*- value	Multivariate regression model after stepwise backward selection* ß (95%CI)	*p*- value
Maternal indicators				
Maternal educational background at intake				
Mid vs. High (reference)	-0.02 (-0.14, 0.10)	0.74	-	
Low vs High (reference)	-0.15 (-0.62, 0.33)	0.55	-	
Household Income at intake <2000 euro vs ≥2000 euro (reference)	0.02 (-0.16, 0.20)	0.84	-	
Marital status at intake Living alone vs Married/living together (reference)	0.22 (-0.06, 0.51)	0.12	0.30 (0.07, 0.52)	<0.01
Maternal smoking during pregnancy Yes vs. No (reference)	0.06 (-0.09, 0.20)	0.47	-	
Maternal alcohol consumption during pregnancy Yes vs. No (reference)	-0.08 (-0.19, 0.04)	0.18	-0.10 (-0.20, -0.01)	0.04
Maternal Body Mass Index before pregnancy (kg/m2)	-0.01 (-0.02, 0.01)	0.33	-	
Energy intake during pregnancy (MJ/day)	0.06 (0.02, 0.11)	<0.01	0.07 (0.03, 0.11)	<0.01
Fat intake during pregnancy (energy percentage/day)	-0.02 (-0.05, 0.004)	0.10	-	
Carbohydrate intake during pregnancy (energy percentage/day)	-0.02 (-0.04, 0.004)	0.10	-	
Dietary fiber intake during pregnancy (grams/MJ/day)	0.13 (0.05,0.20)	<0.01	0.14 (0.07, 0.21)	<0.01
Folic acid supplementation during pregnancy Yes vs No (reference)	0.26 (0.05, 0.47)	0.01	0.21 (0.03, 0.39)	0.02
Maternal age at intake (years)	-0.01 (-0.03, 0.01)	0.35	-	
Parity at intake (0-6)	-0.03 (-0.11, 0.06)	0.56	-	
Maternal history of diabetes, hypertension or hypercholesterolemia at intake Yes vs No (reference)	-0.13 (-0.45, 0.19)	0.43	-0.29 (-0.57, -0.01)	<0.05
paternal indicators				
Paternal educational background at intake				
Mid vs. High (reference)	0.15 (0.02, 0.28)	0.02	0.16 (0.03, 0.30)	0.02
Low vs High (reference)	0.30 (-0.06, 0.66)	0.10	-	
Paternal Body Mass Index at intake (kg/m2)	-0.001 (-0.02, 0.02)	0.91	-	
Paternal history of diabetes, hypertension or hypercholesterolemia at intake Yes vs No (reference)	-0.14 (-0.36, 0.07)	0.18	-	

Table 2.2.4: Predictors of adherence to a 'Health conscious'dietary pattern of children aged 14 months *Continued* (*n*=2420)

	Multivariate regression (fully adjusted model) ß (95%CI)	*p*- value	Multivariate regression model after stepwise backward selection* ß (95%CI)	*p*- value
Paternal age at intake (years)	0.001 (-0.01, 0.02)	0.84	-	
Paternal smoking at intake Yes vs No (reference)	0.06 (-0.06, 0.18)	0.33	-	
Child indicators				
Age of food assessment (months)	-0.01 (-0.03, 0.02)	0.62	-	
Gender female vs. male (reference)	-0.12 (-0.22, -0.01)	0.03	-0.11 (-0.20, -0.01)	0.03
Birth weight (SDS)	0.03 (-0.03, 0.09)	0.35	-	
Breast-feeding in first year of life				
Any partial breast-feeding in first 4 months vs Never breast-feeding (reference)	<0.05 (-0.22, 0.12)	0.59	-	
Any full breast-feeding in first 4 months vs Never breast-feeding (reference)	0.22 (0.02, 0.41)	0.04	0.18 (0.003, 0.36)	<0.05
Timing of introduction of solids in first year of life ≥ 6 months vs < 6 months (reference)	-0.07 (-0.18, 0.05)	0.25	-	
History of food allergy in first year of life Yes vs. no (reference)	0.13 (-0.12, 0.37)	0.31	-	
Daycare or au-pair in first year of life > vs. ≤ 16 hrs/week (reference)	-0.01 (-0.16, 0.15)	0.95	-	
Weight for age at 14 months (*z*-score)	-0.002 (-0.09, 0.09)	0.70	-	
Height for age at 14 months (*z*-score)	0.03 (-0.05, 0.11)	0.51	-	
Watching TV in second year of life ≥2 hours vs. <2 hours per day (reference)	-0.07 (-0.23, 0.10)	0.42	-	

ß, regression coefficient indicating difference in factor score of a 'Health conscious' diet compared to reference group or per unit of a continuous variable; CI, confidence interval.* After using the backward selection procedure with *p*=0.10 as endpoint.

to a 'Western-like' dietary pattern. If we assume that adherence to a 'Western-like' diet may track from pre-school age to child- and adulthood onwards, interventions to promote a healthy diet should start early in the child's life. [41]

Second, we demonstrated that paternal education, household income, maternal and paternal smoking, maternal BMI, parental age, parity, early solid introduction and watching TV are important predictors of high adherence to a 'Western-like' dietary pattern of the child. Maternal BMI has previously reported to be associated with sweets and sugar intake in 1-year olds [32]. Similarly, maternal smoking during pregnancy and viewing TV of more than 2 hours a day showed a significant positive association with unhealthy eating behavior in other studies among children [5, 31, 42]. In infancy, early solid introduction has found to be related to poor dietary quality in the first year of life [43]. The Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) study showed that the number of older siblings is positively associated with adherence to a 'Junk' and 'Snack' dietary pattern[5] which is in line with our results since we found a positive correlation between parity and adherence to a 'Western-like' dietary pattern of the child. Also, the ALSPAC study found that boys were less likely to adhere to a 'Healthy' and 'Traditional' dietary pattern [5]. In our study, we found that girls had lower scores on the 'Western-like' diet along with a 'Health conscious' dietary pattern suggesting differences in food preferences between boys and girls. Low paternal education and low household income was associated with the 'Western-like' dietary pattern which is in accordance with other studies indicating social inequalities in unhealthy eating patterns[3].

We found that high carbohydrate, and low dietary fiber intake of the mother during pregnancy was significantly associated with adherence to a 'Western-like' dietary pattern of the child. An earlier study already showed a strong correlation between maternal macronutrient intake during pregnancy and offspring macronutrient intake [22]. Another study demonstrated that diet quality of thee-years old children is highly associated with maternal diet quality [44]. The latter association appeared to be independent of other maternal characteristics as BMI and educational level [44] which is in line with our study results since the inverse association between maternal dietary fiber intake and a 'Western-like' dietary pattern of the child was still present after adjusting for maternal BMI and socioeconomic background.

Although we did not find a significant association with weight and height *z*-score at 14 months and the dietary patterns, some of the determinants associated with a 'Western-like' dietary pattern have found to be risk-factors of overweight such as parental smoking, socioeconomic background, maternal BMI, maternal diet, early solid introduction, and watching TV [1, 45]. Taking this all into consideration, our study results imply that children with a 'Western-like' dietary pattern might reflect a vulnerable group of children who may be at risk for developing overweight-prone behaviors in later life. Hence, targeting the parents with a low socioeconomic background and unfavorable lifestyle factors may be valuable at a very young age of the child to improve the child's eating habits.

Last, we identified a 'Health conscious' dietary pattern within our study population, which was characterized of high consumption of pasta and rice, potatoes, legumes, fruit, vegetables, fish and vegetable oils. In schoolchildren, high adherence to a dietary pattern characterized by fish, legumes, fruits and leafy vegetables has been found to be associated with a better diet quality [46]. However, not much is known about the determinants and effects of a 'Health conscious' dietary pattern in children. Maternal co-morbidity was inversely associated with a 'Health-conscious dietary pattern'. Similarly, we found that other behavior associated with health awareness of the mother such as folic acid supplementation, high fiber consumption, no alcohol consumption during pregnancy and full breast-feeding were predictors of adherence to a 'Health conscious' dietary pattern of the child. Strikingly, we found that children of fathers with a mid educational level had higher scores on the 'Health conscious' dietary pattern relative to children of fathers with high educational level suggesting that a socioeconomic gradient in healthy eating habits may be less straightforward than in unhealthy eating patterns early in life.

In contrast, we also found that children of mothers living alone were more likely to adhere to a 'Health conscious' dietary pattern. We believe that this might have something to do with other socio-demographic factors such as whether both parents are involved in upbringing or which person is responsible for preparation of the meals. In the ALSPAC study it was demonstrated that cooking performed by a person other than the mother was negatively correlated with a 'Healthy' dietary pattern in three-years olds[5]. In addition, when mother lives together with a partner, unhealthy eating habits of the partner might be more incorporated with the child's diet when the partner is involved in raising the child or the preparation of meals. Unfortunately, we did not have data on paternal dietary habits to further clarify these results.

Several study limitations need to be taken into account to appreciate the results. Although we used the varimax rotation to decrease correlation between the two identified patterns, they still might be intercorrelated to some extent [10, 19]. In addition, meat consumption was a correlated factor in both the 'Western-like' and 'Health conscious' dietary pattern in our study group. The identification of dietary patterns in very young children is challenging because dietary patterns may not be strongly formed yet. Also, it cannot be assumed that dietary patterns are perfectly stable throughout early and mid childhood as previously demonstrated in ALSPAC[47] and thus further longitudinal measurements are needed to assess whether unfavorable dietary patterns track during child- and adulthood.

Dietary pattern analysis involves several decisions such as in the division of food items to food groups, the selected method to define these patterns, and the labelling of these components[10], which may have an influence on the final content of the dietary pattern in our study.

The amount of variance (24.5%) explained by the dietary patterns is small but is similar when compared to previous studies on dietary patterns in young children[5, 43]. Nevertheless, this may have consequences on the generalizability of our results in other populations. Therefore we encourage further study on dietary patterns at this very young age in other populations.

Another limitation of the study is that the FFQ that was used for the collection of maternal nutrition during pregnancy was only validated in an older Dutch population in Rotterdam, the Netherlands [21]. Although we did validate the FFQ of the children against 3d-24h recalls, it has been shown that 24h recalls still underestimate nutrient intake [48]. Unfortunately, we were not able to validate the FFQ against the doubly labelled water method which is considered to be the gold standard to validate total energy intake [48].

Missing data can be an important limitation in cohort studies. We aimed to reduce attrition bias as much as possible. For that reason we used a multiple imputation procedure, which is a very appropriate method do deal with missing data because it requires the least assumptions and exhibit bias when missing data is not completely at random [36]. As a result, the 95% confidence intervals in our study reflect the uncertainty associated with the missing values.

We did not have data on the child's preferences, social context of meal consumption and parents' beliefs about nutrition and health. We expect that these factors can be important predictors of child's adherence to a healthy or unhealthy eating pattern[3]. Therefore, an in-depth study aiming to explore the influence of other social determinants can be worthwhile.

Conclusion

In conclusion, this study demonstrated that a 'Western-like' dietary pattern can already be present in the second year of the child's life and adherence to this diet is associated with other risk-factors for obesity. Targeting parents of these children for health promotion might be useful. Future studies should clarify whether this dietary pattern track during child- and adulthood, and which other social determinants predict adherence to a 'Health conscious' dietary pattern in childhood. Finally, the analyses of this study can form a basis for future studies regarding dietary patterns and various health outcomes in this cohort.

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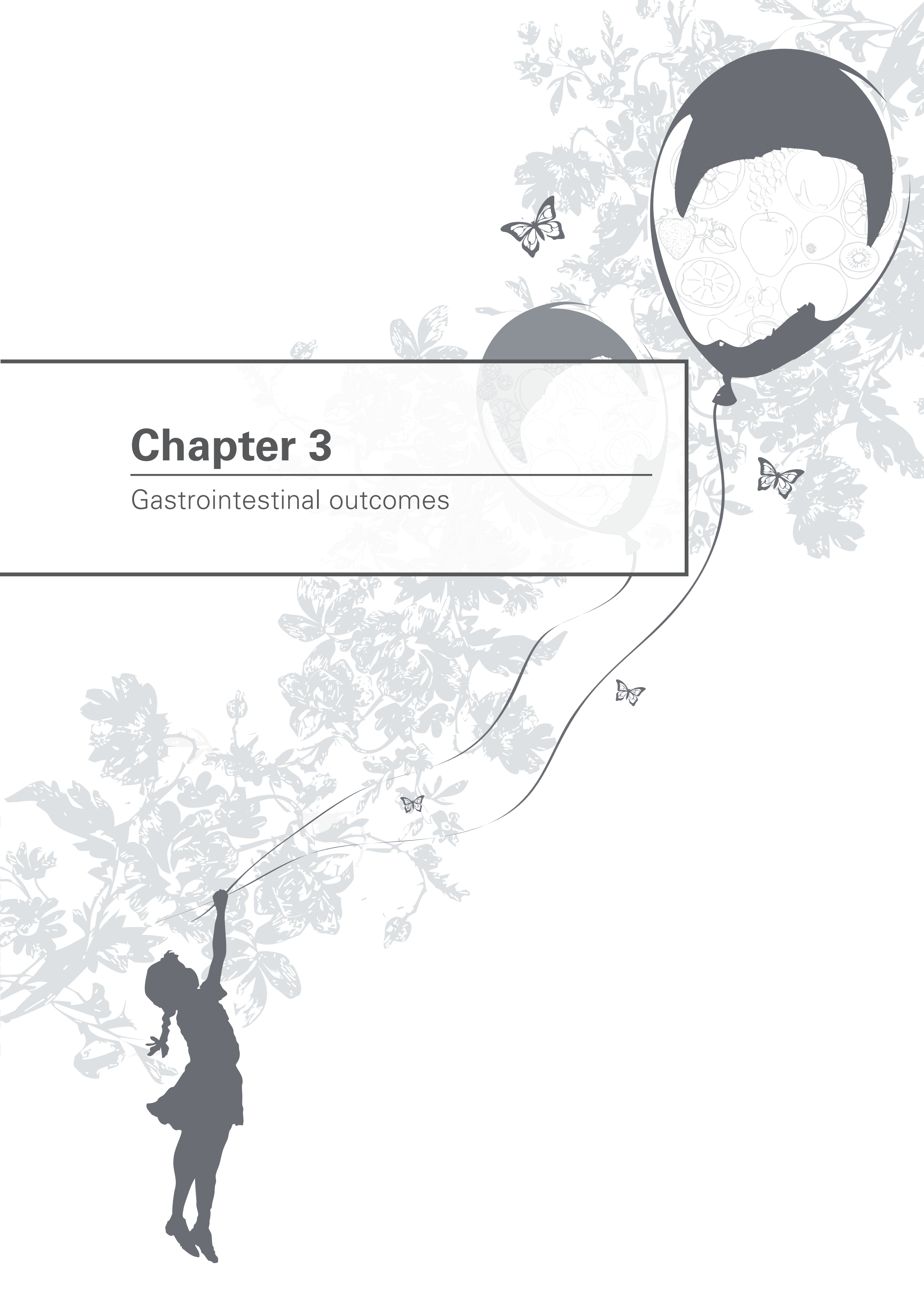
SUPPLEMENTARY MATERIAL

Table S2.2.1: Division of food items into food groups

Food group	Included food items
Refined bread or breakfast cereals	Waffles, rusk, crackers, currant bread, currant bun, white bread or baguette, croissant, cornflakes, low-fiber breakfast cereals
Whole bread or breakfast cereals	Brown or whole-bran bread, brown or whole-bran baguette, oatmeal, muesli, multigrain breakfast cereals
Starchy foods	Pasta, rice, couscous, bulgur.
Dairy	Full creamed, semi-skimmed or skimmed milk, full creamed, semi-skimmed or skimmed flavored milk, full creamed, semi-skimmed or skimmed yoghurt, yoghurt drinks, chocolate-flavored milk, full creamed, semi-skimmed or skimmed custard, milk pudding, mousse, porridge, full creamed, semi-skimmed or skimmed fromage frais, cream, infant milk feeding, cheese.
Fruit	Fruits and fruit compote (excluding fruit drink)
Soy substitutes	Soy milk, soy dessert, flavoured soy milk, soy-based meat substitutes.
Vegetables	Vegetables (including raw, cooked and baked vegetables).
Potatoes	Potatoes (excluding fried or baked potatoes)
Soups and sauces	Soup, mayonnaise (including half fat mayonnaise), salad cream, peanut sauce, ketchup and other sauces added to meals or snacks.
Savory and snacks	Chips, toasts with cheese or pâté, sausages rolls, spring rolls, meat rolls, meat croquettes, sateh, peanuts and nuts, burgers, chicken nuggets, fried chips or fried potatoes (i.e. French fries).
Confectionery	Dutch spiced honey cake, chocolate pasta, chocolate confetti, sweet sandwich fillings, ice cream, (added) sugar, cakes, cookies, biscuits, chocolates, pastry, pancakes, candy's.
Vegetable oils	Olive oil and other oils
Other fats	Full fat and low fat margarines, butter and cooking fats.
Fish	Fish
Shellfish	Shellfish
Meat	All processed and non-processed meat (except meat-containing snacks in between which are included in 'savory and snacks' food group)
Eggs	Eggs (baked or boiled egg)
Legumes	Legumes (i.e. white or brown beans, kidney beans, lentils, chick peas)
Sugar-containing beverages	Soft drinks, fruit drinks, lemonade.
Non-sugar containing beverages	Tea without sugar, water, diet soft drinks (i.e. without sugar)
Composite dishes	Ready-to-eat infant meals and ready-to-eat cooled or frozen meals.

Chapter 3

Gastrointestinal outcomes



Chapter 3.1

Celiac disease auto antibodies and fetal growth

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ABSTRACT

Celiac disease in pregnant women has been associated with growth of the fetus, but little is known about how the level of celiac disease affects fetal growth or birth outcomes. We assessed the associations between levels of antibodies against tissue transglutaminase (anti-tTG, a marker of celiac disease) and fetal growth and birth outcomes for pregnant women. We performed a population-based prospective birth cohort study of 7046 pregnant women. Serum samples were collected during the second trimester of pregnancy and analyzed for levels of anti-tTG. Based on level, women were categorized into 3 groups: negative anti-tTG (≤0.79 U/ml, *n*=6702), intermediate anti-tTG (0.8 to ≤6 U/ml, *n*=308), or positive anti-TG (>6 U/ml, *n*=36). Data on fetal growth and birth outcomes were determined using ultrasound measurements and medical records.

Fetuses of women in the positive anti-tTG group weighed 16g less than those of women in the negative anti-tTG (95% confidence interval [CI], –32 to –1 g) during the second trimester and 74 g less (95% CI, –140 to –8 g) during the third trimester. Infants of women in the intermediate and positive anti-tTG groups weighed 53 g (95% CI, –106 to –1 g) and 159 g (95% CI, –316 to –1 g) less at birth, respectively, than those of women in the negative anti-tTG group. The reduction in birth size among in the intermediate anti-tTG group occurred most frequently among women that carried human leukocyte antigens (HLA) DQ2 or DQ8.

In conclusion, levels of anti-tTG in pregnant women are associated with fetal growth. Fetal growth was reduced to the greatest extent (by 74 g in the 3rd trimester) in women with the highest levels of anti-tTG (>6 U/ml). Birthweight was also reduced in women with intermediate levels of anti-tTG (0.8 to ≤6 U/ml), and further reduced in those with HLAs DQ2 and DQ8.

INTRODUCTION

Celiac disease (CD) is characterized by an immune response to gluten resulting in histological alterations of the small intestine[1]. In the Netherlands, the prevalence of recognized CD is approximately 0.02% in adults. However, the prevalence of unrecognized CD is thought to be even more common in the population (prevalence of ±0.5%)[2]. Screening studies in other European countries and USA even revealed that the total prevalence of CD can be around 1%[3-5].

It has been seen in patients diagnosed with CD that the immune response triggered by gluten leads to the production of auto antibodies; such as those against tissue transglutaminase (anti-tTG), which correlates well with severe mucosal damage of the small bowel[6].

Several observational studies have suggested that CD is associated with different pregnancy outcomes[7-10]. Some studies demonstrated that CD is associated with intrauterine growth restriction[11-13], low birth weight[13-15], and pre-term delivery.[14-16] Other studies showed no relation between CD and these birth outcomes.[12, 17-19]. The proposed link between CD and birth outcomes can be explained by nutrient deficiencies; including iron, folate and vitamin B12 which are associated with both CD-induced malabsorption[20] and low birth weight[21] (figure 3.1.1). Also, studies suggest that the role of anti-tTG levels, specifically inflammatory or immunological, may impair placental development and function, which leads to fetal growth restriction.[22-23] (figure 3.1.1). However, most population-based studies on CD and birth outcomes, studied patients

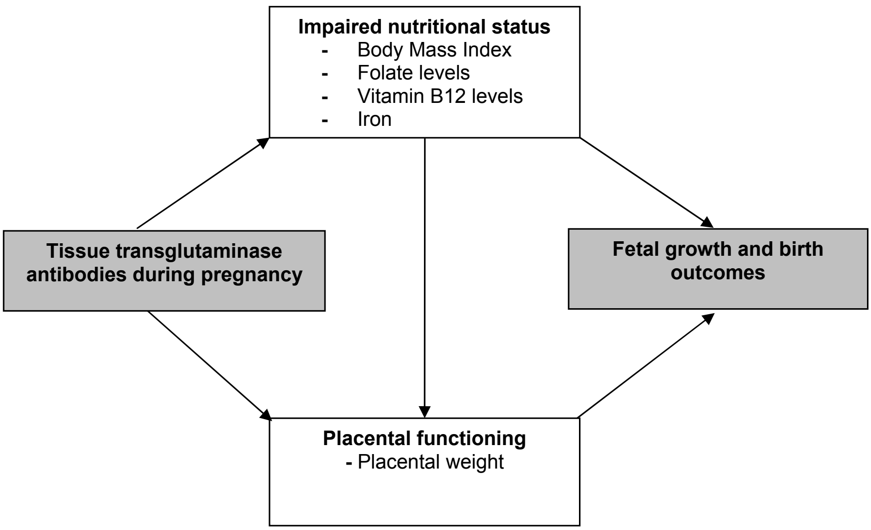

Figure 3.1.1: Simplified conceptual framework for the association between anti-tTG and fetal growth

CHAPTER 3.1

with the diagnosis of CD according to the International Classification of Diseases, and did not take in account the actual levels of anti-tTG that reflect the degree of mucosal damage associated with undiagnosed CD or limited compliance to a gluten free diet[6]. Therefore, the objective of this study was to asses whether maternal celiac disease auto-antibodies during pregnancy, as measured by anti-tTG, are associated with fetal growth, and birth outcomes.

METHODS

Study design

This study was embedded within the framework of the Generation R study, a population-based prospective cohort study from fetal life onwards and has been described in detail previously.[24-25] A total of 8880 mothers with a delivery date between April 2002 and January 2006, were included in this study (figure 3.1.2). Ethic approval for the study was

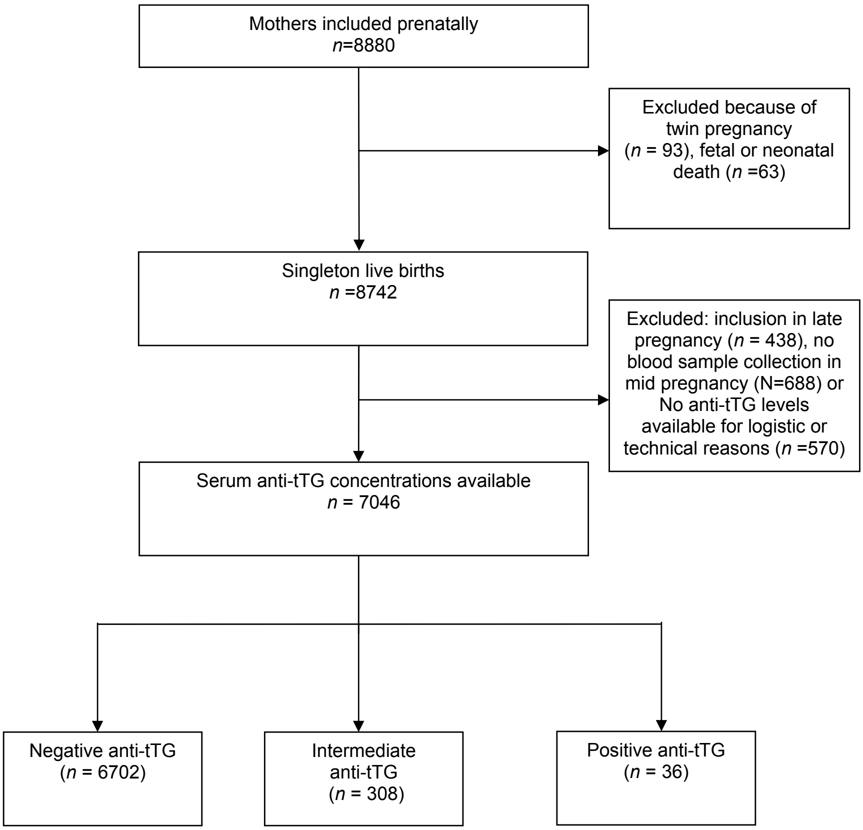

Figure 3.1.2: Flow chart of the study

obtained from the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all participants. Generation R provided data for statistical analyses anonymously.

Celiac disease auto-antibodies

In the second trimester of pregnancy (mean±SD: 20.6±1.2 weeks) venous blood serum samples were drawn and stored at room temperature before being transported to the regional laboratory for storage at -80 °C[25]. In 2010, the serum samples were all transported to the Department of Immunology, Erasmus MC - University Medical Center Rotterdam, the Netherlands, to measure anti-tissue transglutaminase IgA (anti-tTG) concentrations. Concentrations of anti-tTG were assessed using a fluorescence enzyme immunoassay (ELiA Celikey® IgA, Phadia Immunocap 250, Phadia AB, Uppsala, Sweden). The intra- and inter-assay coefficient of variation (CV) was below 10% and 15% respectively. Median anti-tTG concentration of the study population was 0.23 U/ml, varying from 0 U/ml to 4565 U/ml. Serum anti-tTG was available in 80% of the mothers. According to the laboratory, the cut of in clinical practice of 6 U/ml was used to discriminate patients with CD from healthy subjects. The 95th percentile of anti-tTG in our study population (0.79 U/ml) was defined as a cut-off point for intermediate levels of anti-tTG. Thus, levels of anti-tTG were categorized into three groups: 0= Negative anti-tTG (≤0.79 U/ml), 1= Intermediate anti-tTG (between 0.8 and ≤6 U/ml) and 2=Positive TGA (>6 U/ml) (figure 3.1.2).

Detection of Human Leukocyte Antigen Risk Alleles

A tag single nucleotide polymorphism (SNP) approach was used to capture whether mothers carried the HLA-DQ risk type of DQ2 or DQ8 as described in detail previously by Monsuur et al.[26] Mothers were genotyped for these SNPs[26] for HLA-DQ2 (rs2187668, rs2395182, rs4713586 and rs7775228) and DQ8 (rs7454108)[26] using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was performed using the GeneAmp® PCR system 9600 (with a primary incubation at 95°C for 15 minutes, followed by 40 cycles of 94°C (15 seconds) and 60°C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotype and allele frequencies were in Hardy Weinberg equilibrium (rs2187668, p=0.12; rs2395182, p=0.64; rs4713586, p=0.47; rs7775228, p=0.86; rs7454108, p=0.82).

Outcomes

Fetal ultrasound measurements and medical records from the midwives or obstetricians were used to obtain information regarding fetal growth and birth outcomes including birth and placental weight. Ultrasound measurements were used to establish gestational age in the first trimester (mean±SD: 13.6±1.9 weeks) and to assess estimated fetal weight in the second trimester (mean±SD: 20.7±1.2 weeks) and third trimester (mean±SD: 30.4±1.2 weeks). Estimated fetal weight was calculated using the formula of Hadlock[27]. The ultrasound measurements have been found to be reproducible in our cohort as described in detail previously[28]. Subsequently, longitudinal growth curves and gestational-age-adjusted standard deviation (*z*-)scores were constructed based on reference growth curves from the study population with a mean of zero, as described before[29]. Early-pregnancy ultrasound measurements were primarily used to assess gestational age and therefore were not included in the estimated fetal growth measurements.

Small for gestational age (SGA) was defined as a *z*-score < -2.0 (< 2.3th percentile)[30] at birth on the basis of the growth curves derived from our cohort[28]. Low birth weight was defined as birth weight below 2500 grams. Spontaneous prematurity was defined as gestational age at birth below 37.0 weeks. Placental weight was measured in grams. Placental index was calculated by placental weight divided by birth weight.

Covariates

Data on maternal age, educational level, household income, parity, smoking habits, periconception folic acid use, alcohol consumption, gastrointestinal disease, and other maternal co-morbidity was available from prenatal questionnaires. Ethnicity was defined as Western or non-Western according to the following classification: if both parents of the mother were born in a Western country, the ethnicity of the mother was defined as Western; if one of the parents of the mother was born in a non-Western country, that country applied[31]. Maternal social economic background was defined according to educational level as follows; low: no education, primary school or less than 3 years of secondary school, mid: more than 3 years of secondary school, higher vocational training or bachelor's degree, and high: academic education[32].

At enrolment (mean±SD: 15.7±4.4 weeks) maternal height and weight were measured to calculate body mass index (BMI, kg / m^2). Information on fetal gender, and gestational age at birth were obtained from midwifes, and obstetricians. Folate, vitamin B12, and hemoglobin (Hb), concentrations and mean corpus volume (MCV) were assessed from blood plasma samples (EDTA) collected during early pregnancy (mean±SD: 13.5±2.0 weeks of gestation). After sampling, these were transported to the regional laboratory for storage at -80 °C (STAR-MDC, regional laboratory Rotterdam, the Netherlands). In 2008, all EDTA samples were transported to the Department of Clinical Chemistry, Erasmus Medical Center, Rotterdam, the Netherlands. Subsequently, plasma folate and vitamin B12 concentrations were analyzed using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands).

The between-run coefficients of variation for plasma folate were 8.9% at 5.6 nmol / L, 2.5% at 16.6 nmol / L, and 1.5% at 33.6 nmol / L; the coefficients of variation for vitamin B12 were 3.6% at 148 pmol / L, 2.7% at 295 pmol / L, and 3.1% at 590 pmol / L. Biomarker concentrations in early pregnancy were available in 78% of the study population.

Statistical analysis

Statistical analyses were carried out by using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL) and the Statistical Analysis System 9·2 (SAS Institute Inc, Cary, NS).

The best fitted model for assessing anti-tTG continuously was by using fractional polynomials[33] (Figure S3.1.1). However, since the main effects were found for intermediate and positive anti-tTG levels, further analyses were performed after treating anti-tTG levels as categorical variables for clinical and interpretation purposes (three groups: negative, intermediate and positive anti-tTG). First, independent Student's t-test and Chi-square tests were used to test for differences in characteristics between the three groups of anti-tTG levels. Second, linear regression analysis was performed to assess whether intermediate and positive anti-tTG levels were associated with fetal growth, birth weight (grams and *z*-score), and placental weight (grams). To longitudinally assess non-linear associations between anti-tTG and fetal growth *z*-scores, random coefficient analysis[34] with an unstructured covariance structure using restricted cubic splines was performed. The interior knots (n=4) of the restricted cubic splines were positioned at fixed and equally spaced percentiles (5%, 35%, 65% and 95%) as recommended by Harrell Jr.[35] The *F*-test was used to test whether the fetal growth curves for intermediate and positive anti-tTG were significantly different from the negative anti-tTG group. We created multivariate models for the linear regression and spline models with stepwise adjustment for maternal age, household income, ethnicity, marital status, maternal educational background, maternal smoking, maternal co-morbidity, maternal folic acid supplementation, parity, gestational age, and gender. Because of the small numbers in the positive anti-tTG group, final adjustment of potential confounders was restricted to those who attained a ≥10% alteration in effect estimate as suggested by Greenland et al.[36]

Additionally, to assess whether BMI, Hb/MCV, folate, and vitamin B12 concentrations mediated the association between anti-tTG and fetal growth, birth weight and placental weight, these variables were added separately to the final multivariate models (Supplementary figure S3.1.2 and S3.1.3). Logistic analyses were performed to assess the association between anti-tTG and the risk of small for gestational age, low birth weight and preterm birth.

To reduce bias, associated with missing data, multiple imputation of the outcome variables and covariates (n=5 imputations) was performed. The multiple imputation procedure was based on the correlation between each variable with missing values with other subject characteristics as described in detail by Sterne et al[37]. Analyses were then performed in each data set separately to obtain the desired effect sizes and standard errors. Regression coefficients were pooled by taking the average of the coefficients of the 5 imputed datasets. The pooled standard error was then calculated by using Rubin's rules.[38]The pooled results of the 5 imputed datasets were reported in this paper as regression coefficients (ß) or Odds Ratios (OR's) and 95% Confidence Intervals (95%CI). A *p*-value <0.05 was considered as statistically significant.

Results

Maternal and child characteristics are shown in table 3.1.1. Mean±SD age of the mothers at enrollment was 30±5 years. Out of 7046 mothers, 0.5% had positive anti-tTG levels during pregnancy and 4.4% had intermediate anti-tTG levels (Table 3.1.1; Figure 3.1.2). Of mothers with positive anti-tTG levels, 93% of the total study group and 100% of the Western mothers carried the HLA-DQ2 or –DQ8 molecule. Out of mothers with intermediate anti-tTG, 61-62% carried the HLA-DQ2 or DQ-8 molecule (Figure 3.1.3).

Non-response analyses showed that mothers with no blood samples available in second trimester of pregnancy were slightly younger (29 vs. 30 years; $p<0.01$), had lower folate levels in the first trimester of pregnancy (16 vs. 18 years; $p=0.01$), used folic acid less often before conception (37% vs. 40% $p<0.01$), and were more often lower educated (13% vs. 11% low education; $p=0.03$) than mothers with blood samples available in the second trimester of pregnancy. No difference was found in smoking habits, maternal chronic conditions, maternal BMI, fetal gender and birth weight and birth outcomes between mothers with blood samples available in the second trimester and those without it (data not shown).

Positive and intermediate anti-tTG levels and fetal growth and birth outcomes

Estimated fetal weights in the second and third trimester were significantly lower in mothers with positive anti-tTG (Table 3.1.2), but not in those with intermediate anti-tTG after adjustment for potential confounders.

Longitudinal analyses on fetal weight *z*-score from second trimester until birth are illustrated by the spline curves in figure 3.1.4. Fetal growth development was significantly different in mothers with intermediate anti-tTG levels, than in mothers with negative anti-tTG (figure 3.1.4; $p=0.04$). However, this association between intermediate anti-tTG and fetal growth was mostly noticeable at birth and not during second and third trimester of pregnancy (figure 3.1.4; table 3.1.2).

Table 3.1.1: Maternal and child characteristics according to anti-anti-tTG levels (*n*= 7046)

	Negative anti-tTG n=6702 (95.1%)	Intermediate anti-tTG n=308 (4.4%)	Positive anti-tTG n= 36 (0.5%)
Mother			
Maternal age (mean±SD; years)	30±5	29±5	31±6
BMI at intake (mean±SD; kg/m2)	25±5	25±4	24±4
Plasma folate levels (median; range; nmol/L)	16.2 (2.8, 47.8)	16.2 (0.13, 45.3)	13.4 (5.8, 36.5)
Plasma folate <8 nmol/L (n; %)	1012 (15%)	42 (14%)	7 (19%)
Plasma B12 levels (median; range; pmol/L)	173 (44, 1476)	178 (44, 589)	187 (80, 982)
Plasma B12 < 145 pmol/L (n; %)	2328 (35%)	107 (35%)	12 (33%)
Hemoglobin (mean±SD; mmol Fe/L)	7.5±0.68	7.5±0.68	7.5±0.68
Hb < 6.83 Fe/L	842 (13%)	52 (17%)	8 (22%)
Mean Corpus Volume (mean±SD; fL)	88.0±4.9	87.9±5.0	87.8±4.9
MCV < 80 fL	418 (6.2%)	23 (7.5%)	3 (8.3%)
Ethnicity (n; %)			
Non-Western	2823 (42%)	157 (51%)*	12 (33%)*
Western	3879 (58%)	151 (49%)	24 (66%)
Educational level (n; %)			
Low	822 (12%)	32 (10%)	1 (3%)
Mid	3142 (47%)	146 (47%)	13 (36%)
High	2738 (41%)	130 (42%)	19 (53%)
Household income (n; %)			
≤ 2000 Euro	2979 (44%)	146 (47%)	11 (31%)
> 2000 Euro	3723 (56%)	162 (52%)	25 (69%)
Smoking during pregnancy (n; %)	1756 (26%)	66 (21%)	4 (11%)*
Folic acid supplementation (n; %)			
No	2028 (30%)	101 (33%)	8 (22%)
Start 1st 10 weeks	2109 (31%)	95 (31%)	12 (33%)
Start preconception	2565 (38%)	112 (36%)	16 (45%)
Nulliparous (n; %)	2958 (44%)	126 (41%)	14 (39%)
Gastrointestinal disease (n; %)			
None or not reported	6666 (99.5%)	306 (99.4%)	34 (94.4%)
Celiac disease	0 (0%)	1 (0.3%)	1 (2.8%)
Lactose-intolerance	7 (0.1%)	0 (0%)	0 (0%)
Inflammatory bowel disease	5 (0.1%)	1 (0.3%)	1 (2.8%)
Bowel complaints without known organic cause	24 (0.3%)	0 (0%)	0 (0%)
Any other chronic condition (n; %)	726 (11%)	32 (10%)	7 (19%)
Placental weight (mean±SD: grams)	637±146	617±146*	582±183*
Child			
Male gender (n; %)	3389 (51%)	148 (48%)	16 (44%)
Estimated fetal growth (mean±SD: grams)			
Second trimester	381±94	381±89	369±98
Third trimester	1613±251	1594±221	1595±222
Birth weight (mean±SD: grams)	3418±559	3307±563*	3300±613
Gestational age (mean±SD: weeks)	39.9±1.8	39.6±1.6*	39.8±2.6
Small for gestational age (n; %)	127 (2%)	9 (3%)*	4 (11%)*
Low birth weight (n; %)	328 (5%)	20(6%)	6 (17%)*
Spontaneous prematurity (n; %)	196 (3%)	13(4%)	0 (0%)

*Significantly different from negative anti-tTG levels

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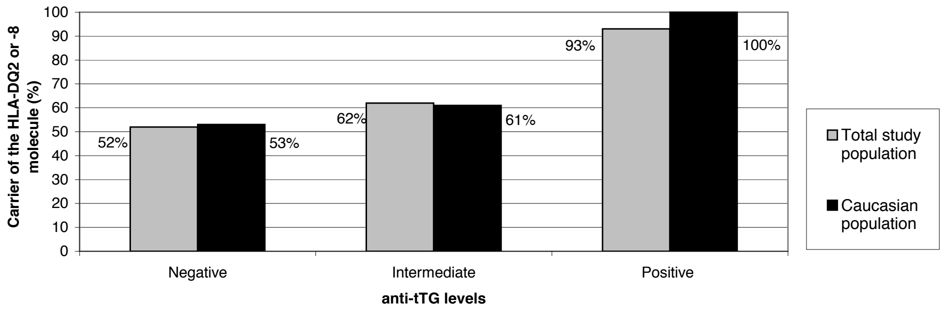

Figure 3.1.3: HLA-DQ2.5, -DQ2.2, or –DQ8 status according to anti-tTG levels

Table 3.1.2: Association between anti-tTG levels and estimated fetal weight and birth weight (*n*=7046)

	Univariate (ß; 95%CI)	**Multivariate**** (ß; 95%CI)
	Second trimester estimated fetal weight in grams* **	
Negative	Reference	Reference
Intermediate	-0.5 (-11, 10)	-2 (-7, 4)
Positive	-13 (-44, 18)	-16 (-32, -1)*
	Third trimester estimated fetal weight in grams* **	
Negative	Reference	Reference
Intermediate	-19 (-48, 10)	- 5 (-27, 16)
Positive	-19 (-102, 65)	-74 (-140, -8)*
	Birth weight in grams* **	
Negative	Reference	Reference
Intermediate	- 111 (-176, -47)*	-53 (-106, -1)*
Positive	-118 (-306, 69)	-159 (-316, -1)*
	Second trimester estimated fetal weight standard deviation score	
Negative	Reference	Reference
Intermediate	-0.05 (-0.20, 0.10)	-0.04 (-0.20, 0.10)
Positive	-0.40 (-0.80, -0.10)*	-0.46 (-0.82, -0.10)*
	Third trimester estimated fetal weight standard deviation score	
Negative	Reference	Reference
Intermediate	-0.05 (-0.17, 0.07)	-0.02 (-0.14, 0.11)
Positive	-0.27 (-0.62, 0.09)*	-0.40 (-0.76,-0.03)*
	Birth weight standard deviation score	
Negative	Reference	Reference
Intermediate	-0.18 (-0.30, -0.06)*	-0.16 (-0.28, -0.04)*
Positive	-0.27 (-0.61, 0.08)	-0.41 (-0.77, -0.05)*

p*<0.05;Adjusted for maternal age, educational level, folic acid supplementation, smoking and any chronic condition during pregnancy, and fetal gender,***Additionally adjusted for gestational age at measurement; ß: between-group difference in mean fetal weight or birth weight relative to reference group; 95%CI: 95% Confidence interval.

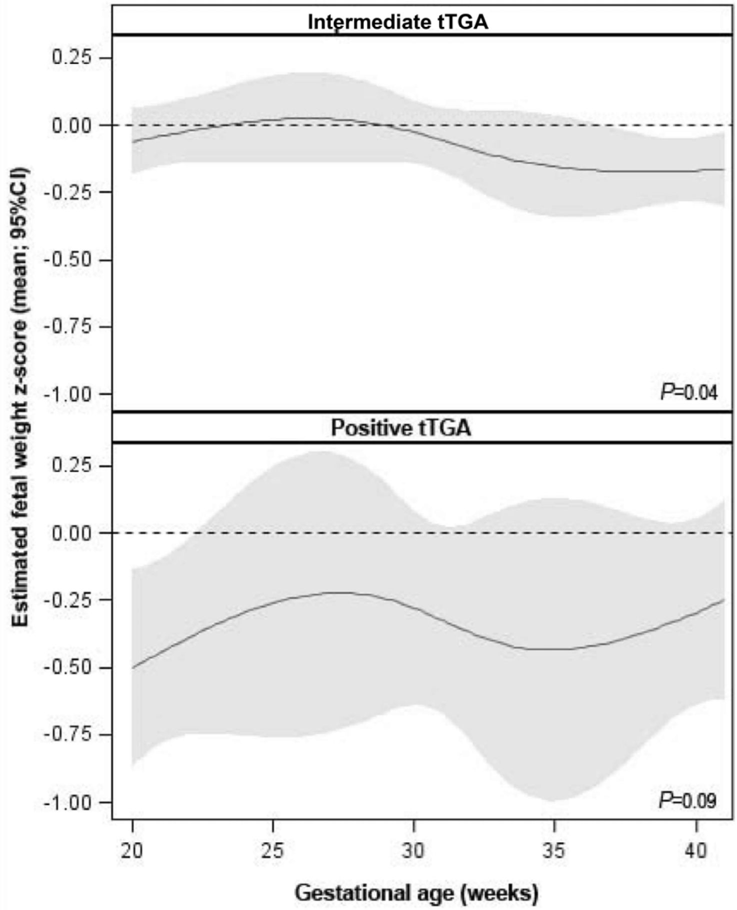

Figure 3.1.4: Difference in fetal growth standard deviation score during pregnancy for intermediate and positive anti-tTG levels relative to mothers with negative anti-tTG (N=7046; *p*=0.02 for difference in growth curves between positive and intermediate levels relative to negative anti-tTG levels; shaded area represents 95% CI; dotted line represents the reference group: negative anti-tTG).

After stratification by HLA-DQ2/-DQ8 status, the association between intermediate anti-tTG and birth weight was predominantly present in mothers carrying the HLADQ2/-DQ8 molecule (table 3.1.3).

Fetuses of mothers with positive anti-tTG, tended to have a different growth curve with lower *z*-scores relative to mothers with negative anti-tTG; which was mainly due to impaired growth in the second trimester (*p*=0.09; figure 3.1.4). Both mothers with intermediate anti-tTG and mothers with positive anti-tTG gave birth to neonates with a significantly lower birth weight than mothers with negative anti-tTG levels (table 3.1.2). The prevalence of low birth weight was significantly higher in mothers with intermedi-

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Table 3.1.3: Association between intermediate anti-tTGIgA levels and birth weight for according to maternal HLA-DQ2/-DQ8 status (*n*=7046)

	Univariate (ß; 95%CI)		Multivariate* (ß; 95%CI)	
	No HLA-DQ2/-DQ8 carrier	HLA-DQ2/-DQ8 carrier**	No HLA-DQ2/-DQ8 carrier	HLA-DQ2/-DQ8 carrier**
Birth weight in grams				
Negative	Reference	Reference	Reference	Reference
Intermediate	-100 (-207, 7)	-106 (-194, -18)*	-34 (-122, 54)	-76 (-149. -4)*
Birth weight standard deviation score				
Negative	Reference	Reference	Reference	Reference
Intermediate	-0.15 (-0.35, 0.05)	-0.20 (-0.36, -0.04)*	-0.12 (-0.32, 0.09)	-0.21 (-0.37, -0.04)*

*Adjusted for maternal age, folic acid supplementation, smoking and any chronic condition during pregnancy, maternal age, educational level and fetal gender, ß: between-group difference in mean birth weight relative to reference group; 95%CI: 95% Confidence interval. **Positive anti-tTG (*N*=36) were excluded from these analyses since ≥ 93% of these subjects carried the HLA-DQ2/-DQ8 molecule.

ate or positive anti-tTG than in mothers with negative anti-tTG (OR: 1.59; 95%CI: 1.04, 2.42; *p*=0.03 for between-group difference; multiple adjustment N/A). Also, the prevalence of neonates born small for gestational age was significantly higher in mothers with intermediate or positive anti-tTG than in those with negative anti-tTG (OR: 2.07; 95%CI: 1.12, 3.81; *p*=0.02 for between-group difference; multiple adjustment N/A). The prevalence of spontaneous pre-term birth was not significantly different in mothers with intermediate and positive anti-tTG, relative to mothers with negative anti-tTG (OR: 1.30; 95%CI: 0.73, 2.03; *p*=0.38 for between-group difference; multiple adjustment N/A). At birth, placental weight was significantly lower in mothers with intermediate anti-tTG and with positive anti-tTG levels (difference between intermediate and negative anti-tTG: -20; 95%CI: -38, -2; *p*=0.03 and difference between positive and negative anti-tTG: -66; 95%CI: -120, -13; *p*=0.02, after adjustment for maternal age, educational level, folic acid supplementation, smoking and any chronic condition during pregnancy, gestational age and fetal gender). No significant difference was found in placental index in mothers with positive and intermediate anti-tTG relative to mothers with negative anti-tTG (Supplementary table S3.1.1).

In addition to the final multivariate models, additional adjustment for maternal Hb/MCV, folate, vitamin B12 and BMI did not markedly change the effect estimates for anti-tTG and birth weight or the estimates for anti-tTG and placental weight (Supplementary figure S3.1.2 and S3.1.3).

DISCUSSION

This prospective observational study showed that both positive and intermediate anti-tTG concentrations during pregnancy have consequences on fetal growth and birth outcomes. The effects of intermediate anti-tTG were mostly present in mothers carrying the HLA-DQ2/-DQ8 molecule.

A debate has started if general screening on CD is warranted in the population, since large subsets of CD patients remain often undiagnosed[2, 11, 39]. It can be discussed if truly symptom-free CD patients would benefit from screening and whether it would be cost-effective[40-41]. On the other hand, it is suggested that screening on CD may be particularly be performed in women to prevent unfavorable pregnancy outcomes.[7-9]

Impaired fetal growth development has found to be associated with metabolic adaptations in favor of the development of adult diseases such as cardiovascular disease, hypertension and type 2 diabetes[42]. Also, low birth weight has shown to be associated with lower neuropsychological performance[42]. In addition, two prospective cohort studies showed that undiagnosed CD during pregnancy increased the risk of intrauterine growth retardation and low birth weight relative to controls; whereas this risk disappeared when CD was diagnosed before pregnancy[14-15]. Similarly, a historical cohort study demonstrated that offspring of mothers with no hospitalization for CD prior to pregnancy, had a higher risk of fetal growth restriction than when birth occurred after first hospitalization for CD [13].

Several hypotheses have been proposed to explain the association between CD and birth outcomes. First, it is known that maternal nutritional status, such as iron, folate and vitamin B12 deficiency, and maternal BMI during pregnancy, markedly affects fetal growth[21, 43-44]. Studies in CD patients have shown that impaired nutritional status can be present in these subjects, however this may vary within different age groups[45]. Moreover, malnutrition is not a very consistent feature in women with CD and adverse birth outcomes[16, 18]. Our study confirmed that intermediate and positive anti-tTG affect fetal growth, but this was independent of maternal nutritional status as measured by maternal BMI, Hb/MCV, folate and vitamin B12 levels.

Second, some recent studies implied an independent influence of anti-tTG on placental development and function. In addition, expression of anti-tTG was found in the human placenta[46]. Di Simone et al (2010)[23] showed that anti-tTG induces apoptosis of trophoblasts which are essential in placental development[23]. Likewise, another study confirmed that anti-tTG impairs placental function by binding to trophoblasts specifically[22]. Indeed, in the study by Ludvigsson et al.[14], placental weight was significantly lower in women with undiagnosed CD relative to controls, but the effect was not present in women with diagnosed CD[14]. We also found a significantly lower placental weight in women with intermediate and positive levels of anti-tTG. Although maternal nutritional status may also affect placental growth[43], the association between anti-tTG and placental weight was independent of maternal BMI, Hb/MCV, folate and vitamin B12 levels in our study.

Strikingly, we found that intermediate anti-tTG levels during pregnancy had consequences on birthweight. Although it is unclear if mothers with these intermediate anti-tTG levels are potential CD patients, these results may have consequences on current cut-off points of anti-tTG in women of childbearing age. In addition, growing evidence suggests that different types of CD exist, which is also known as the 'iceberg of CD' and may include subjects with latent or potential CD having positive or intermediate serology and no villous atrophy; or a later development of villous atrophy[41]. However, it is difficult to judge our results in terms of the 'iceberg of CD', since the sensitivity of celiac disease auto antibodies has found to be questionable in subjects with minimal intestinal lesions[47]. Nevertheless, we found that the effect of intermediate anti-tTG was predominantly true in those carrying the HLA-DQ risk alleles for CD (-DQ2/DQ8). This finding is unique and may imply that mothers with intermediate anti-tTG who gave birth to children with lower birth weight may have a subclinical state that may be potential CD patients in future. Hence, further studies on the nature and consequences of intermediate anti-tTG levels in those carrying HLA-DQ2 or –DQ8 are needed.

This is the first population-based study that took the effect of different anti-tTG concentrations into account. Also, the broad range of available data including multiple fetal growth measurements, Hb/MCV, folate and B12 levels during pregnancy and placental weight provide insight in potential mechanisms that play a role in the association between CD and fetal growth and birth outcomes. However, to appreciate these results, limitations of this study should be taken into account. In contrast to other studies, we did not find any difference in prevalence of pre-term birth between the anti-tTG strata and pre-term delivery. Both Ludvigsson et al. and Khashan et al. found a significant increased risk of pre-term birth in mothers with untreated CD during pregnancy[14-15]. Although the prevalence of pre-term birth in these countries is comparable to The Netherlands[48], the different results might be explained by differences in policy and diagnosis of pre-eclampsia and other pregnancy complications. In addition, our study only included spontaneous pre-term birth and results may differ within studies when mothers with pre-term delivery by indication are included. Also, 63 (1.5%) of the mothers in our study experienced a miscarriage or neonatal death. Data on anti-tTG, fetal growth, gestational age and birth weight were not available for these pregnancies. It is expected that embryos and fetuses who died in utero had fetal growth restriction[49], and those infants who died neonatally may be born prematurely[50]. Since women with CD experience miscarriages more often[8], our effect estimates for fetal growth and pre-term birth could be an underestimation of the 'true' effect due to 'survivor bias'.

Although, our results were not explained by maternal BMI, Hb/MCV, folate and vitamin B12 levels, other nutrients may still explain a part of the link between CD and fetal growth restriction. For example, vitamin D and calcium deficiencies are frequently seen in CD[45], but it is unclear whether these nutrients also affect fetal growth[43].

Also, we were not able to perform comprehensive analyses regarding the association between anti-tTG and the prevalence of low birth weight, small for gestational age and pre-term birth because of the small numbers of mothers with positive anti-tTG levels.

Different results on CD and birth weight have been described for mothers with diagnosed CD versus untreated CD[13-15]. However, only two mothers in our study reported a previous diagnosis of CD, thereby precluding conclusions concerning fetal growth and untreated versus treated CD. Finally, we did not have biopsies of the small intestine in mothers with positive anti-tTG or additional data on anti-endomysial antibodies which remains the standard for diagnosing celiac disease in adults. Although the sensitivity and specificity of anti-tTG is high[51], final conclusions concerning clinical CD should, therefore, be made with caution.

Conclusion

In conclusion, both intermediate and positive anti-tTG levels during pregnancy are associated with fetal growth restriction, and lower birth and placental weight. This relationship is not explained by indices of maternal nutritional status, as measured by status of Hb/MCV, folate and vitamin B12, and BMI during pregnancy. Evaluating clinical cut-offs of anti-tTG in women of childbearing age in order to prevent fetal growth restriction may be worthwhile to discuss.

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SUPPLEMENTARY MATERIAL

Table S3.1.1: Association between anti-tTG and placental Index

	Univariate (ß; 95%CI)	Multivariate* (ß; 95%CI)
Negative	Reference	Reference
Intermediate	0.04 (-0.42, 0.50)	-0.05 (-0.53, 0.42)
Positive	-1.21 (-2.45,0.04)	- 1.07 (-2.37, 0.25)

*Adjusted for maternal age, folic acid supplementation, smoking and any chronic condition during pregnancy, maternal age, educational level and fetal gender, ß: between-group difference in mean placental index divided by 100 relative to reference group; 95%CI: 95% Confidence interval.

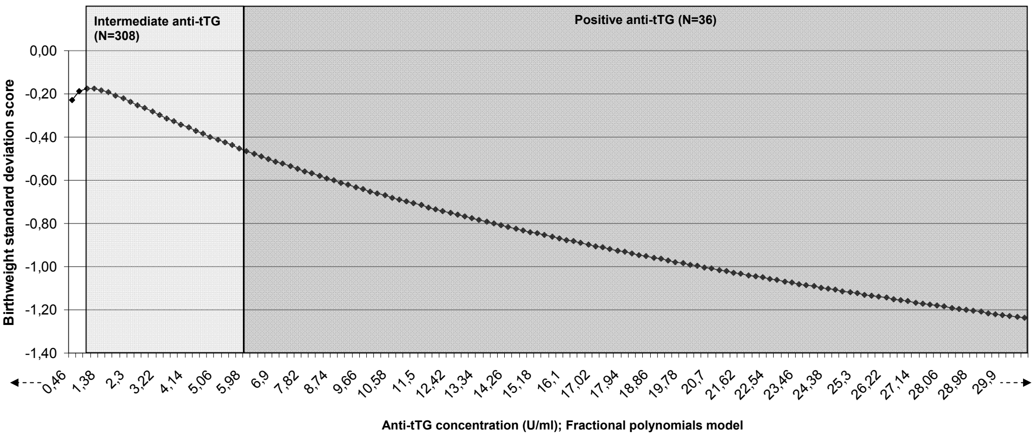

Figure S3.1.1: Fractional polynomials model between anti-tTG levels and birthweight standard deviation score (Y=B0+B1*1/√anti-tTG+B2*In(anti-tTG)*Ln(anti-tTG); *p*<0.01).

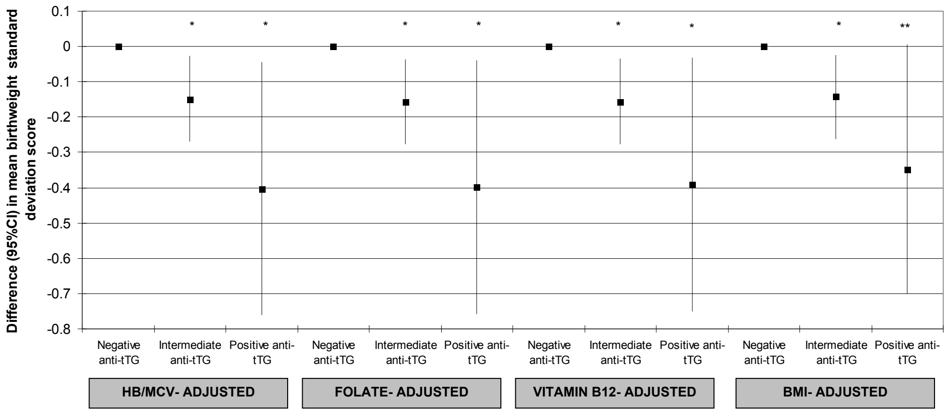

Figure S3.1.2: Maternal nutritional status as explanation for the association between anti-tTG levels and birthweight standard deviation score (*n*=7046; **p*<0.05; ***p*=0.05).

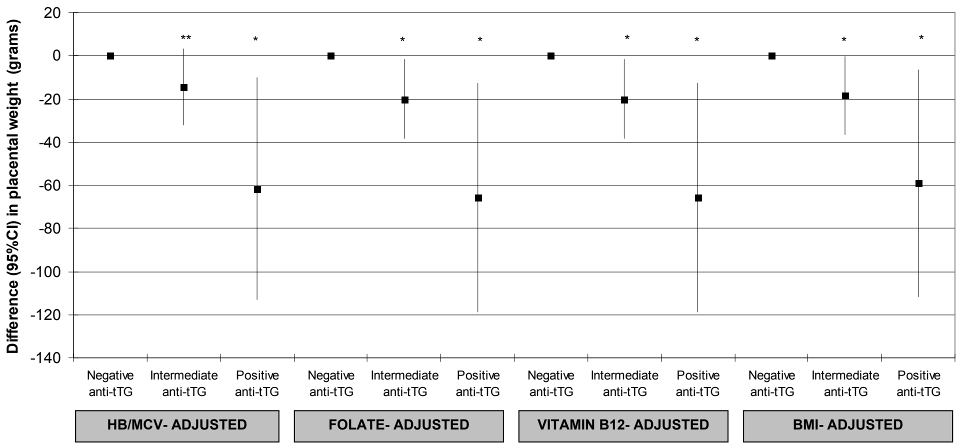

Figure S3.1.3: Maternal nutritional status as explanation for the association between anti-tTG levels and placental weight (grams) (*n*=7046; **p*<0.05).

Chapter 3.2

Infant nutritional factors and functional constipation

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Am J Gastroenterol. 2010 Apr;105(4):940-5.

ABSTRACT

Food allergy and celiac disease may lead to childhood constipation. Early introduction of food allergens and gluten in the first year of life has been suggested to play a role in these food intolerances but it is unclear whether this also holds true for development of childhood constipation. The aim of this study was to assess the association between the timing of introduction of food allergens and gluten early in life and functional constipation in childhood.

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood. Functional constipation at 24 months of age was defined in 4651 children according to the Rome II: defecation frequency less than 3 times a week or the presence of mainly hard feces for at least 2 weeks.

At the age of 24 months, 12% of the children had functional constipation. Children with functional constipation got introduced to gluten before or equal to the age of 6 months more often than children without functional constipation (37% and 27% respectively). After adjustment for birth weight, gestational age, gender, ethnicity, maternal education and family history of atopy and intestinal disorders, functional constipation was significantly associated with early gluten introduction (aOR: 1.35; 95%CI: 1.10, 1.65). No association was found between timing of introduction of cow's milk, hen's egg, soy, peanuts and tree nuts with functional constipation. A history of cow's milk allergy in the first year of life was significantly associated with functional constipation in childhood (aOR: 1.57; 95%CI: 1.04, 2.36). These results suggest that early gluten introduction in the first year of life provide a trigger for functional constipation in a subset of children. In case of functional constipation, there also might be a role for cow's milk allergy initiated in the first year of life.

INTRODUCTION

Functional constipation is a widespread symptom in children. Although the reported prevalence vary widely because of different definitions (2-23%)[1-3], it can have a great impact on the child's quality of life[4] . Studies have shown that the frequency of irritable bowel syndrome (IBS) in adults is higher in those who had a history of childhood constipation suggesting that risk factors may start early in life[5, 6].

The pathophysiology of childhood constipation seems multi-factorial. Genetic predisposition[7], history of gastroenteritis[8], inadequate oral intake[9, 10], low-birth weight and prematurity[11] and obesity[12] have all been suggested as potential determinants of this common clinical problem.

Another determinant of interest is food hypersensitivity. Several studies showed that in a subset of children, constipation may be a symptom of cow's milk allergy[13-17]. Iacono et al. showed that in 68% of the cases improvement was reached after dietary elimination of cow's milk in children with functional constipation[15].

A food protein that may also play a role within this respect is gluten, that is started to be consumed in the first year of the child's life[18]. Recently a hypothesis has been submitted by Verdu et al. (2009) suggesting that gluten might generate gastrointestinal symptoms even in the absence of clinical celiac disease (CD), a gluten-induced chronic disease associated with intestinal inflammation and villous atrophy leading to malabsorption[19, 20]. In addition, several studies suggested that the timing of gluten introduction may play a role in the development of CD[21]. It is, however, unclear whether gluten introduction in the first year of life plays a role in functional gastrointestinal symptoms such as functional constipation.

In case of food allergy, studies have also addressed the possibility to reduce any sensitization to food allergens by delaying the introduction of the main allergens as cow's milk, egg, peanuts, nuts and soy early in life[22, 23]. However, the association between allergen introduction and functional constipation in childhood is unclear.

In this epidemiological study we aim to assess the association between the timing of introduction of food allergens and gluten in the first year of life and the prevalence of functional constipation in children aged 24 months.

METHODS

Participants and study design

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood and has been described in detail previously[24, 25]. In total, 7893 mothers with a delivery date between April 2002 and January

2006 gave consent for follow-up. The study was approved by the medical ethical review board of the Erasmus Medical Center, Rotterdam, The Netherlands.

Functional constipation

At the age of 24 months, stool pattern of the child was assessed by using a questionnaire (*n*=5500; response: 70%). Accordingly, functional constipation was defined in this study if at least one of the following symptoms of ROME II[26] was reported: I) defecation frequency <3 times a week for at least 2 weeks or II) predominantly hard feces for the majority of stools for at least 2 weeks.

To avoid the influence of metabolic disorders and clustering, children were excluded in the analyses in case of the following: I) twinborn (*n*=238), II) siblings within the Generation R cohort (*n* =343) III) presence of a congenital heart condition (*n* =47), IV) anemia in the past year (*n* =58) or V) growth retardation defined as height < -2SD based on the Netherlands growth curves of 12-24 months children (*n* =163)[27].

Covariates

At the child's age of 6 and 12 months, mothers filled in a questionnaire including topics regarding the child's general health (ie. medication use, co-morbidity) and the consumption of food products and breast-feeding.

The presence of cow's milk allergy was obtained by questionnaires at the age of 6 and 12 where parents were asked whether their child had doctor-attended cow's milk allergy.

Prenatal questionnaires completed by the mother and father included information on ethnicity, mother's educational level and maternal smoking, family history of atopy and family history of any chronic bowel condition. Level of maternal education was defined as follows: I) low: no education, primary school or less than 3 years of secondary school, II) midlow: more than 3 years of secondary school, III) midhigh: higher vocational training or bachelor's degree, and IV) high: academic education[28]. Ethnicity of the child was defined as follows: if both parents were born in The Netherlands, the ethnicity was defined as Dutch, if one of the parents was born in another country than The Netherlands, that country counted; if parents were born in the different countries other than The Netherlands, the country of the mother counted[29].

From obstetric records assessed in mid-wife practices and hospital registries data on gender, birth weight, gestational age and birth outcomes were available[25].

Weight and height at the age of 24 months were available from the child health centers. Body Mass index (BMI) was then calculated and being overweighed was defined according to age- and gender dependent BMI-thresholds for young children from Cole et al (2000)[30].

Introduction of food allergens and gluten in the first year of life

At the child's age of 6 and 12 months, parents were asked at what age they had introduced the following products in the infant's diet for the first time: milk, yoghurt, porridge, egg, bread or biscuits, peanuts, nuts and soy products. The reported introduction of these food products were cross-checked with a short food-frequency questionnaire in children aged 6 and 12 months consisting of food-products frequently consumed according to a Dutch food consumption survey in infants[31]. For example, if the parent indicated at the age of 12 months that they had never introduced peanuts in their infant's diet but at the infant's age of 6 months the parent filled in that the infant consumed peanut butter more than once, then the introduction of this allergen was considered to be before or equal to 6 months of age. In case of gluten introduction, it was additionally cross checked with the consumption of bread and biscuits but also with the type of porridge (based on wheat or oats instead of rice) which was consumed at the age of 6 and 12 months. In addition, if the parent indicated that porridge was introduced in the infant's diet before the age of 6 months but at the age of 6 months porridge was only based on rice, this product was considered as not gluten containing and vice versa.

Furthermore, in case of the introduction of cow's milk and soy, the timing of introduction was also cross checked with the type of bottle feeding used at the age of 6 and 12 months (soy based or whether or not based on fully hydrolyzed whey protein). Data on breast-feeding was not included in the introduction of cow's milk but analyzed separately as described below.

Breast-feeding

Breast-feeding duration was assessed according to five variables: ever breast-feeding, cessation of breast-feeding and receiving any breast-feeding at the age of 2, 6 and 12 months. Data on ever breast-feeding were collected from delivery reports and data on breast-feeding cessation or continuation were derived from postnatal questionnaires at 2, 6 and 12 months. Subsequently, breast-feeding was categorized into 6 groups: I) never breast-feeding, II) partial breast-feeding with duration of less than 4 months and not thereafter, III) partial breast-feeding until 6 months of age, IV) exclusive breast-feeding until 4 months of age and not thereafter, V) exclusive breast-feeding less than 4 months, partial thereafter and VI) exclusive breast-feeding until 6 months of age. An approximation of exclusive breast-feeding was performed according to whether the child received breast-feeding without any other bottle feeding, milk or solids according to the short food frequency questionnaire described previously in this section. Partial breast-feeding indicates infants receiving both breast-feeding, bottle feeding and/or solids in this period. After the age of 6 months all infants received complementary feeding.

Statistical methods

Firstly, univariate analyses were performed by using Chi-square tests for categorical variables and the student T-test for continuous variables (normally distributed). Secondly, logistic regression analysis was performed with functional constipation as dependent variable. Introduction of food allergens and breast-feeding in the first year of life were analyzed separately as independent variables and adjusted for major confounders. The selection of potential confounders in the multivariate model was carried out by the alteration in odds ratios (OR). In case of ≥10% alteration in OR's, the potential confounder was kept in the multivariate model. Statistical interaction by a history of cow's milk allergy and/or being overweight was evaluated by adding the product term of the covariate and subgroup (covariate x subgroup) as an independent variable to the model.

Out of 4919 parents who completed the questionnaire at 24 months, 4651 children were available with data on functional constipation after exclusion and were defined as the population for analysis. Since complete data on covariates at both 6, 12 and 24 months of age were available for only 3009 children; there was some missing data for covariates (0.5–25%). For that reason covariates were multiple imputed (*n*=5 imputations) based on the correlation between the variable with missing values with other patient characteristics[32]. Data were imputed according to the Markov Chain Monte Carlo (MCMC) method (assuming no monotone missing pattern) and the imputations were repeated for 5 times to obtain the 5 copies of the filled-in data set. Data were analyzed in each data set separately to obtain desired parameter estimates and standard errors. Subsequently the results of the 5 imputed analyses were pooled and reported in this paper as odds ratio's (OR's) and 95% confidence interval (95%CI). A p-value <0.05 was considered as statistically significant.

The statistical analyses were carried out by using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Study population

Maternal and child characteristics of the study population are presented in table 3.2.1. Out of 4651 children, 12% had symptoms of functional constipation at the age of 24 months.

In children with at least two weeks of constipation related symptoms at the age of 24 months, 22% had ever used laxatives in the past year compared to 1% children with no constipation or symptoms for a shorter duration than two weeks (p<0.01).

Table 3.2.1: Maternal and child characteristics and functional constipation (*n*=4651).

		No Constipation (*n*=4080)	Functional constipation (*n*=571)	*p*-value
Mother				
Educational level of mother *n* (%)				<0.01
	Low	620 (15%)	154 (27%)	
	Midlow	1170 (29%)	195 (34%)	
	Midhigh	1030 (25%)	118 (21%)	
	High	1259 (31%)	104 (18%)	
Maternal smoking *n* (%)		983 (24%)	171 (30%)	<0.01
Child				
Male *n* (%)		2093 (51%)	240 (42%)	<0.01
Ethnicity *n* (%)				<0.01
	Dutch/other Western	3100 (76%)	321 (56%)	
	Non-Western	980 (24%)	250 (44%)	
Birth weight mean±SD)		3479±535	3382±572	<0.01
Gestational age at delivery (mean±SD)		40.0±1.6	39.7±1.9	0.01

Introduction of food allergens and gluten

Early introduction of soy and nuts in the infant's diet were significantly associated with functional constipation but this was mainly explained by confounders as gender, mother's educational level, ethnicity, birth weight, gestational age, maternal smoking, family history of atopy and family history of intestinal disorders (table 3.2.2).

The timing of food allergens such as peanuts, cow's milk and hen's egg in the first year of life was not significantly associated with functional constipation (table 3.2.2).

Gluten introduction before the age of 6 months was reported in 37% and 27% of the children with and without functional constipation respectively. Children who consumed gluten before the age of 6 months had a significantly higher prevalence of functional constipation at the age of 24 months which remained statistically significant in the multivariate model (table 3.2.2). The result did not differ whether the child had a history of cow's milk allergy in the first year of life (p>0.25 for statistical interaction) or was overweight (p>0.50 for statistical interaction).

Breast-feeding

Whether mothers had ever breast-fed their child was not significantly associated with functional constipation in childhood compared to children who were never breast-fed (OR: 0.91; 95%CI: 0.64, 1.30 after adjustment for gender, birth weight, gestational age, mothers educational level, ethnicity, maternal smoking, family history of atopy and family history of intestinal disorders). The odds ratio's for functional constipation slightly decreased as the duration of breast-feeding was longer but this did not reach statistical significance (table 3.2.3). The results did not differ whether the child had a

Table 3.2.2: Associations between the introduction of food allergens and gluten and functional constipation (*n*=4651).

	n (%)	Univariate model OR (95%CI)	Multivariate model* OR (95%CI)
Introduction of cow's milk ≤ 6 months**	3264 (70%)	1.20 (0.95, 1.51)	1.09 (0.86, 1.39)
Introduction of gluten ≤ 6 months	1345 (29%)	1.54*** (1.26, 1.89)	1.35*** (1.10, 1.65)
Introduction of soy ≤ 6 months	926 (20%)	1.28*** (1.03, 1.60)	1.13 (0.90, 1.41)
Introduction of peanuts ≤ 6 months	373 (8%)	1.29 (0.82, 2.02)	1.01 (0.65, 1.55)
Introduction of tree nuts ≤ 6 months	288 (6%)	1.49*** (1.01, 2.19)	1.20 (0.83, 1.74)
Introduction of hen's egg ≤ 6 months	550 (12%)	1.26 (0.97, 1.65)	1.04 (0.79, 1.38)

OR: odds ratio; 95%CI: 95% Confidence interval. OR's are compared to introduction > 6 months of age. *Adjusted for, gender, mother's educational level, ethnicity, birth weight, gestational age, maternal smoking, family history of atopy and family history of intestinal disorders. **Excluding breast-feeding, including bottle feeding containing casein and whey proteins.*** P<0.05.

Table 3.2.3: Associations between breast-feeding and functional constipation (*n*=4651).

	n (%)	Univariate model OR (95%CI)	Multivariate model* OR (95%CI)
Duration of breast-feeding			
Never	420 (9%)	Reference	Reference
Partial breast-feeding until 4 months, not thereafter	2181 (47%)	0.95 (0.69, 1.32)	0.91 (0.66, 1.25)
Exclusive breast-feeding until 4 months, not thereafter	455 (10%)	0.82 (0.51,1.31)	0.83 (0.50, 1.36)
Exclusive breast-feeding until 4 months, partial thereafter	798 (17%)	0.73 (0.50, 1.07)	0.74 (0.51, 1.07)
Partial breast-feeding until 6 months	735 (16%)	0.93 (0.65, 1.33)	0.80 (0.55, 1.16)
Exclusive breast-feeding until 6 months	62 (1%)	0.63 (0.21, 1.85)	0.63 (0.19, 2.02)

OR: odds ratio; 95%CI: 95% Confidence interval; *Adjusted for gender, mother's educational level, ethnicity, birth weight, gestational age, maternal smoking, family history of atopy and family history of intestinal disorders.

history of cow's milk allergy in the first year of life ($p>0.10$ for statistical interaction) or was overweight ($p>0.30$ for statistical interaction).

Parental report of cow's milk allergy in first year of life

Compared to children without functional constipation, a history of cow's milk allergy was more frequently found in children with functional constipation at the age of 24 months (6% and 9% respectively). Logistic regression analyses revealed that a history of cow's milk allergy in the first year of life was significantly associated with functional constipation in childhood (OR: 1.48; 95%CI: 1.03, 2.11) which remained statistically significant after adjustment for major confounders as gender, mother's educational level, ethnicity, birth weight, gestational age, maternal smoking, family history of atopy and family history of intestinal disorders (OR: 1.57; 95%CI: 1.04, 2.36).

DISCUSSION

This study demonstrates that early introduction of gluten was significantly associated with functional constipation. No significant association was found between early introduction of food allergens, breast-feeding and functional constipation independently of gender, social economic background, ethnicity, birth weight, gestational age and maternal smoking which was not different within strata of a history of cow's milk allergy or being overweight.

To our knowledge this is the first study that describes the association between early nutritional factors and functional constipation in childhood in a large cohort of healthy children.

The association between early gluten introduction and functional constipation may be explained in several ways. Firstly, early gluten introduction might reflect early complementary feeding in general since gluten containing cereals are frequently consumed on a daily basis in young children in the Netherlands[33]. Early complementary feeding may alter the intestinal flora[34]. In addition, Amarri et al[35] demonstrated that in healthy breast-fed infants changes in intestinal microbiota occurred for some intestinal bacteria after introduction of solid foods. It is acknowledged that there is little evidence that altered gut flora may contribute to functional constipation in adults but studies on the gut flora in young children with functional constipation are inconsistent with respect to interventions implying to influence the intestinal flora (ie. probiotica)[36, 37].

Secondly, several studies imply a relationship between early gluten introduction and celiac disease (CD) [38, 39, 40]. CD may present with symptoms of constipation. Ford et al (2008) showed that the prevalence of CD is higher in subjects with Irritable Bowel Syndrome (IBS) relative to subjects with no gastrointestinal symptoms[20]. Early gluten introduction has been proposed to be a risk factor for CD in Swedish epidemiological studies[39]. Particularly in the first months of life, the infant's intestine is still developing[34]. Introduction of gluten during these months may disrupt gut homeostasis which may establish gluten sensitivity or auto-immunity associated with CD in vulnerable subjects. However, taking into account only a 10% difference in early gluten introduction between children with and without functional constipation and a prevalence of 0.5-1% of clinical CD in The Netherlands[41], it still leaves a scientific challenge with respect to our study results. In addition, Verdu et al (2009) recently proposed that gastrointestinal symptoms might be a feature of gluten sensitivity but not necessarily clinical CD. The authors proposed that even in the absence of CD, gluten may induce symptoms comparable as in functional bowel disorders, which may shed some light on our study results. Studies showed that, even in the absence of classic mucosal injury as seen in CD, improvement of gastrointestinal symptoms could be reached after a gluten-free diet[19].

According to the hypothesis of Verdu et al[19], there might be some ground that gluten could be responsible for constipation. However, a gluten-free diet as therapy in constipated patients with no villous atrophy is still controversial and should be further investigated in clinical studies, as does the timing of introduction of gluten in the first year of life.

It is remarkable that our study shows that parental report of doctor-attended cow's milk allergy in the first year of life is still strongly associated with constipation in childhood. Although several studies suggested that cow's milk allergy could be a cause of functional constipation[3, 17, 42, 43], cow's milk allergy usually resolves within the first few years of life, with already two-third of patients becoming tolerant by the age of 2 years[44, 45]. In our study, it could be the case that in a proportion of children, cow's milk allergy remains but that cow's milk is not fully eliminated in the child's diet, since the diet becomes more diverse after the age of 1 year[33, 46] that makes symptoms of cow's milk allergy persist in childhood. Also, there could be a shift in features of cow's milk allergy over time with different clinical manifestations later in life compared to symptoms at commencement[16] through which the allergy seems to pass undeservedly.

However, to appreciate these results some limitations of the study have to be discussed.

We did not have other evidence of cow's milk allergy in the first year than the parental report if the child had doctor-attended cow's milk allergy. It is known that self-report of food allergy overestimates the true prevalence of food allergy[47]. If these children were more likely to have persistent constipation at 24 months then this information bias may have led to overestimation of our study results.

Also, our data did not allow assessing any effect of very early gluten introduction. As known from the Swedish studies.

Furthermore, to define our outcome we used criteria from ROME II[26] We were not able to fully specify our outcome according to the most recent evidence based ROME III criteria[48]. Although the prevalence of functional constipation in our study is comparable with other studies in the general population or school samples[2], our results preclude conclusions on allergen introduction in subsets of more severe functional constipation.

Finally, we did not have data on psychological factors and lifestyle in this study population. Since this study is of epidemiological design, residual confounding by lifestyle and psychological aspects could remain thereby not permitting any final conclusions with respect to the causality of the described associations.

Conclusion

In conclusion, this study addresses the possibility that early gluten-introduction in the first year of life provide a trigger that may explain a part of the spectrum influencing functional constipation in childhood. The results do not support a role for the time of introduction of cow's milk, soy, hen's egg, peanuts and tree-nuts in the development of functional constipation in childhood. The study also described the potential influence of cow's milk allergy commenced in the first year of life and the development of functional constipation in childhood. Further clinical studies should clarify whether more attention should be paid to gluten consumption in the first year of life in a subset of children and if constipated children may have prolonged cow's milk allergy

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Chapter 3.3

Dietary patterns, overweight, sedentary behavior and functional constipation.

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Matern Child Nutr. 2012 Jan 30 [Epub ahead of print].

ABSTRACT

The influence of childhood nutrition on the development of constipation beyond the period of weaning and breast-feeding is relatively understudied. In addition, eating patterns in childhood can be highly correlated with overweight and sedentary behavior which may also have an influence on constipation. The aim of this study was to assess whether common dietary patterns, sedentary behavior, and childhood overweight are associated with constipation in childhood. The study was embedded in a population-based prospective birth cohort. Information on dietary intake was obtained by a food frequency questionnaire at the child's age of 14 months (*n*=2420). The adherence scores on a 'Health conscious' and 'Western-like' diet were extracted from Principal Component Analysis. At the age 24, 36 and 48 months, information on constipation and sedentary behavior, and weight and height was obtained by parental-derived questionnaires and from the child health centers respectively. Adherence to a 'Western-like' dietary pattern was associated with a higher prevalence of constipation up to 48 months (aOR; 95%CI: 1.39; 1.02, 1.87), which was not mediated by overweight or sedentary behavior. Adherence to a 'Health Conscious' dietary pattern was only associated at short-term, with a lower prevalence of constipation at 24 months (aOR: 95%CI: 0.65; 0.44, 0.96). No association was found between overweight, sedentary behavior and constipation. Our results suggest that specific dietary patterns in early childhood could be associated with higher or lower risks for constipation but these effects are time-dependent. Overweight and sedentary behavior seem not have a major role on constipation in childhood.

INTRODUCTION

Constipation is frequently seen in the pediatric population with a prevalence varying from 0.7-30%[1]. Several factors including infant nutrition as potential causes for constipation in childhood have been suggested. Previously, we have found that timing of introduction of gluten and a history of cow's milk allergy are associated with constipation in childhood [2]. Another study demonstrated that constipation was more frequent in infants who were not exclusively breast-fed [3]. The influence of childhood nutrition on the development of constipation shortly beyond the period of weaning and breast-feeding is relatively understudied. In adults and schoolchildren, it is known that increasing dietary fiber and fluid intake can be effective in amelioration of symptoms of constipation [4-5]. However, in pre-school children this approach has been controversial since the results of studies regarding dietary fiber and constipation in very young children are inconsistent [6-7]. Several studies have demonstrated that the prevalence of overweight and sedentary behavior is increased in children with functional bowel disorders [8-11]. As dietary fiber intake and physical activity can be important determinants of both constipation and overweight [5, 12] but unhealthy eating patterns also cluster with sedentary behavior [13], the association between diet and constipation can be easily mediated by overweight or with the level of physical activity.

Although most studies regarding nutrition and health focused on single nutrients, the fact that people do not eat isolated nutrients but a variety of foods that may have a biological interaction in the human body, should be considered. For instance, dietary fiber intake may have an interaction with carbohydrate and dietary fat absorption [14]. Accordingly, a new approach within nutritional research has been developed by using dietary pattern analysis taking into account that the intake of foods can be highly intercorrelated [15]. A benefit of this approach is that cumulative effects of nutrients and nutrient interactions can be detected much easier than the effect of a single nutrient since effects of single nutrients are often very small [15]. Finally, dietary patterns capture the totality of a child's diet and give greater insight in overall lifestyle choices since dietary patterns incorporate with non-dietary behaviors as well [13]. Studying dietary patterns in relation to constipation can improve understanding of dietary practice in young children and may provide guidance for nutritional recommendations in children with constipation.

The aim of this study was to determine in a population-based sample whether adherence to common dietary patterns in early childhood is associated with constipation between 24 and 48 months of age. A second aim was to explore whether overweight and TV-watching, as proxy for sedentary behavior, are associated with constipation in childhood.

METHODS

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood and has been described in detail previously [16]. In total, 5088 mothers with a delivery date between April 2002 and January 2006 provided consent for postnatal follow-up and received a food frequency questionnaire (FFQ) for their child at the age of 14 months. Ethic approval for the study was obtained from the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam.

Dietary assessment

Out of 5088 mothers who received the FFQ to assess the child's dietary intake, 3643 (72%) completed the FFQ (mean±SD: 14±2 months) and were eligible for analysis. The FFQ was developed in cooperation with the division of Human Nutrition of Wageningen University, the Netherlands and based on an existing validated food questionnaire developed and described in detail previously [17]. This FFQ was modified on the basis of foods frequently consumed in early childhood according to a Dutch food consumption survey among young children [18]. The FFQ was validated against three-days 24h recalls in Dutch children aged 14 months with the following intra-class correlation coefficients for macronutrients: total energy: 0.4, total protein: 0.7, total fat: 0.4, carbohydrates: 0.4, and dietary fiber: 0.7. The FFQ consisted of 211 food items and included questions on the frequency of consumption of these food items over the last month, the amount and type of the food item, and preparation methods. Dietary pattern analysis was restricted to children from parents who were both born in the Netherlands (*n*=2420)[19] after random exclusion of siblings within the Generation R cohort.

Dietary patterns

All 211 food items from the FFQ data of all Dutch children (*n*=2420) were classified into 21 food groups (Table S3.3.1). Subsequently, we applied principal component analysis (PCA) on 21 food groups (in grams per day) of the children to construct overall dietary patterns by explaining the largest proportion of variation in the food group intake[15]. To reduce correlation between the factors, the varimax method by maximizing the sum of the variance of the loading components was used [20]. To reduce bias as a result of multiple testing and to better identify common dietary patterns, only the dietary patterns with an Eigenvalue of ≥ 1.5 were extracted (*n*=2) which accounted for 24.5% of the variability in food consumption within our study population. Dietary pattern 1 represented a 'Health conscious' dietary pattern characterized of high intake of fruit, vegetables, legumes and fish (table 3.3.1). Dietary pattern 2 represented a 'Western-like' dietary pattern comprising high intakes of savory and snacks, other fats, confectionery and sugar-containing

Table 3.3.1: Correlation of foods and macronutrients for 'Western-like' and 'Health conscious' dietary pattern scores in Dutch children aged 14 months (retaining r>0.2 or r<-0.2)

	Mean intake grams/day	Western-like dietary pattern	Health conscious dietary pattern
Food group		**Factor loading**	**Factor loading**
Refined bread and breakfast cereals	15	0.57	-
Whole bread and breakfast cereals	62	-	-
Starchy foods	23	-	0.62
Dairy	626	-	-
Fruit	162	-	0.32
Soy substitutes	4	-	-
Vegetables	52	-	0.74
Potatoes	34	-	0.61
Soups and sauces	9	0.23	-
Savory and snacks	4	0.59	-
Confectionery	28	0.72	-
Vegetable oils	1	-	0.50
Animal fats	11	0.58	-
Fish	8	-	0.22
Shellfish	0.3	-	-
Meat	26	0.27	0.21
Eggs	2	-	-
Legumes	4.0	-	0.59
Sugar-containing beverages	198	0.59	-
Non-sugar containing beverages	56	-	-
Composite dishes	102	-	-
Eigen value*		3.4	1.7
Variance explained (%)		16.3	8.2

Nutrients		**Pearson's correlation coefficient**	**Pearson's correlation coefficient**
Energy (kcal)	1275 kcal	0.5	0.3
Proteins (grams)	41	0.3	0.4
Fat (grams)	40	0.6	0.2
Saturated fat (grams)	14	0.3	-
Monounsaturated fat (grams)	12	0.3	-
Polyunsaturated fat (grams)	7	0.4	0.2
Carbohydrates (grams)	188	0.5	0.3
Mono- and disaccharides (grams)	111	0.5	-
Polysaccharides (grams)	76	0.4	0.5
Dietary fiber (grams)	18	-	-

PCA was used as an extraction method in which the Pearson's correlation coefficients represent the relative contribution of that food group to the identified dietary pattern. *The Eigen value was used as indicator of the amount of variation explained by each dietary pattern.

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beverages (table 3.3.1). Nutrient characteristics of the dietary patterns are presented in table 3.3.1. Accordingly, each participant was assigned two personalized adherence scores (*z*-scores) for these dietary components, which is a linear composite of the optimally weighted food items by factor loadings constructed for the two dietary patterns derived from the PCA.

Overweight and sedentary behavior

Height and weight were measured with standardized methods at visit to the child health center at the age of 24, 30 and 36 months (response: 69%, 75%, and 65% respectively). Body Mass Index (BMI) was calculated as weight (kg)/height $(m)^2$ and overweight and obesity was defined according to the age-and gender dependent cut off points for childhood BMI of the International Obesity task Force [21]. Information on TV watching as a proxy for sedentary behavior was obtained by questionnaire at the child's age of 24, 36 and 48 months (response rates: 70%, 64% and 63% respectively) and was categorized into ≥ and < 2 hours a day according to the American Academy of Pediatrics [22].

Constipation during childhood.

At the age of 24, 36 and 48 months, stool pattern of the child was assessed by using a parental-derived questionnaire (response rates: 70%, 64% and 63% respectively) consisting of the following questions: 'Has your child had the following for at least 2 weeks: 1) A bowel movement twice or less per week (yes vs no), and 2) predominantly hard/firm feces (yes vs no). The outcome of constipation was considered as present if at least one of the above symptoms of ROME II was reported [23].

Covariates

Several medical, behavioral and socio-demographic characteristics obtained from a combination of pre- and postnatal questionnaires, community midwife and hospital registries were used as potential confounder or used as predictor in the multiple imputation procedure.

In early, mid, and second trimester of pregnancy (response: 91%, 81%, and 77% respectively) information by questionnaire was obtained on maternal educational level (low: no education, primary school or less than 3 years of secondary school; mid: more than 3 years of secondary school or higher vocational training or bachelor's degree, and high: academic education), household income (≥2000 euro vs. <2000 euro per month), marital status (living together vs living alone), maternal smoking (yes vs no), maternal alcohol use (yes vs no), folic acid supplementation (yes vs no), history of intestinal disorders, atopic disease, diabetes mellitus, hypertension, and hypercholesterolemia (yes vs no), and parity.

During visits at one of the community midwife research centres in first, second and third trimester (response: 76%, 93%, and 93% respectively), maternal anthropometrics were measured. Information on pregnancy complications was obtained from medical records as described in detail previously [16] which was available in 99% of the enrolled mothers. In all children, information about sex, birth weight and gestational age was available from the obstetric records from the hospitals and midwife practices.

From postnatal questionnaires at the age of 6 and 12 months (response: 73% and 71% respectively), data was available on timing of introduction of solids (> vs. ≤ 6 months of age; after the age of 6 months all children received complementary feeding), breast-feeding duration, and infant history of food allergy in the first year of life as described in detail previously[2]. Postnatal questionnaires at the age of 24, 36, and 48 months (response: 76%, 72%, and 73% respectively) included information on wheezing, atopic dermatitis, and infectious disease, and day-care attendance in the previous year. Information about the child anthropometrics prior to 24 months was collected at each routine visit to the child health centres at the age of 6, 11, 14, and 18 months (response varied from 60% to 82%). The level of parental stress was assessed by the Nijmeegse Ouderlijke Stress Index-Kort (NOSIK[24]), the Dutch version of the Parental Stress Index-Short Form [25] at the child age of 18 months (response: 75%).

Statistical analysis

Univariate analyses were performed by using Chi-square tests for categorical variables and the student T-test for continuous variables to compare differences in diet score and prevalence of overweight between children with and without constipation. Subsequently, to assess how the child's dietary patterns, overweight and sedentary behavior were associated with functional constipation, logistic generalized estimating equations (GEE) with an exchangeable correlation structure was performed. Briefly, GEE analysis assesses the longitudinal association between variables by correction for the within subject's dependence as a result of the repeated observations on constipation, overweight and sedentary behavior[26].

The primary independent variables in the GEE model were I) adherence score (*z*-scores) to the 'Health conscious' and 'Western-like' dietary pattern after stratification into tertiles with the first tertile (lowest z-score) as reference category, II) Overweight, divided into being overweight and being obese with normal weight as reference category or III) Sedentary behavior defined as TV watching of at least 2 hours a day with less than 2 hours a day as reference category. All models were adjusted for time and the analyses concerning the dietary patterns were all adjusted for total energy intake [27] and age of food assessment. We created multivariate models with stepwise adjustment for potential confounders as maternal age, household income, marital status, maternal educational background, maternal BMI, maternal smoking, maternal alcohol consumption, maternal co-morbidity, maternal folic acid supplementation, maternal history of intestinal disorders, parity, birth weight, gestational age, gender, timing of introduction of solids, breast-feeding duration and history of food allergy. These confounders were selected on the basis of variables associated with constipation or dietary patterns in young children described in previous studies [1-2, 28].

In case of ≥10% alteration in effect estimate, the potential confounder was kept in the final multivariate model as described by Greenland et al [29]. Additionally to assess whether overweight and TV-watching had any influence on the association between the dietary patterns and constipation, these variables were added separately to the final multivariate models.

To reduce bias associated with missing data, multiple imputation of the data (*n*=5 imputed datasets). The multiple imputation was based on the correlation between each variable with missing values (varying from 0% to 28%; table 3.3.2) with the following subject characteristics:

maternal age, household income, marital status, maternal educational background, maternal BMI, maternal smoking, maternal alcohol consumption, maternal co-morbidity, maternal folic acid supplementation, maternal history of intestinal disorders, history of atopic disease, pregnancy complications (i.e. diabetes gravidarum, hypertension), parity, birth weight, gestational age, gender, all anthropometric measurements, timing of introduction of solids, breast-feeding duration, watching TV, history of food allergy, symptoms of wheezing, atopic dermatitis, infectious disease and constipation in previous year, any daycare attendance, parental stress score, total energy intake, dietary pattern *z*-score (used as predictor only). This procedure have been described in detail by Sterne et al [30]. Data were imputed according to the Markov Chain Monte Carlo method since no monotone missing pattern was found. GEE analysis was then performed in each data set separately to obtain the desired effect sizes and standard errors. Results of the 5 imputed datasets were pooled by taking the average of the regression coefficients. The pooled standard error was then calculated by using Rubin's rules[31]: $\sqrt{[W+(1+1/m)*B]}$ with W= mean variance of the effect size within the imputed datasets; B=variance of the effect sizes between the imputed datasets; m= number of imputed datasets (*n*=5) which takes into account the uncertainty associated with missing data[30]. Analyses were performed in the original data and after the multiple imputation procedure. Since we found similar effect estimates the final results in our paper are presented as the pooled odds ratio (OR) with its 95% Confidence Intervals (95%CI) after the multiple imputation procedure. A *p*-value <0.05 was considered as statistically significant. Statistical analyses and the multiple imputation procedure were performed by using SPSS 17.0 for Windows.

Table 3.3.2: Child and mother characteristics of the study population (*n*=2420)

	Original data		Imputed data	
	n	%	*n*	%
Mother				
Maternal educational background				
Low	39	2%	41	2%
Mid	1662	69%	1703	70%
High	658	27%	677	28%
Missing	61	2%	-	-
Household income per month				
< 2000 euro	294	12%	301	12%
≥ 2000 euro	1804	75%	2119	88%
Missing	322	13%	-	-
Marital status				
Married/living together	2239	93%	2298	95%
No partner	121	5%	122	5%
Missing	60	3%	-	-
Smoking during pregnancy	432	18%	508	21%
Missing	399	17%	-	-
Alcohol consumption during pregnancy	1159	48%	1420	59%
Missing	296	12%	-	-
Body Mass Index at intake (mean±SD; kg/m2)	24	±4	24;	±4
Missing	214	9%	-	-
Perinatal folic acid supplementation	1685	70%	2230	92%
Missing	593	25%	-	-
Maternal age at intake (mean±SD; years)	32.0	±4.2	32.0	±4.2
Missing	-	-	-	-
Nulliparous	1454	60%	1498	62%
Missing	59	2%	-	-
History of intestinal disorders	70	3%	78	3%
Missing	232	10%	-	-
History of diabetes mellitus, hypertension or hypercholesterolemia	51	2%	243	10%
Missing	683	28%	-	-
Child				
Male gender	1201	50%	1201	50%
Missing	-	-	-	-
Birth weight (mean±SD; grams)	3503	±570	3503	±570
Missing	-	-	-	-
Gestational age (mean±SD; weeks)	39.9	±1.7	39.9	±1.7
Missing	-	-	-	-
Breast-feeding				
Never breast-feeding	231	10%	302	13%
Partial breast-feeding until 4 months of age	1314	54%	1439	59%
Exclusive breast-feeding until 4 months of age	630	26%	679	28%
Missing	245	10%	-	-
Timing of introduction of solids ≤ 6 months of age	1620	67%	1628	67%
Missing	14	1%	-	-

Table 3.3.2: Child and mother characteristics of the study population *Continued* (*n*=2420)

	Original data		Imputed data	
	n	**%**	***n***	**%**
History of food allergy in first year of life	144	6%	152	6%
Missing	87	4%	-	-
Institutional and non-institutional care in first year of life >16 hrs/week	1301	54%	1640	68%
Missing	524	22%	-	-
Body Mass index in 2nd year of life (mean±SD; kg/m^2)	16.5	±1.4	17.1	±1.3
Missing	536	22%	-	-
TV watching ≥ 2 hrs a day in 2nd year of life	223	9%	245	10%
Missing	166	7%	-	-

Results

Child and mother characteristics are presented in table 3.3.2. The prevalence of constipation ranged from 8% till 13% and significantly increased between 24 and 48 months (*p*<0.01 for difference in prevalence of constipation between 24 and 36 months and between 24 and 48 months).

The prevalence of overweight remained stable around 10% between 24 and 36 months of age (*p*=0.34 for difference in prevalence relative to 24 months) but slightly decreased to 8% at 48 months (*p*=0.01 for difference in prevalence between 24 and 48 months).

The prevalence of overweight was almost similar in children with and without constipation (8% vs. 11%; *p*= 0.46, 13% vs. 10%; *p*= 0.10 and 8% vs. 9%; *p*=0.60 at the age of 24, 36 and 48 months respectively*)*. TV-watching of at least 2 hours a day was slightly more frequent in children with constipation than in children without constipation at the age of 36 months (10% vs. 11%; *p*=0.49 , 4% vs. 7%; *p*= 0.02, 5% vs. 6%; *p*= 0.70 at the age of 24, 36 and 48 months respectively) .

Mean±SD score of a 'Western-like' dietary pattern score was 0.07±1 vs 0.01±1, 0.13±1 vs 0.02±1, and 0.05±0.8 vs 0.01±1 in children with constipation relative to those without symptoms at the age of 24, 36, and 48 months respectively (*p*= 0.35, *p*=0.04, and *p*=0.55 for 24, 36, and 48 months respectively). Mean±SD score of a 'Health conscious' dietary pattern score was -0.14±1 vs 0.01±1, -0.07±1 vs 0.01±1, and -0.05±1 vs 0.01±1 in children with constipation relative to those without symptoms at the age of 24, 36, and 48 months (*p*=0.04, *p*=0.22, and *p*=0.45 for 24, 36, and 48 months respectively).

Mean±SD dietary fiber intake was 17±9 g/day vs 18±9 g/day children with and without constipation respectively at the age of 24, 36, and 48 months, which was not significantly different between groups (*p*>0.5 for between group difference). Mean total energy intake was similar among children with and without constipation (mean difference in total energy intake 8- 12 kcal per day at the age of 24, 36, and 48 months;

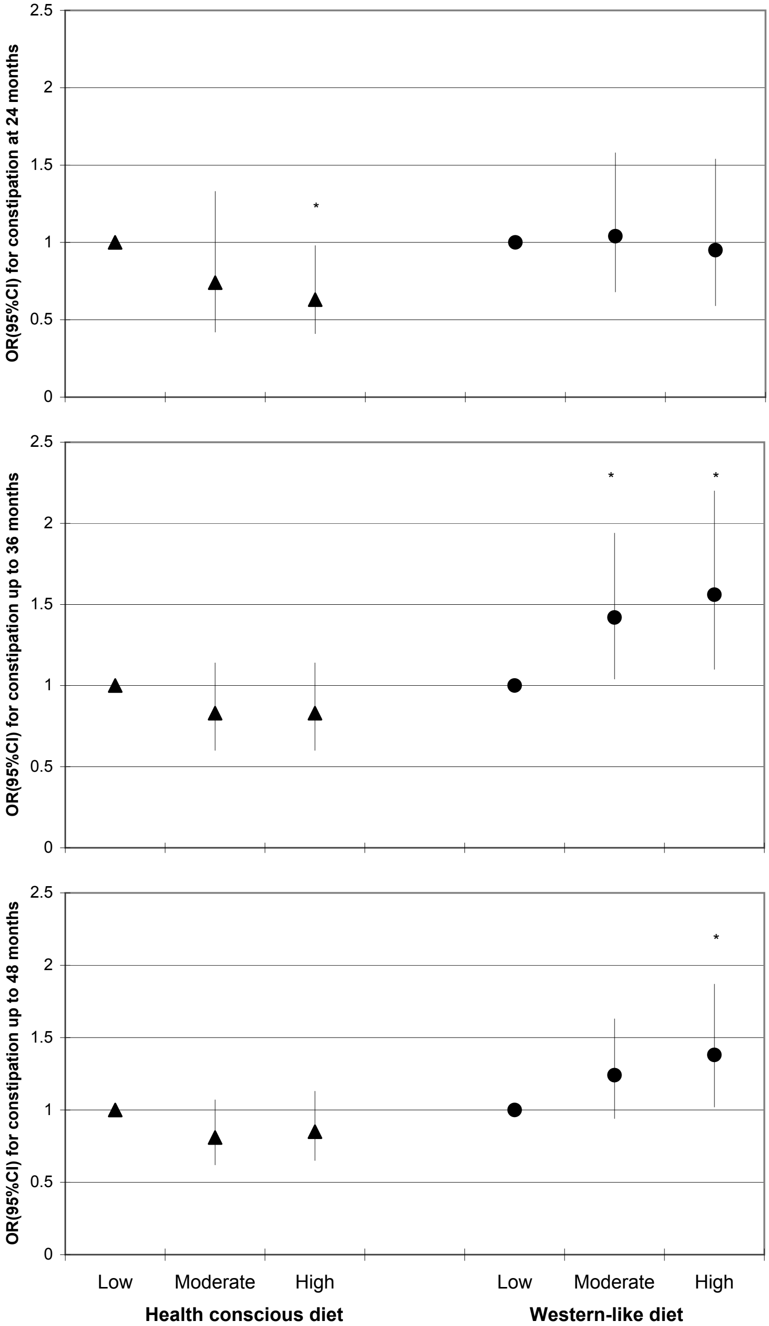

Figure 3.3.1: Time-specific associations (i.e. at 24 and up to 36 and 48 months) between dietary patterns and constipation (*n*=2420; * *p*<0.05 after the multiple imputation procedure)

p>0.7). Children with constipation consumed slightly higher energy percentage from saturated fat than children without constipation at the age of 36 months but this was not statistically significant (11en% vs 10en%, *p*=0.15). Also, a slightly lower energy percentage from polysaccharides and a slightly higher energy percentage from mono- and disaccharides was consumed by children with constipation relative to those without it (24en% vs 23en%, *p*=0.06, and 35en% vs. 34en%, *p*=0.12 at the age of 48 months).

No difference was found between other macronutrient consumption and constipation at the age of 24, 36, and 48 months (data not shown). Also, no difference was found in total food volume per day between children with and without constipation (data not shown)

On short-term, high adherence to the 'Health conscious' dietary pattern was significantly associated with a lower prevalence of constipation at the age of 24 months whereas no association was found between high adherence to a 'Western-like' dietary pattern and constipation at the age of 24 months (figure 3.3.1).

Longitudinal analyses revealed that adherence to the 'Health conscious' dietary pattern did not remain significantly associated with constipation up to 36 and 48 months

Table 3.3.3: Longitudinal analyses on the child's dietary patterns, overweight, and sedentary behavior and childhood constipation after the multiple imputation procedure (*n*=2420).

	Univariate OR (95%)	Multivariate** OR (95%CI)
Health conscious diet		
Low adherence (*n*=807)	Reference	Reference
Moderate adherence (*n*=807)	0.87 (0.69, 1.10)	0.81 (0.62, 1.07)†
High adherence (*n*=806)	0.83 (0.64, 1.07)	0.85 (0.65, 1.13)†
Western-like diet		
Low adherence (*n*=807)	Reference	Reference
Moderate adherence (*n*=807)	1.16 (0.91, 1.47)	1.24 (0.94, 1.63)†
High adherence (*n*=806)	1.31 (1.02, 1.70)*	1.39 (1.02, 1.87)*†
Nutritional status		
Normal weight (*n*=2161)	Reference	Reference
Overweight (*n*=237)	0.98 (0.98, 1.07)	0.95 (0.64, 1.40)
Obese (*n*=22)	1.37 (0.67, 2.79)	1.01 (0.69, 1.46)
Sedentary behavior		
TV watching < 2 hours a day (*n*=2173)	Reference	Reference
TV watching ≥ 2 hours a day (*n*=247)	1.11 (0.97, 1.55)	1.07 (0.74, 1.57)

OR: Odds Ratio; 95%CI: 95% Confidence Interval derived after the multiple imputation procedure; * *p*<0.05; **Adjusted for time, maternal smoking, maternal alcohol consumption, maternal history of intestinal disorders, maternal BMI, household income, parity, gender birth weight, gestational age, duration of breast-feeding, timing of introduction of solids and history of food allergy. †Additionally adjusted for age of food assessment and total energy intake.

of age (table 3.3.3, figure 3.3.1) whereas high adherence to a 'Western-like' dietary pattern was longitudinally associated with a significant higher prevalence of constipation up to 36 months and up to 48 months (table 3.3.3; figure 3.3.1). Additional adjustment for other potential confounders as maternal folic acid supplementation, maternal education, maternal co-morbidity, and marital status did not alter these results (data not shown).

No association was found between overweight and constipation and between TV-watching and constipation (table 3.3.3). Additional adjustment for overweight and TV-watching did not have any influence on the results between adherence to a 'Health conscious' or 'Western-like' dietary pattern and constipation between 24 and 48 months of age (data not shown).

DISCUSSION

This study shows that high adherence to a 'Western-like' dietary pattern is longitudinally associated with constipation, which was independent of the presence of overweight or sedentary behavior in pre-school children. Interestingly, adherence to a 'Health conscious' dietary pattern was only associated with a lower risk of constipation at 24 months whereas no association was found between overweight and constipation and between sedentary behavior and constipation.

The association between high adherence to a 'Western-like' dietary pattern and an increased prevalence of constipation in childhood can be explained by several components. This dietary pattern was characterized by foods with high fat content (table 3.3.1). In healthy adults, it is known that infusion of fat into the small intestine reduces gastric emptying and is associated with lower intestinal motility that may be a trigger for constipation [32]. Furthermore, studies show that foods high in fat content causes gut problems in subjects with the irritable bowel syndrome (IBS) [33-34].

High intake of confectionery and sugar containing beverages have been shown to be associated with poor diet quality [35]. In children, high intake of confectionery and sugar containing beverages may lead to early satiety which may cause poor compliance to meals containing starchy foods and vegetables. In addition, another study in children aged 9-18 months demonstrated that children who were frequently fed confectionery and sugar containing beverages had less frequent intakes of healthy foods as fruit, vegetables, potatoes and bread [36]. Hence, this leads to a lower dietary fiber intake but may also reflect a less regular eating pattern.

Strikingly, we did not find a longitudinal association between adherence to a 'Health conscious' dietary pattern and constipation in childhood since the effect only concerned constipation at the age of 24 months. The 'Health conscious' dietary pattern was characterized by high intake of fruits, vegetables, potatoes, starchy foods and legumes. Since these food products have high dietary fiber content, we also expected a longitudinal protective effect of high adherence to this dietary pattern and childhood constipation. The role of dietary fiber in very young children with constipation is controversial. There are concerns that a high-fiber diet in children under the age of 5 years may lead to growth faltering due to decreased energy density of the diet and altered mineral absorption [37]. However, these concerns are not well supported by evidence [37]. Besides, studies have shown that constipation in children was associated with low dietary fiber intake [5, 38] and low consumption of fruit and vegetables[39]. Although the odds ratios implied that this dietary pattern was overall associated with a lower prevalence of constipation, this effect was not statistically significant in the long-run. An explanation for this short term impact of the 'Health conscious' diet might be that a healthy diet at pre-school age may change more towards a diet with components of a 'Western-like' dietary pattern when the child gets older. Indeed, from the Bogalusa Heart Study it is known that the intake of sugar-sweetened beverages, snacks and confectionary increases during childhood with an overall decrease in diet quality over the years[40]. This may weaken our association between a 'Health conscious diet' and a lower prevalence of constipation in later childhood in our study.

Several studies reported an increased prevalence of overweight or obesity in children with constipation [8-10, 41]. Lower prevalence of overweight is associated with high dietary fiber consumption [12] whereas high prevalence of overweight is associated with high consumption of sugar containing beverages [42]. Nonetheless, the association between the dietary patterns and constipation was not influenced by the presence of overweight in our study. Also, we were not able to confirm the association between overweight and constipation. This might be explained by the fact that most studies concerning overweight and constipation in children have been performed in a secondary-care setting [8-10, 41] and the association between overweight and constipation might not be so apparent in primary care- or population-based studies.

Although increasing physical activity has shown to be effective in the amelioration of symptoms of constipation in adults [43], other studies regarding the association between physical activity and constipation in adults show inconsistent results.[44, 45] However, the role of sedentary behavior or physical activity in constipation is very much understudied in the pediatric population. Only two studies on physical activity and constipation in children have been published. One study reported that sedentary time during a school day rather than moderate physical activity time was significantly associated with low defecation frequency in children aged 10-18 years [11]. In another study among children aged 7-10 years, constipation was more prevalent in children with high physical activity levels [38]. Although these studies can be barely compared with our study group because it concerns different age groups, we found no association between sedentary behavior, as measured by at least 2 hours of TV watching per day, and constipation. Nonetheless, we did not have comprehensive data on physical activity thus our study precludes final conclusions on physical activity and constipation in children.

The strength of this study is the use of a large-scale and population-based study group. However, a possible drawback of this study can be that most data was obtained by parental-derived questionnaires and no additional information from medical records or physical examinations was available. Therefore, some subjects may be misclassified concerning the outcome of constipation. However, only if this misclassification is also related to the child's diet, sedentary behavior or overweight, this misclassification would have markedly influenced our results. We did not have data on potential metabolic or physiological causes of constipation. Although the prevalence of these diseases can expected to be low in our population, potential influence of e.g. food hypersensitivity or celiac disease on constipation can not be fully ruled out. Also, we did not have data on constipation at the age of 14 months. Parents of children with constipation may be more likely to change their child's diet towards a 'Health conscious diet'. However, only if this was also related to the presence of constipation at the age of 24 months onwards, this would have influenced our results.

Another challenge is the identification of dietary patterns in young children. This involves several decisions such as in the division of food items to food groups and the selected method to define these patterns and the labeling of these components that may have a influence on the final content of the dietary pattern in this study population [15]. The amount of variance (24.5%) explained by the dietary patterns is small but are comparable with previous studies [28, 46]. Nevertheless, this may have consequences on the generalizability of our results on diet and constipation in other populations. Also, the dietary patterns may vary among other ethnic groups and culture; therefore replication of our study in other ethnic populations is necessary.

Conclusion

In conclusion, a 'Western-like' dietary pattern is longitudinally associated with an increased prevalence of constipation up to 48 months, which was not mediated by the presence of overweight or sedentary behavior. A time-specific protective effect on constipation seems applicable when the child adheres to a 'Health conscious' dietary pattern.

Clinicians should not focus on one specific nutrient in case of childhood constipation, but a combination of dietary changes as eliminating fat-rich foods, sugar-containing beverages, confectionery and refined grains may be worth trying to explore in children with constipation. Further studies are needed to clarify whether the association between overweight and constipation is applicable to primary-care settings and to what extent physical activity and the 'Health conscious' diet play a role in childhood constipation in the long run.

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SUPPLEMENTARY MATERIAL

Table S3.3.1: Division of food items into food groups

Food group	Included food items
Refined grains	Waffles, rusk, crackers, currant bread, currant bun, white bread or baguette, croissant, cornflakes, low-fiber breakfast cereals
Whole grains	Brown or whole-bran bread, brown or whole-bran baguette, oatmeal, muesli, multigrain breakfast cereals
Starchy foods	Pasta, rice, couscous, bulgur.
Dairy	Full creamed, semi-skimmed or skimmed milk, full creamed, semi-skimmed or skimmed flavored milk, full creamed, semi-skimmed or skimmed yoghurt, yoghurt drinks, chocolate-flavored milk, full creamed, semi-skimmed or skimmed custard, milk pudding, mousse, porridge, full creamed, semi-skimmed or skimmed fromage frais, cream, infant milk feeding, cheese.
Fruit	Fruits and fruit compote (excluding fruit juice)
Soy substitutes	Soy milk, soy dessert, flavoured soy milk, soy-based meat substitutes.
Vegetables	Vegetables (including raw, cooked and baked vegetables).
Potatoes	Potatoes (excluding fried or baked potatoes)
Soups and sauces	Soup, mayonnaise (including half fat mayonnaise), salad cream, peanut sauce, ketchup and other sauces added to meals or snacks.
Savory and snacks	Chips, toasts with cheese or pâté, sausages rolls, spring rolls, meat rolls, meat croquettes, sateh, peanuts and nuts, hamburgers, chicken nuggets, fried chips or fried potatoes (i.e. French fries).
Confectionery	Dutch spiced honey cake, chocolate pasta, chocolate confetti, sweet sandwich fillings, ice cream, (added) sugar, cakes, cookies, biscuits, chocolates, pastry, pancakes, candy's.
Vegetable oils	Olive oil and other oils
Other fats	Full fat and low fat margarines, butter and cooking fats.
Fish	Fish
Shellfish	Shellfish
Meat	All processed and non-processed meat (except meat-containing snacks in between which are included in 'savory and snacks' food group)
Eggs	Eggs (baked or boiled egg)
Legumes	Legumes (i.e. white or brown beans, kidney beans, lentils, chick peas
Sugar-containing beverages	Soft drinks, fruit juice, lemonade.
Non-sugar containing beverages	Tea without sugar, water, diet soft drinks (i.e. without sugar)
Composite dishes	Ready-to-eat infant meals and ready-to-eat cooled or frozen meals.

Chapter 3.4

Cortisol diurnal rhythm and stress reactivity in constipation and abdominal pain

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J Pediatr Gastroenterol Nutr. 2011 Oct;53(4):394-400.

ABSTRACT

Cortisol as a marker for the individual stress response may play a role in functional bowel disorders.

The aim of this study was to assess whether diurnal cortisol rhythm and cortisol stress reactivity were associated with functional constipation and abdominal pain in infancy. This study was embedded in a subset of the Generation R Study, a prospective cohort study from fetal life onwards in Rotterdam, The Netherlands. Data of infants between 14 and 24 months of age (*n*=483) were used. Salivary cortisol diurnal rhythm and salivary cortisol stress reactivity after a Strange Situation Procedure were assessed at the age of 14 months. Data on functional constipation was available according to the ROME II criteria and data on abdominal pain on the basis of the Abdominal Pain Index were available from questionnaire data at 24 months. In the second year of life, 13% of the infants had functional constipation and 17% had abdominal pain. Only 4% had symptoms of both functional constipation and abdominal pain. Diurnal cortisol rhythm did not differ significantly between children with and those without functional constipation and abdominal pain. Cortisol stress reactivity was slightly higher in infants with abdominal pain than those without it but this was not statistically significant (aOR: 1.41; 95%CI: 0.46, 4.31). No association was found between the cortisol stress reactivity and functional constipation. Our results suggest that cortisol as a marker for stress does not play a major role in functional constipation or abdominal pain in infancy.

INTRODUCTION

Functional bowel disorders comprise of a large range of gastrointestinal symptoms such as irritable bowel syndrome (IBS), functional constipation and abdominal pain. These symptoms are frequently seen in Western countries[1]. The etiology of these disorders is multifactorial[1] and is a challenge for health care professionals.

Over recent years, various studies have suggested that functional bowel disorders underlie a complex interaction between psychosocial and physiologic factors through the hypothalamic pituitary-adrenal (HPA) axis[2]. The HPA axis regulates the synthesis and secretion of glucocorticoids, which helps to control the metabolism of energy substrates[2]. The most important glucocorticoid in humans is cortisol, which is secreted by the adrenal cortex in response to adrenocorticotrophic hormone (ACTH), which is itself released by the hypothalamus as an effect of the corticotrophic-releasing hormone (CRH)[3]. Studies show that psychological stressors activate the HPA axis[3]. This can have a direct effect on the motor function of the gastrointestinal tract[4, 5]. Also, chronic gastrointestinal pain can further enhance activation of the HPA-axis leading to a vicious cycle that might explain the persistence of the symptoms[5, 6]. While some studies have indeed shown elevated CRH levels and cortisol response in adults with IBS[7-9], others have claimed the opposite or provided evidence that cortisol responses are blunted in adults with IBS[10, 11].

However, psychological stressors may affect individuals differently, and since the HPA axis is still developing during childhood[6], results on IBS and stress in adults cannot be extrapolated to the pediatric population with functional bowel disorders. Because studies with respect to HPA-axis activity and functional bowel disorders in children are extremely scarce, we tested whether infants with functional constipation and abdominal pain, have an abnormal profile of cortisol after awakening, throughout the day and in response to a mental stressor.

METHODS

Participants and study design

This study was embedded in the Generation R Study; a prospective cohort study from early fetal life onwards which has been described in detail previously[12, 13]. An ethnically homogeneous subgroup of Dutch infants was randomly selected from the total cohort to prevent possible confounding or effect modification by ethnicity. Infants were born between February 2003 and August 2005, and n=1108 parents gave consent for postnatal follow-up of their child. The study was approved by the medical ethical review committee at Erasmus University Medical Centre, Rotterdam, the Netherlands.

Collection of salivary cortisol samples

At the age of 14 months, parents visited the Generation R research center. Prior to this visit parents were asked to collect five saliva samples (Salivette sampling devices, Sarstedt, Rommelsdorf, Germany) from their infant, and to note the sampling times during a normal routine weekday at home: immediately after awakening (mean±SD: 07:50 am±56 min), 30 minutes later (mean±SD: 08:25 am± 56 min), between 11 am and 12 pm (mean±SD: 11:52 am±0:32 min) , between 3 and 4 pm (mean±SD: 15:49 pm±39 min) and at bedtime (mean±SD: 19:33 pm±57 min). Parents received detailed written instructions with pictures concerning the saliva sampling and were asked to keep the samples stored in a freezer until they visited the research centre.

To assess how the infant copes with stress, the Strange Situation Procedure (SSP) was used during the visit in the Generation R research centre. This is a validated procedure described in detail by Ainsworth et al[14]. Briefly, the procedure consisted of seven episodes of 3 minutes each and is designed to evoke mild stress in the infant evoked by I) the unfamiliar lab environment, II) a female stranger entering the room and engaging with the infant, and III) the parent leaving the room twice[14]. The SSP took place for all participants between 8:40 am and 15:41 pm at weekdays (mean±SD: 11:31 am±2 hrs). The saliva samples were collected at three time points: prior to, directly after the SSP, and 15 minutes later by a research assistant. The infants were supposed not to eat or drink 30 minutes before sampling.

Missing data after the SSP was due to technical or procedural problems. Reasons of non-response were lack of time and failure to obtain saliva samples because the infant was not familiar with pacifiers.

Samples were centrifuged and stored at -80ºC and were sent on dry ice in a single delivery to the laboratory of the Department of Biological Psychology at the Technical University of Dresden. Subsequently, the cortisol levels were assessed by using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficient of variation were <7% and 9%.

To assess the cortisol stress reactivity, a delta was calculated between the last sample (15 minutes post-SSP) and the first sample (pre-SSP). The second assessment, just after the SSP, was not used, as it was too close to the onset of stress. Since we assumed that the direction of response of a body function depends to a large degree on the initial level, analyses were adjusted for cortisol levels assessed prior to the SSP.

To assess the total cortisol secretion during the day and to take account of the differences between separate cortisol measurements within each child and the time of the measures from baseline, the area under the curve (AUC) was estimated by calculating the curve of the cortisol measurement in nmol/L on the y-axis and the time between the measurements on the x-axis. To adjust for differences in the duration of the day of measurement, the AUC was divided by the number of hours between the first and the final saliva collection. This method has been described in detail by Pruessner and colleagues[15] and Watamura et al[16], and has successfully been used in previous studies[17, 18]. The cortisol awakening response (CAR) was estimated as the difference between the cortisol concentrations at awakening and 30 minutes thereafter as described by Kunz-Ebrecht et al[19]. As a measure of circadian cortisol decline, the slope was calculated by fitting a linear regression line for each child that predicted the cortisol values from time since awakening by using the first and last saliva samples and at least one other cortisol sample.

Functional constipation

In the second year of life, each child's stool pattern was assessed by using a questionnaire. Accordingly, functional constipation in the second year of life was defined according to symptoms of ROME II[20]. To avoid the influence of metabolic disorders, infants were excluded in the analyses in case of the following: I) presence of a congenital heart condition, II) anemia in the past year or III) growth retardation defined as height < -2SD based on the Netherlands growth curves of 12-24 months infants[21].

Abdominal pain

A binary definition was defined as the presence or absence of any abdominal pain in the previous 3 months in the second year of life. Additionally, the severity of abdominal pain was classified according to an adapted version of the Abdominal Pain Index as described previously by Walker et al[22]. Parents were asked about the frequency of the pain episodes that was rated over the previous 3 months on a 5 point-scale (ranging from 0=not at all to every day[5]). The daily frequencies of the pain episodes were assessed on a 4-point scale (none (1), 1-2 times a day, 3-6 times a day, and throughout the day (4)). The duration of the pain episode was rated on a 4-point scale (a few minutes (1), about half an hour, a few hours, all day (4)). Finally, parents indicated the intensity of the abdominal pain on a 10-point scale (1= no pain and 10=the most pain possible). The five pain ratings were summed and considered as the index of abdominal pain.

Covariates

Prenatal questionnaires completed by the mother, included information on mother's educational level, parity, maternal BMI, maternal smoking and maternal alcohol consumption. Data on gender, birth weight, gestational age and birth outcomes were available from obstetric records assessed in mid-wife practices and hospital registries[13]. Breast-feeding duration was available from questionnaire data filled in when the child was aged 6 and 12 months. The level of parental stress in the child's second year of life was assessed using the Nijmeegse Ouderlijke Stress Index—Kort (NOSIK)[23], the Dutch version of the Parenting Stress Index—Short Form, which has been shown to be reliable and valid[24]. The NOSIK comprises two domains consisting of 25 items; parenting stress due to parent factors and parenting stress due to child factors. Only the items on the parent domain were available in this study (*n*=15). Items were assessed on a 4-point scale and the scores were summed and divided by the number of items that has been filled in. Higher scores indicate greater levels of parental stress.

Population of analysis

From the 882 infants who participated in the Generation R Focus Study and visited our research centre between June 2004 and November 2006, information on more than one home saliva samples was available in 483 infants. During the SSP procedure, 442 infants had more than one saliva samples available and were eligible for analysis. Non-response analysis showed that the prevalence of functional constipation and abdominal pain was not different between infants with and without cortisol measurement (8.3% vs. 8.1% and 7.6% vs 7.6% respectively). Mothers of infants with no cortisol measurements were slightly more often smokers during pregnancy (29% vs 18%) and slightly more often lower educated (3% vs 1% low education). Infants with no cortisol measurement were slightly more often girls (52% vs 44%) and were slightly more often breast-fed for longer than 6 months (46% vs 36%). No difference between infants with and without cortisol measurement was found on birthweight (3517 vs 3509 grams), gestational age (40.1 vs 40.1 weeks), parity (61% vs 68% nulliparous) and parental stress score (0.26 vs 0.25). To prevent bias associated with attrition, missing data of the infants who had at least more than one saliva sample available (either from home sampling or during the SSP, *n*=483) were multiple imputed (*n*=5 imputations) on the basis of the correlation between each variable with missing values and the other patient characteristics as described previously by Sterne et al[25]. To obtain the desired effect sizes and standard errors, data were analyzed in each data set separately. Subsequently, the results of the 5 imputed analyses were pooled and reported in this paper.

Statistical analysis

Differences in characteristics between infants with and without functional constipation and abdominal pain were tested with Chi^2 test for categorical variables and the Mann-Whitney U test for continuous variables. To assess how diurnal cortisol rhythm and cortisol reactivity were associated with functional constipation and abdominal pain, logistic regression analyses were performed with functional constipation and abdominal pain as dependent variables. Linear regression analyses were performed with the abdominal pain index as dependent variable (normally distributed). Tests for linear trend were carried out fitting the indicators of cortisol diurnal rhythm and stress response as a continuous variable. To test for nonlinear trends, a quadratic term was added to the model that included the linear term. Since both the linear term and the quadratic term were not statistically significant (Table S3.4.1), analyses were performed after stratification of AUC, CAR, cortisol slope and delta stress into tertiles.

Additional adjustment for potential confounders such as gender, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration and parental stress, were on the basis of literature followed by the change in effect size (i.e. ≥10% change in regression coefficient). Effect modification by gender was evaluated by adding the product-term of cortisol variables and gender (e.g. AUC*gender) as independent variable to the model.

Results were reported as odds ratios (ORs) and 95% confidence interval (95%CI) for the analyses on abdominal pain and functional constipation and as regression coefficients (β) and 95% confidence interval (95% CI) for the analyses on abdominal pain index. A *p*-value <0.05 was considered as statistically significant. Statistical analyses were carried out by using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Patient characteristics

Patient characteristics are presented in table 3.4.1. Out of 483 infants with cortisol data, 13% and 17% had functional constipation and abdominal pain respectively. Four percent of the infants had symptoms of both functional constipation and abdominal pain. The mean±SD index for abdominal pain was 6.61±1.61. The mean±SD age of cortisol sampling during the SSP and at home throughout the day was 14.5±0.87 and 14.4±1.07 months.

Diurnal rhythm

Mean±SD cortisol levels at time of awakening was 15.80±9.70 nmol/L in the total study group. Between 11 and 12 pm this decreased to 7.26±6.45 nmol/L, declining further to 3.42±5.80 nmol/L at bedtime.

Similar diurnal patterns of cortisol were found in infants with functional constipation and abdominal pain (figure 3.4.1). Compared to infants with no functional constipation or abdominal pain, no significant difference was found in AUC, cortisol awakening response and circadian cortisol decline (table 3.4.2). There was no significant association found between indices of diurnal cortisol rhythm and the abdominal pain index (table 3.4.3). Additional adjustment for age of cortisol sampling, gender, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration and parental stress did not change these results (data not shown).

Table 3.4.1: Maternal and child characteristics (*n*=483)

		Abdominal pain			Functional constipation		
		Yes *n*=80	No *n*=403	*p*-value	Yes *n*=62	No *n*=421	*p*-value
Mother							
Nulliparous *n* (%)		46 (58%)	227 (56%)	0.48	31 (50%)	242 (57%)	0.16
Educational level of mother *n* (%)				0.09			0.41
	Low	7 (9%)	31 (8%)		6 (10%)	33 (8%)	
	Mid	65 (81%)	274 (68%)		44 (71%)	295 (70%)	
	High	7 (9%)	97 (24%)		13 (21%)	93 (22%)	
Maternal smoking *n* (%)		21 (26%)	105 (26%)	0.63	14 (23%)	112 (27%)	0.36
Maternal alcohol consumption *n* (%)		41 (51%)	216 (54%)	0.40	28 (45%)	230 (55%)	0.26
NOSIK score median (range)		0,18 (0.0, 1.8)	0,18 (0.0, 1.0)	0.46	0,18 (0.0, 1.8)	0,18 (0.0, 1.0)	0.57
Body Mass Index (mean±SD)		24±3.4	25±4.3	0.04	24±3.4	24±4.3	0.47
Child							
Male N (%)		44 (55%)	200 (50%)	0.40	33 (53%)	211 (50%)	0.58
Birth weight, grams (mean±SD)		3400±580	3459±534	0.28	3456±488	3453±547	0.61
Gestational age, weeks (mean± SD)		40.0±1.7	40.0±1.7	0.64	39.8±1.5	40.0±1.7	0.41
Body Mass Index (mean±SD)		17.3±1.7	17.3±1.4	0.65	17.3±1.5	17.3±1.4	0.42

Table 3.4.2: Logistic regression analyses on categories of diurnal cortisol rhythm and stress reactivity in children with and without functional constipation and abdominal pain (*n*=483).

	Abdominal pain OR (95%CI)	Functional constipation OR (95%CI)
Delta stress*		
≤ -1.40 nmol/L	Reference	Reference
-1.39 – 2.53 nmol/L	1.08 (0.39, 2.99)	0.92 (0.40, 2.09)
≥ 2.53 nmol/L	1.41 (0.46, 4.31)	0.88(0.38, 2.04)
CAR		
≤ -2.94 nmol/L	Reference	Reference
-2.93 – 0.28 nmol/L	1.13 (0.43, 2.93)	1.35 (0.55, 3.31)
≥ 0.29 nmol/L	0.72 (0.27, 1.97)	0.93 (0.40, 2.17)
Slope		
≤ -1.28 nmol/L	Reference	Reference
-1.27 – -0.68 nmol/L	1.21 (0.28, 2.50)	1.39 (0.54, 3.62)
≥ -0.67 nmol/L	1.66 (0.74, 3.72)	1.19 (0.39, 3.59)
AUC		
≤ 6.01 nmol/L	Reference	Reference
6.02 – 9.45 nmol/L	0.98 (0.40, 2.44)	0.97 (0.25, 3.70)
≥ 9.46 nmol/L	1.04 (0.40, 2.71)	1.37 (0.44, 4.23)

OR: odds ratio; 95%CI: 95% Confidence interval; *Adjusted for baseline cortisol levels.

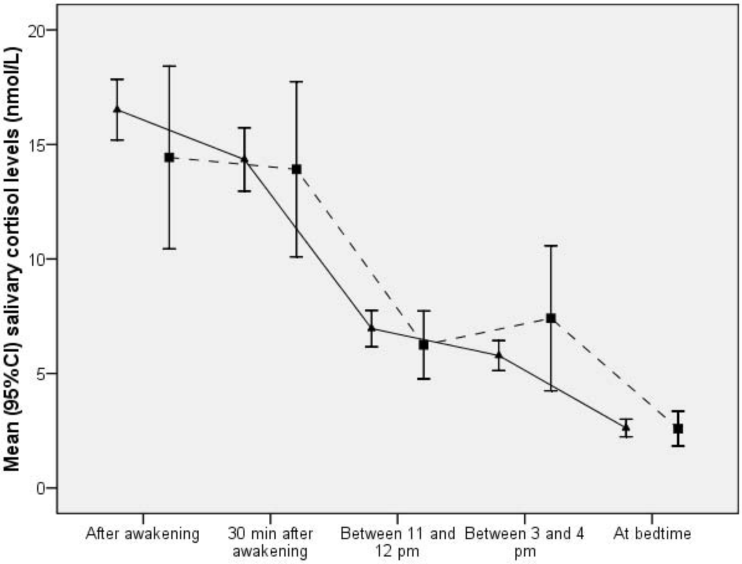

Figure 3.4.1a: Circadian cortisol rhythm according to infants with and without functional constipation (-▲- No constipation; --■-- Functional constipation).

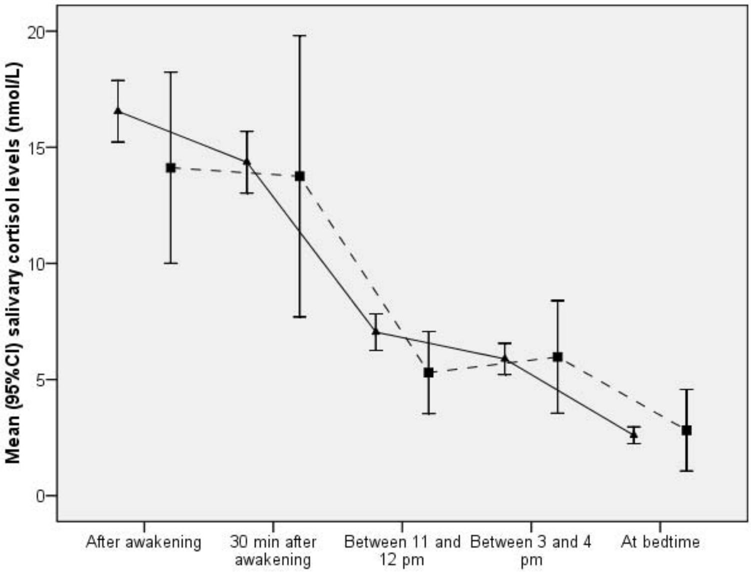

Figure 3.4.1b: Circadian cortisol rhythm according to infants with and without abdominal pain (-▲- No abdominal pain; --■-- Abdominal pain).

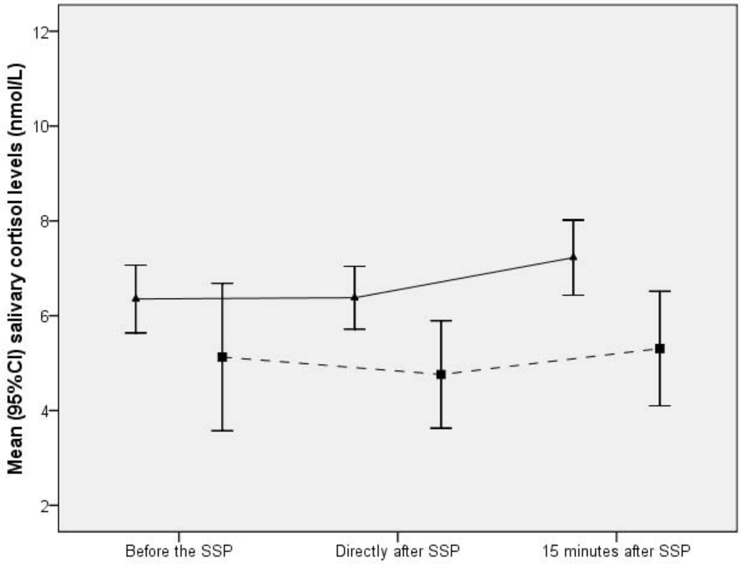

Figure 3.4.2a: Salivary cortisol response after a Strange Situation Procedure (SSP) in infants with and without functional constipation (-▲- No constipation; --■-- Functional constipation).

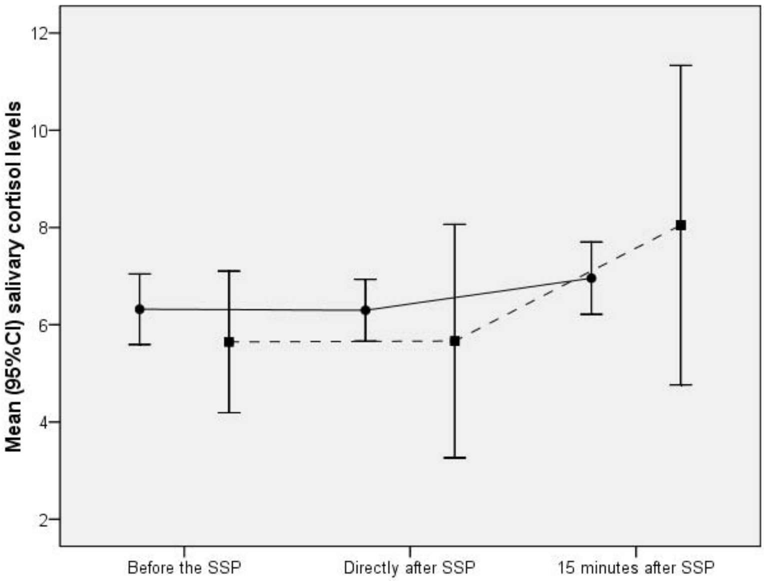

Figure 3.4.2b: Salivary cortisol response after a Strange Situation Procedure (SSP) in infants with and without abdominal pain (-▲- No abdominal pain; --■-- Abdominal pain).

Stress response

Before the SSP, mean±SD levels in the study group was 6.26±5.21 nmol/L. Directly after the SSP, mean±SD cortisol levels remained relatively stable to 6.19±5.21 nmol/L but increased to 7.18±6.29 nmol/L 15 minutes after the SSP. The increase in cortisol levels after the SSP was higher in infants with functional constipation and abdominal pain (figure 3.4.2) but this was not statistically significant after adjustment for baseline cortisol levels (table 3.4.2). No statistically significant association was found between stress reactivity and the abdominal pain index (table 3.4.3). Additional adjustment for age of cortisol sampling, gender, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration and parental stress did not alter the results (data not shown). No statistical interaction with gender was found in the analyses between cortisol stress response, diurnal rhythm and functional constipation and abdominal pain (data not shown).

DISCUSSION

In this study, we demonstrated that the diurnal rhythm of cortisol and stress reactivity is neither significantly associated with functional constipation nor with abdominal pain.

The effect of the HPA axis in young infants with functional bowel disorders has been studied relatively little. Even the results reported in adults are conflicting. For example, while Fitzgerald et al[26] recently showed that controls had a higher cortisol response after acute stress than women with IBS did, Chang et al[27] showed that IBS patients had higher cortisol levels than controls, but that there was no association with baseline cortisol levels. Similarly, an earlier study in children with recurrent abdominal pain found that cortisol levels were blunted relative to the levels in controls[28].

A recent study among young children with IBS demonstrated that cortisol stress reactivity is related more to adverse life events than to the presence of IBS in children[29], suggesting that the association between cortisol and functional bowel disorders in previous studies might be confounded by psychological status. Several studies have indeed suggested that the prevalence of depression and anxiety disorders is higher in adults with functional bowel and chronic life stress can contribute to functional bowel disorders[30-33]. Although the amount of stress is difficult to quantify in very young children, Dorn et al showed that scores on social stress were found in children with anxiety scores were similar to those children with recurrent abdominal pain[34].

Since blunted and increased cortisol levels have both been shown in patients with functional bowel disorders, we expected to find differences in cortisol levels in infants with functional constipation and abdominal pain compared to those without these com-

Table 3.4.3: Linear regression analyses on categories of diurnal cortisol rhythm, stress reactivity and the abdominal pain index (*n*=483).

	Abdominal Pain Idex β (95%CI)
Delta stress*	
≤ -1.40 nmol/L	Reference
-1.39 – 2.53 nmol/L	0.16 (-0.58, 0.89)
≥ 2.53 nmol/L	0.01 (-1.02, 1.03)
CAR	
≤ -2.94 nmol/L	Reference
-2.93 – 0.28 nmol/L	0.12 (-0.58, 0.82)
≥ 0.29 nmol/L	0.04 (-0.66, 0.74)
Slope	
≤ -1.28 nmol/L	Reference
-1.27 – -0.68 nmol/L	0.29 (0.09, 0.48)
≥ -0.67 nmol/L	0.10 (-0.44, 0.63)
AUC	
≤ 6.01 nmol/L	Reference
6.02 – 9.45 nmol/L	0.03 (-0.58, 0.63)
≥ 9.46 nmol/L	0.02 (-0.70, 0.73)

β: Regression coefficient indicating the mean difference in abdominal pain score compared to the reference group; 95%CI: 95% Confidence interval; *Adjusted for baseline cortisol levels.

plaints. However, HPA-response in people with functional bowel disorders might vary according to their psychological condition. For instance, IBS patients without psychiatric co-morbidity are more sensitive to stress than those with severe depression[35]. Similarly, because the HPA-axis is still developing early in life and has a high intra-individual instability[36], the influence of the HPA axis in early childhood constipation and abdominal can be difficult to explore. Intervention studies also suggest that colon motility increases not decreases, after administration of CRH to IBS subjects[37]. If cortisol levels were inversely associated with functional constipation in a subset of our study group but elevated cortisol levels could also occur in infants who had these symptoms, the association might be cancelled out.

Other neurological pathways such as the autonomic nervous system have also been suggested to play a role in functional bowel disorders. Not only has increased activity of the autonomic nervous system been found in adults with IBS[38, 39], differences in autonomic activity in response to stress have also been found in children with and without chronic abdominal pain[40]. Since the autonomic nervous system respond to stress much faster than the HPA-axis[41], this may have a more prominent role in functional bowel disorders, but this needs further elucidation.

The strength of this study is that the study population was not selected on the basis of the medical care they had received. Due to reverse causality, a selected population may increase bias because children with abdominal pain seeking medical care may already have elevated cortisol levels due to the constant pain or symptoms.

Despite this strength of the study, different methodological considerations must be taken into account when interpreting our results. First, we used criteria from ROME II[20] to define functional constipation and we were not able to specify this outcome according to the most recent evidence-based ROME III criteria[42]. As a result, our results preclude conclusions on the role of the HPA-axis in more severe functional constipation according to the ROME III criteria.

Second, studies have also shown that cortisol reactivity to stress collapses with increasing age and it has been proposed that the difference in cortisol levels in response to stress become smaller as a child ages[41]. As we had only 17% cases with abdominal pain, the small difference in cortisol stress response that we were not able to detect as statistically significant might be influenced by the small sample size. On the other hand, differences in cortisol stress reactivity are in accordance with results from other studies in the same age group but these studies were much smaller than our study group[43,44]. Third, the cortisol stress response and its physical changes is thought to be time-limited[6]. The time-lag between cortisol sampling and the assessment of gastrointestinal symptoms in our study might, therefore, explain our results as the association between cortisol secretion and functional bowel disorders might only be applicable when it is measured in short succession. At last, differences between our study and results from other studies may be due to different types of cortisol measurement (salivary, urinary, total serum cortisol). Nevertheless, it is thought that only the free (unbound) forms of cortisol are biologically active and we used salivary cortisol measurements which highly correlate with free (unbound) serum cortisol levels in healthy subjects[45].

Conclusion

In conclusion, these data do not support the hypothesis that cortisol plays a significant role in functional constipation and abdominal pain in infants aged 24 months. Further studies should clarify whether other branches of the brain-gut axis may be involved in these conditions and whether there is any influence of adverse life events.

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SUPPLEMENTARY MATERIAL

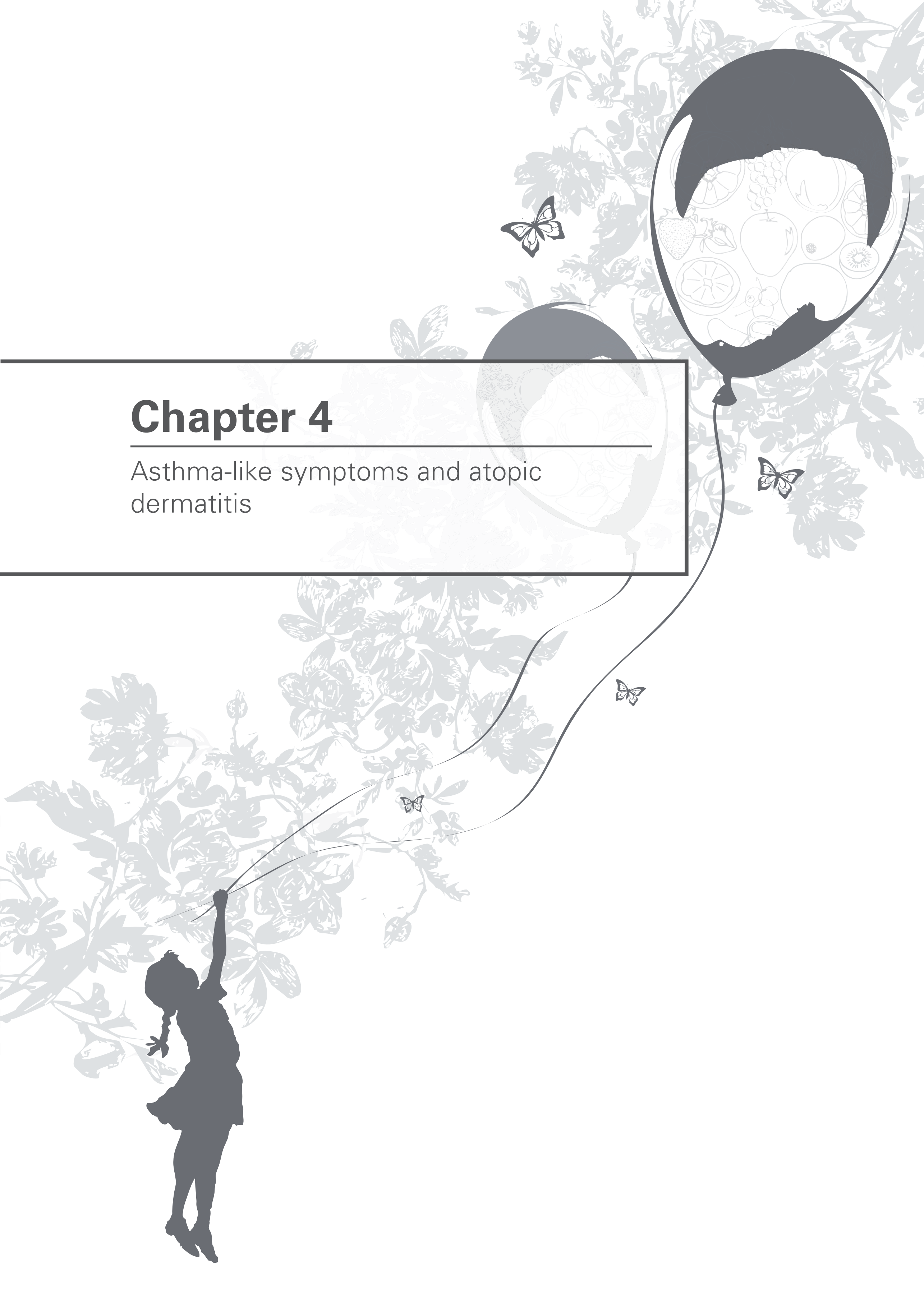
TableS3.4.1: Analyses on diurnal cortisol rhythm and stress reactivity in children with and without functional constipation and abdominal pain by using a linear term and a quadratic term (*n*=483).

	Abdominal pain OR (95%CI)	Functional constipation OR (95%CI)	Abdominal pain index β (95%CI)
Delta stress*			
Linear term	1.04 (0.98, 1.10)	0.99 (0.93,1.05)	-0.005 (0.63, 1.56)
Quadratic term	1.00 (0.99, 1.00)	0.99 (0.99, 1.00)	0.00 (-0.002, 0.002)
CAR			
Linear term	0.98 (0.91, 1.05)	0.99 (0.93, 1.06)	0.001 (0.93, 1.08)
Quadratic term	1.00 (0.99, 1.01)	1.00 (0.98, 1.01)	0.00 (-0.07, 0.07)
Slope			
Linear term	1.40 (0.82, 2.40)	1.15 (0.60, 2.00)	0.077 (0.65, 1.79)
Quadratic term	0.98 (0.63, 1.52)	0.61 (0.22, 1.66)	-0.013 (-0.17, 0.14)
AUC			
Linear term	1.02 (0.63, 1.65)	1.18 (0.64, 2.17)	0.008 (0.71, 1.44)
Quadratic term	1.04 (0.54, 1.99)	1.22 (0.37,4.05)	-0.19 (0.60, 1.63)

OR: odds ratio per unit of the cortisol measure (ie. deltastress, CAR, slope or AUC); 95%CI: 95% Confidence interval; *Adjusted for baseline cortisol levels. β: Regression coefficient indicating the mean difference in abdominal pain score per unit of the cortisol measure.

Chapter 4

Asthma-like symptoms and atopic dermatitis



Chapter 4.1

Folate and vitamin B-12 during pregnancy and wheezing and atopic dermatitis in the offspring.

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J Nutr. 2012 Apr;142(4):731-8.

ABSTRACT

Recent studies suggest that in utero exposure of methyl donors influences programming of the fetal immune system in favor of development of allergic disease. The aim of this study was to assess whether the *MTHFR* C677T polymorphism, folic acid supplementation, and circulating folate and vitamin B12 concentrations during pregnancy were associated with wheezing, shortness of breath, and atopic dermatitis in offspring. The study was a population-based birth cohort from fetal life until 48 months (n = 8742). The use of folic acid supplementation during pregnancy was assessed by questionnaire. Plasma folate and serum vitamin B12 concentrations and the *MTHFR* C677T polymorphism were available from blood collected in early pregnancy. Atopic dermatitis, wheezing, and shortness of breath in the offspring were assessed by parental-derived questionnaires at 12, 24, 36, and 48 months. Maternal folate >16.2 nmol/L and vitamin B12 >178 pmol/L were positively associated with the development of atopic dermatitis [aOR: 1.18 (95%CI: 1.05, 1.33) and aOR: 1.30 (95%CI: 1.06, 1.60) for the highest quartiles of folate and vitamin B12 concentrations, respectively] but not with wheezing and shortness of breath. Maternal *MTHFR* C677T polymorphism and folic acid supplementation were not associated with wheezing, shortness of breath, and atopic dermatitis. No interactions were found by age, family history of atopy, folic acid supplementation, *MTHFR* C677T polymorphism, or maternal smoking (p-$_{interaction}$ > 0.10). High folate and vitamin B12 levels during pregnancy are associated with increased prevalence of atopic dermatitis in the offspring. Potential risks of high folate and vitamin B12 concentrations on allergic outcomes should be evaluated when discussing mandatory fortification programs.

INTRODUCTION

In 1976 Smithells et al.[1] suggested an association between low maternal folate status and the occurrence of Neural Tube Defects (NTD). In the early 1990s, this hypothesis was supported by two randomized trials showing that folic acid supplementation reduced the risk of NTD[2, 3]. The firm scientific evidence of these studies has led to the general advice for women planning pregnancy to use a folic acid supplement from at least 1 months before until 3 months after conception in addition to consuming a healthy diet[4, 5]. Furthermore, fortification of food with folic acid to reduce the number of NTD has been introduced in the United States and Canada and is now increasingly being considered by other countries as well[4].

Despite long-term research, the precise mechanisms underlying the beneficial effects of folic acid remain unknown[5]. Folate has many functions and along with vitamin B12 it plays a critical role in the homocysteine pathway, which is important for protein, lipid, and DNA synthesis. In addition, folate provides, with vitamin B12 as cofactor, methyl groups for the synthesis of methionine and its derivate, *S*-adenosyl-methionine. The latter is the most important methyl donor in the human body and is involved in epigenetic mechanisms[5]. Initial evidence has shown that varying dietary inputs to the methionine/folate cycle during the periconceptional period can lead to widespread epigenetic alterations in the offspring influencing health-related phenotypes[6]. Also, the *MTHFR* C677T polymorphism may play a role, because the TT genotype renders individuals more susceptible to epigenetic alterations in response to low folate status associated with this genotype[7].

Because folic acid may have a function on an epigenetic level, potential adverse effects of folic acid have also been debated[8]. Hollingsworth et al.[9] found differential methylation in lung tissue between the offspring of mice fed either a high-methyl or low-methyl donor diet. They showed that an increased supply of folic acid and vitamin B12 in mice was associated with an increased risk of allergic airway disease that was mediated through epigenetic mechanisms. Likewise, other studies have demonstrated that epigenetic mechanisms may influence gene expression regulating airway inflammation[10] and polarization of Th1/Th2 cells[11]. Also, recent epidemiological studies on the association between folic acid supplementation during pregnancy and the increased risk of allergic disease support the hypothesis that epigenetics may be involved in the development of allergies [12, 13].

In view of these findings and of the current recommendations and policy considerations concerning folic acid fortification in several countries, we aimed to assess whether folic acid supplementation, plasma folate, and serum vitamin B12 concentrations during pregnancy were associated with the development of wheezing, shortness of breath, and atopic dermatitis in the offspring up to 48 months of age. A second aim was to assess whether the maternal *MTHFR* C677T polymorphism was associated with wheezing, shortness of breath, and atopic dermatitis. The last aim was to assess whether any interaction with age, family history of atopy, folic acid supplementation, maternal smoking, and *MTHFR* C677T polymorphism was observed for the above associations.

METHODS

Study design.

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood and was previously described in detail[14]. In total, 8742 mothers with a delivery date between April 2002 and January 2006 provided consent for prenatal or postnatal follow-up and were included in this study (Figure S4.1.1). Ethic approval for the study was obtained from the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam, The Netherlands. Written informed consent was obtained from all participants.

Assessment of periconceptional folic acid exposure.

At enrollment, we assessed folic acid exposure during pregnancy by asking the following question by questionnaire: "Have you taken folic acid, either as a single supplement or as part of multivitamin supplement during the first trimester?" Self-reported maternal folic acid intake was classified as follows as previously described [15]: *1*) no exposure, defined as no folic acid use at all; *2*) periconceptional exposure, defined as the start of a folic acid supplement prior to conception; and *3*) start of folic acid supplement within first 10 weeks of conception (i.e., from the moment that pregnancy was recognized but in any case before 10th week of pregnancy). Approximately 15% of the mothers used folic acid as part of a multivitamin supplement. The doses of folic acid in these multivitamins were comparable with single supplements of folic acid (between 0.4 and 0.5 mg/d), which is the recommended dose of folic acid for women in the preconceptional phase in The Netherlands. No valid information on dietary intake of B vitamins from food was available.

Plasma folate and serum vitamin B12 concentrations.

Nonfasting blood plasma (EDTA) and serum samples were collected during the first trimester of pregnancy (13.5 ± 2.0 week of gestation). After sampling, these were transported to the regional laboratory for storage at −80°C (STAR-MDC, regional laboratory). Processing aimed to be finished within a maximum of 3 hours after venous puncture. In 2008, all EDTA samples were transported to the Department of Clinical

Chemistry, Erasmus Medical Centre, Rotterdam. Subsequently, plasma folate and serum vitamin B-12 concentrations were analyzed using microparticle-enhanced immunoassay on the AxSYM and Architect system (Abbott Diagnostics). The between-run CV for plasma folate were 8.9% at 5.6 nmol/L, 2.5% at 16.6 nmol/L, and 1.5% at 33.6 nmol/L, with an analytic range of 1.8–45.3 nmol/L. This CV for serum vitamin B12 was 3.6% at 142 pmol/L, 7.5% at 308 pmol/L, and 3.1% at 633 pmol/L, with an analytic range of 44–1476 pmol/L.

MTHFR C677T polymorphism.

Analyses of the maternal *MTHFR* C677T polymorphism (CC, CT, or TT genotype) were restricted to Western mothers (n = 4955) (Figure S4.1.1). Ethnicity was defined according to the classification of Statistics Netherlands, meaning that if both parents of the mother were born in The Netherlands, the ethnicity of the mother was defined as Dutch; if one of the parents was born in a country other than The Netherlands, that country applied; if parents of the mother were born in different countries other than The Netherlands, the country of the mother applied[16]. In addition, Western ethnicity was defined as Dutch, European (excluding Turkey), or American (excluding Latin American). Genotype and allele frequencies in this Western population were in Hardy Weinberg equilibrium (p = 0.68). Mothers were genotyped for the *MTHFR* C677T polymorphism (rs1801133) using a TaqMan allelic discrimination assay (Applied Biosystems) and Abgene QPCR ROX mix. The genotyping reaction was amplified using the GeneAmp PCR system 9600 [95°C (15 min), then 40 cycles of 94°C (15 s) and 60°C (1 min)]. The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems).

Assessment of childhood atopic dermatitis, wheezing, and shortness of breath.

Information regarding atopic dermatitis, wheezing, and shortness of breath was obtained using core questions from the validated International Study of Asthma and Allergies in Childhood[17] and doctor attendance for atopic dermatitis at the ages of 12, 24, 36, and 48 months. Parents were asked the following questions from the International Study of Asthma and Allergies in Childhood questionnaire: "Has your child had problems with a wheezing chest during the last year?," "Has your child had problems with tightness of the chest or shortness of breath during the past year?," and "Has your child had an itchy rash that came and went during the past year?"

The observed agreement (k) for wheezing and shortness of breath between measurement by questionnaire and by physician interview was 75% [k = 0.30) and 81%

(k = 0.39), respectively, as previously described[18]. The observed agreement for atopic dermatitis measured by questionnaire and physician interview was 79% (k = 0.44).

Covariates.

Several medical, behavioral, and socio-demographic characteristics obtained from a combination of pre- and postnatal questionnaires and community midwife and hospital registries as previously described in detail[14] were considered as potential confounders in the analyses of folate, vitamin B-12, and wheezing, shortness of breath, and atopic dermatitis. The selection of confounders was based on previous studies on determinants of folic acid supplementation[5] and asthma development[19]. The following variables were considered as potential confounders: *1*) maternal age at pregnancy; *2*) maternal BMI at inclusion; *3*) maternal educational level (low: no education, primary school, or <3 years of secondary school; mid: >3 years of secondary school; high: higher vocational training, bachelor's degree, or academic education)[20]; *4*) maternal ethnicity (Western: Dutch, European, or American vs. non-Western: Cape Verdian, Moroccan, Antillean, Surinam, Turkish, African, Asian, or Latin American)[16]; *5*) parity; *6*) infant's sex; *7*) infant's birth weight and gestational age at birth; *8*) any maternal smoking during pregnancy; *9*) any maternal alcohol consumption during pregnancy; *10*) duration of breast-feeding; *11*) any attendance of day care of the child in the first 24 months of the infant's life (yes vs. no); and *12*) parental atopic constitution, defined as any parental history of doctor-attended atopic dermatitis, asthma, hay fever, or allergy to house dust.

Statistical analysis.

First, independent Student's *t* test and Chi-square tests were used to test differences in characteristics between mothers who used folic acid and women who did not use a folic acid supplement during pregnancy.

To assess how folic acid supplementation during pregnancy and plasma folate and serum vitamin B12 concentrations were associated with wheezing, shortness of breath, and atopic dermatitis, logistic Generalized Estimating Equations (GEE) analyses were performed. Briefly, GEE analysis assesses the association by correction for the within-subject dependence as a result of the repeated observations on wheezing, shortness of breath, and atopic dermatitis[21]. Because the within-subject correlation coefficient for the outcome variables at the four time points were comparable (r = 0.2–0.4 for wheezing and shortness of breath and r = 0.3–0.5 for atopic dermatitis), an exchangeable working correlation structure was used for the GEE models. The primary independent variables in the GEE model were use of folic acid during pregnancy (0 = no, 1 = start within 10 weeks of conception, and 2 = periconceptional start), plasma folate concentration (after stratification into quartiles with the first quartile as reference), serum vitamin B12 concentration (after stratification into quartiles with the first quartile as reference) or *MTHFR* C677T polymorphism (CC genotype as reference). All crude models were adjusted for time (12, 24, 36, and 48 months). Subsequently, we created multivariate models including adjustment for potential confounders as maternal age, maternal BMI, ethnicity, family history of atopic constitution, parity, maternal educational level, maternal smoking, maternal alcohol consumption, gender, parity, daycare attendance, breast-feeding duration, and birth weight SD score. Additionally, to assess whether the associations among folate and vitamin B12 and wheezing, shortness of breath, and atopic dermatitis were different by age of the child or by the *MTHFR* C677T polymorphism, folic acid supplementation, maternal smoking, or parental atopic constitution, the statistical interaction was evaluated by adding the product term of the covariate (e.g., folate concentrations) and stratum (folate × stratum) as an independent variable to the models.

Non-response analysis showed that mothers who had no postnatal data on the child's health had lower mean±SD folate (14.7±8.3 nmol/L) and vitamin B-12 (183±92 pmol/L) concentrations during pregnancy relative to mothers who filled out the questionnaires concerning the child's health (19 ± 9 nmol/L and 191 ± 93 pmol/L, respectively; $p < 0.01$) and used folic acid supplements less frequently during pregnancy (54 vs. 81%; $p < 0.01$). To reduce potential bias associated with attrition, a multiple imputation procedure for all variables used in this study was performed ($n = 5$ imputations) (Figure S4.1.1; Table S4.1.1 and S4.1.2). The multiple imputation was based on the correlation between each variable with missing values with other participant characteristics as previously described[22]. GEE analyses were then separately performed in each of the five datasets to obtain the desired effect sizes and standard errors. OR's were pooled by taking the mean of the effect sizes of the five imputed datasets. The pooled standard errors were then calculated by using Rubin's rules[23]. The pooled regression results of the five imputed datasets were reported in this paper as OR and 95% CI or as mean±SD values. $p < 0.05$ was considered significant. Statistical analyses were carried out by using SPSS 17.0 for Windows.

RESULTS

Overall, 69% of the mothers used folic acid during early pregnancy. A history of asthma or other allergies was present in about one-half of the parents and was more often present in mothers who used folic acid during pregnancy (Table 4.1.1). The folate concentration during pregnancy was 17.6 ± 9.1 nmol/L and that of vitamin B12 was 188 ± 93 pmol/L. Mothers who took folic acid during pregnancy had significantly higher concentrations of plasma folate and serum vitamin B-12 and had a lower BMI relative to those without folic acid supplementation during pregnancy (Table 4.1.1). Folic acid

Table 4.1.1 Mother and child characteristics according to folic acid supplementation (*n*=8742)

	Folic acid exposure during pregnancy		
	no (*n*=2738)	yes (*n*=6004)	*p*-value
Child characteristics			
Gender, % boy	52%	50%	0.05
Birth weight, grams (mean±SD)	3338±565	3457±556	<0.01
Gestational age, weeks (mean±SD)	39.7±2.0	39.9±1.8	<0.01
Any daycare in first 24 months of life, %	92%	91%	0.37
Parental atopic constitution, %	46%	53%	<0.01
Breast-feeding, %			0.09
No breast-feeding	17%	12%	
Partial until 4 months	57%	60%	
Exclusive until 4 months	26%	28%	
Mother characteristics			
Western ethnicity, %	27%	70%	<0.01
Maternal age, years (mean±SD)	28±6	31±5	<0.01
Educational level. %			<0.01
Low	27%	6%	
Mid	55%	43%	
High	17%	51%	
Nullipara, %	44%	65%	<0.01
BMI, kg/m^2 (mean±SD)	26±5	24±4	<0.01
Plasma folate, nmol/L (mean±SD)	9.6±4.3	20.8±8.6	<0.01
Plasma vitamin B12, pmol/L (mean±SD)	177±91	195±96	<0.01
MTHFR C677T polymorphism, %*			0.96
CC	45%	46%	
CT	44%	43%	
TT	11%	10%	
Maternal smoking during pregnancy, %	34%	26%	<0.01
Maternal alcohol use during pregnancy, %	27%	45%	<0.01

*Restricted to Western mothers only (n=4955).

use during pregnancy was more often seen in mothers who were older, had a high educational background, were of Western ethnicity, and who had no multiple parities (Table 4.1.1). Children of mothers who used folic acid during pregnancy had a slightly higher birth weight than children of mothers who did not use folic acid supplementation (Table 4.1.1). Folate levels were lower in Western mothers who had the TT genotype (18.1 ± 8.2 nmol/L) than in mothers with the CC (19.6 ± 8.8; $p = 0.01$) or CT (19.2 ± 8.8; $p = 0.02$) genotype.

The highest prevalence of wheezing and shortness of breath during childhood was at the age of 12 months (39 and 34%, respectively) and this decreased to a prevalence of 22 and 18% at 24 months ($p < 0.01$). Wheezing prevalence remained stable at 15% at the ages of 36 and 48 months ($p = 0.91$) as did the prevalence of shortness of breath (12–13% at 36 and 48 mo) ($p = 0.21$). The prevalence of atopic dermatitis remained relatively stable during the first 24 months of life (28 and 30%) ($p = 0.27$), slightly decreased to 20% at the age of 36 months (p = 0.01), and remained stable afterwards at 25% (p = 0.18). Both periconceptional folic acid supplementation and folic acid supplementation within the first 10 weeks of conception were not significantly associated with an increased prevalence of wheezing, shortness of breath, or atopic dermatitis during childhood after adjustment for confounders (Table 4.1.2). No overall association was found between folate and vitamin B12 concentration during pregnancy and symptoms of wheezing and shortness of breath in childhood (Table 4.1.3 and Table 4.1.4). However, maternal plasma folate concentrations during pregnancy of at least 16.2 nmol/L were significantly associated with an overall increased prevalence of atopic dermatitis in children up to 48 months (Table 4.1.3). Also, maternal serum vitamin B12 concentrations during pregnancy of at least 178 pmol/L were significantly associated with an increased prevalence of atopic dermatitis in the offspring in the first 48 months of life (Table 4.1.4). Both associations were not explained by potential confounders (Tables 4.1.3 and 4.1.4; Table S4.1.3).

Time-specific effects of folate and vitamin B12 concentration on wheezing, shortness of breath, and atopic dermatitis revealed that the OR of maternal folate and vitamin

Table 4.1.2: Association between folic acid supplementation during pregnancy and wheezing, shortness of breath, and atopic dermatitis in the offspring (*n*=8742).

Maternal folic acid use	No use	Start within 10wk of conception	Start periconceptional
	(Reference)	OR 95%CI	OR 95%CI
Wheezing			
Crude*	1.00	0.94 (0.81, 1.07)	0.83 (0.66, 1.05)
Multivariate**	1.00	1.02 (0.90, 1.16)	0.99 (0.89, 1.09)
Shortness of breath			
Crude*	1.00	1.16 (0.86, 1.55)	1.02 (0.84, 1.24)
Multivariate**	1.00	1.16 (0.85, 1.57)	1.04 (0.84, 1.29)
Atopic dermatitis			
Crude*	1.00	1.16 (0.91, 1.48)	1.16 (0.98, 1.37)
Multivariate**	1.00	1.15 (0.90, 1.47)	1.17 (0.97, 1.40)

*Presents overall crude OR (95% confidence interval) adjusted for time/age. **Presents adjusted OR (95% CI) controlled for time, maternal ethnicity, parental atopic constitution, parity, maternal BMI, maternal age, breast-feeding duration, daycare attendance, maternal educational level, prenatal maternal smoking and alcohol consumption and fetal gender, birth weight standard deviation score derived from Generalized Estimation Equations.

Table 4.1.3: Association between maternal folate concentrations during pregnancy and wheezing, shortness of breath, and atopic dermatitis in the offspring (*n*=8742).

Maternal folate concentration	≤ 10.3 nmol/L	10.3 16.2 nmol/L	16.2 - 23.2 nmol/L	≥ 23.2 nmol/L
	(Reference)	OR 95%CI	OR 95%CI	OR 95%CI
Wheezing				
Crude*	1.00	0.93 (0.82, 1.06)	0.88 (0.70, 1.09)	0.86 (0.67, 1.11)
Multivariate**	1.00	1.00 (0.90, 1.11)	0.98 (0.85, 1.12)	1.02 (0.89, 1.18)
Shortness of breath				
Crude*	1.00	1.01 (0.84, 1.22)	0.97 (0.76, 1.23)	0.96 (0.74, 1.23)
Multivariate**	1.00	1.03 (0.87, 1.22)	0.98 (0.78, 1.23)	0.98 (0.79, 1.22)
Atopic dermatitis				
Crude*	1.00	1.13 (1.00 - 1.26)***	1.20 (1.05 -1.36)***	1.21 (1.07 - 1.38)***
Multivariate**	1.00	1.10 (0.98, 1.25)	1.16 (1.03, 1.32)***	1.18 (1.05, 1.33)***

*Presents overall crude OR (95% confidence interval) adjusted for time/age. **Presents adjusted OR (95% CI) controlled for time/age maternal ethnicity, parental atopic constitution, parity, maternal BMI, maternal age, breast-feeding duration, daycare attendance, maternal educational level, prenatal maternal smoking and alcohol consumption and fetal gender, birth weight standard deviation score derived from Generalized Estimation Equations. ***$p < 0.05$ for comparison with reference category.

B-12 concentration on wheezing, shortness of breath, and atopic dermatitis did not differ between the different age groups (Figures S4.1.2–S4.1.4; $p\text{-}_{interaction} > 0.10$).

The association between folate and vitamin B12 concentrations and wheezing, shortness of breath, and atopic dermatitis did not significantly differ within strata of parental atopic constitution, maternal smoking, folic acid supplementation, and *MTHFR* C677T polymorphism ($p\text{-}_{interaction} > 0.10$) (data not shown).

No overall significant association was found between the *MTHFR* C677T polymorphism and the development of wheezing, shortness of breath, or atopic dermatitis in the offspring of Western mothers (Table 4.1.5), which was not different according to the age of the child ($p_{\text{-interaction}} > 0.10$).

Table 4.1.4: Association between maternal vitamin B12 concentrations during pregnancy wheezing, shortness of breath, and atopic dermatitis in the offspring (*n*=8742).

Maternal vitamin B12 concentration	≤ 130 pmol/L	130 – 178 pmol/L	178 – 227 pmol/L	≥ 227 pmol/L
	(Reference)	OR 95%CI	OR 95%CI	OR 95%CI
Wheezing				
Crude*	1.00	0.93 (0.82, 1.06)	0.94 (0.85, 1.06)	0.91 (0.81, 1.03)
Multivariate**	1.00	0.95 (0.84, 1.07)	0.96 (0.87, 1.06)	0.95 (0.85, 1.08)
Shortness of breath				
Crude*	1.00	1.01 (0.89, 1.14)	1.05 (0.92, 1.21)	1.06 (0.90, 1.24)
Multivariate**	1.00	1.01 (0.89, 1.14)	1.05 (0.91, 1.22)	1.07 (0.98, 1.27)
Atopic dermatitis				
Crude*	1.00	1.16*** (1.01, 1.34)	1.17 (1.01, 1.35)***	1.34 (1.07, 1.67)***
Multivariate**	1.00	1.14 (1.00, 1.31)	1.15 (1.00, 1.32)***	1.30 (1.06, 1.60)***

*Presents overall crude OR (95% CI) adjusted for time/age. **Presents overall adjusted OR (95% CI) controlled for time, maternal ethnicity, parental atopic constitution, parity, maternal BMI, maternal age, breast-feeding duration, daycare attendance, maternal educational level, prenatal maternal smoking and alcohol consumption and fetal gender, birth weight standard deviation score derived from Generalized Estimation Equations. ***$p < 0.05$ for comparison with reference category.

Table 4.1.5: Association between maternal *MTHFR* C677T polymorphism and wheezing, shortness of breath, and atopic dermatitis in the offspring (*n*=4955).

MTHFR C677T polymorphism	CC	CT	TT
	(Reference)	OR* 95%CI	OR* 95%CI
Wheezing	1.00	1.03 (0.90, 1.18)	0.96 (0.75, 1.23)
Shortness of breath	1.00	0.99 (0.82, 1.20)	0.95 (0.77, 1.16)
Atopic dermatitis	1.00	0.94 (0.82, 1.07)	1.00 (0.82, 1.21)

*Presents overall crude OR (95% CI) adjusted for time/age.

DISCUSSION

This study demonstrated that high folate and vitamin B12 concentrations in the first trimester of pregnancy, but not maternal *MTHFR* C677T polymorphism and folic acid supplementation, were significantly associated with the development of atopic dermatitis, but not with asthma-like symptoms in early childhood.

Results from previous studies on folate and asthma or allergic outcomes are conflicting. Haberg et al.[12] and Whitrow et al.[13] showed an increased risk of early asthma-like symptoms and asthma in the offspring of mothers who took folic acid supplements in the first and third trimesters of pregnancy, respectively. Additionally, Haberg et al.[24] confirmed that folate concentrations in the second trimester of pregnancy were associated with an increased risk of asthma in the offspring. In contrast, Martinussen et al. [25] showed no association between folic acid supplementation in the first trimester of pregnancy and asthma in the offspring. Results from the KOALA birth cohort study[26], however, showed no association with folic acid supplementation during pregnancy, but high intracellular folic acid levels in late pregnancy tended to be associated with a lower risk of asthma in the offspring at the age of 6–7 y. Also, in the Avon Longitudinal Study of Parents and Children (ALSPAC) study, no evidence for an association between folic acid supplementation and asthma development was found in the second and third trimesters of pregnancy[27]. Recently, the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study demonstrated a weak association between folic acid supplementation in the third trimester and asthma-like symptoms at the age of 1 year but not at other ages[28]. To our knowledge, only a few studies reported data on (atopic) dermatitis. A recent prospective cohort study reported no association between maternal folate consumption in the second trimester, assessed by food questionnaire, and asthma-like symptoms and atopic dermatitis at the age of 16–24 months[29] (29). Also, the PIAMA study showed no association with eczema[28]. Magdalijns et al.[26] showed an increased risk of eczema in the offspring of mothers using folic acid supplements for the entire pregnancy, but this association remained nonsignificant after adjustment of confounders. Although differences in measurement methods and the timing and amount of folate exposure make it difficult to compare our results with previous studies, assessment of folic acid supplementation by questionnaire can be prone to information bias and adherence bias, ensuring that association studies on this topic are challenging and vary in results. Also, we found no significant association with folic acid supplementation but we did find an association with folate and vitamin B12 concentrations, advocating that assessing maternal folate and vitamin B12 concentrations during pregnancy in future studies can be a promising approach to shed light on the associations previously observed.

Pregnancy is a critical period for the programming of the fetal immune system insofar as emerging evidence shows that regulation of neonatal Th1/Th2 balance is under epigenetic control[10]. Epigenetics refers to the heritable modifications of phenotypes and gene expression that are not coded in the DNA sequences[10]. Folate together with vitamin B12 plays a crucial role in providing methyl donors for methylation of DNA[5]. Methylation of DNA substitutes can lead to the activation or inactivation of certain genes that control cell growth or proliferation and determine when and where a gene is expressed, e.g., during neonatal immune development[10, 11]. In mice it was demonstrated that in utero exposure to methyl donors such as folic acid and vitamin B12 is associated with altered lymphocyte development. This skewed the fetal immune system toward a Th2 profile, which favored the development of allergic disease[9]. Also, neonates at high risk for allergy are born with altered DNA methylation in their dendritic cells that process antigens and may change the Th1/Th2 balance[30]. Other studies have found that during early immune development, increased DNA methylation plays a role when naive T-cells differentiate into Th2 cells[31, 32]. It has been hypothesized that a decreased methylation of DNA in particular may support the switch of T-cells into a Th1 phenotype[33]. When assuming that folate and vitamin B12 enhance gene-specific methylation of DNA, the latter hypothesis suggests that increasing folate concentrations during pregnancy might lead to adverse effects in utero besides the protective effect on NTD[2, 3]. Nevertheless, Magedelijns et al.[26] showed that high intracellular folic acid concentrations in late pregnancy in particular was associated with a lower risk of asthma, whereas Haberg et al.[24] showed that high folate concentrations in the second trimester of pregnancy are associated with a higher risk of asthma in the offspring. Thus, the timing of folate exposure during pregnancy that increases the risk of allergic disease might be important and needs further elucidation. Other studies with respect to postnatal folate intake during childhood or adulthood show a protective effect of folate on allergic disease instead of an increased risk as seen in studies on folate intake during pregnancy[34, 35]. Increased folate levels may be associated with a healthy lifestyle that may protect against allergic disease; thus, the inverse association between folate and allergic disease can be easily influenced by residual confounding[36]. Nevertheless, the difference in effect of pre- and postnatal folate intake on allergic disease could support the hypothesis that infants are especially susceptible to an adverse immune effect of methyl donors in utero. Unfortunately, we did not have data on the B vitamin status of the children to ascertain whether effects of B vitamins in our study were restricted to exposure during pregnancy.

A strength of this study is the prospective study design and the fact that the population was not selected according to their allergy risk. In the United States, food fortification with folic acid is mandatory[37], whereas in The Netherlands, food is fortified with folic acid only on a voluntary basis. This makes it easier to assess the effect of folate during pregnancy in The Netherlands, because certain heterogeneity of the exposure is necessary[38]. Also, we used actual concentrations of folate and vitamin B12 during pregnancy, which is less susceptible to information and attrition bias than assessment of folic acid supplementation during pregnancy, which may explain the fact that we found a significant association only with folate and vitamin B12 concentration and not with folic acid supplementation during pregnancy. Another strength of this study was that we explored several potential interactions, including with *MTHFR* C677T polymorphism and family history of atopic disorders. Limitations of this study include the relatively short follow-up, which precludes an asthma diagnosis. Earlier studies on maternal folate and allergy focused on asthma as the outcome[12, 13, 29]. We did not have data on asthma diagnosis, and wheezing and shortness of breath during childhood are not optimal predictors of childhood asthma[39]. Hence, our results do not allow for conclusions regarding folate and childhood asthma. Nevertheless, because ~50% of the children with atopic dermatitis develop asthma later in life[40], follow-up studies on maternal folate and vitamin B12 and asthma in the offspring is worthwhile. This epidemiological study does not permit conclusions on causality regarding maternal folate, vitamin B12, and atopic dermatitis in childhood. In addition, residual confounding is a common phenomenon in epidemiological studies that may have influenced our results. Because intake of folate may also be associated with a healthy lifestyle, health consciousness might be a residual confounder in our study. If health-conscious mothers are more alert to health problems in their child, this residual confounding may have lead to overestimation of the association between folate and atopic dermatitis. Most data were obtained by parental-derived questionnaires and no additional information from medical records or physical examinations was available. Therefore, some children may be misclassified concerning the outcome of atopic dermatitis or wheezing or shortness of breath. However, only if this misclassification was also related to maternal folate or vitamin B12 status would this misclassification have influenced our results.

We used principles of Mendelian randomization by using the *MTHFR* C677T polymorphism as a genetic determinant of folate status. Although this has been considered to provide the highest epidemiological evidence because it assumes that genes are not confounded by other factors, we did not find any evidence that the maternal *MTHFR* C677T polymorphism had a role in the development of wheezing, shortness of breath, and atopic dermatitis. These results are in accordance with Thuesen et al.[41] and the ALSPAC study[27]. People homozygous for the minor T allele have reduced folate concentrations[42]. Because we found an association between high folate concentration and atopic dermatitis, we expected to at least find an inverse association between TT genotype and atopic dermatitis. However, the assumptions of Mendelian randomization when the *MTHFR* C677T polymorphism is used has recently been challenged[43]. Also, the deficiency state associated with the TT genotype might influence dietary behavior of these mothers, which may cancel out the association between the *MTHFR* C677T polymorphism and atopic dermatitis. Unfortunately, we had no data available on maternal dietary intake of folate to further elucidate the latter suggestion. In addition, the difference in folate concentrations between mothers with the TT genotypes relative to the CC genotype was ~2 nmol/L and these levels were still in the third tertile of folate concentrations of the study group (16.2–23.2 nmol/L).

In the majority of the longitudinal cohort studies, missing data may lead to decreased external validity of the study, because attrition frequently leads to a population with fewer women with low education and an unhealthy lifestyle. Indeed, we found that mothers who had no data on the child's health had lower folate and vitamin B12 concentrations during pregnancy. To reduce attrition bias, we performed the final analyses after a multiple imputation procedure, which is considered to be a very appropriate method to deal with missing data, because it requires the fewest assumptions and reduces potential bias when missing data are not completely at random[22]. As a result of this procedure, the 95% CI in our study results reflected the uncertainty associated with missing data.

We had data only on folate concentrations measured in plasma, which fluctuate with recent changes in folate intake or temporary alteration in folate metabolism, whereas folate levels measured in RBC provide a more accurate indication of folate status in the preceding 3 months[44]. Therefore, conclusions on the relationship between maternal folate status very early in pregnancy and allergic outcomes should be made with caution.

Conclusion

In conclusion, this study showed that high maternal plasma folate and serum vitamin B12 concentrations are associated with the development of atopic dermatitis but not with wheezing and shortness of breath in childhood. Further studies are needed to confirm this association with the outcomes of IgE sensitization and asthma diagnosis at older ages as well as additional measurements on gene-specific DNA methylation. With regard to policy decisions concerning mass interventions such as mandated folic acid fortification, potential adverse effects on allergic disease in childhood should be evaluated.

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SUPPLEMENTARY MATERIAL

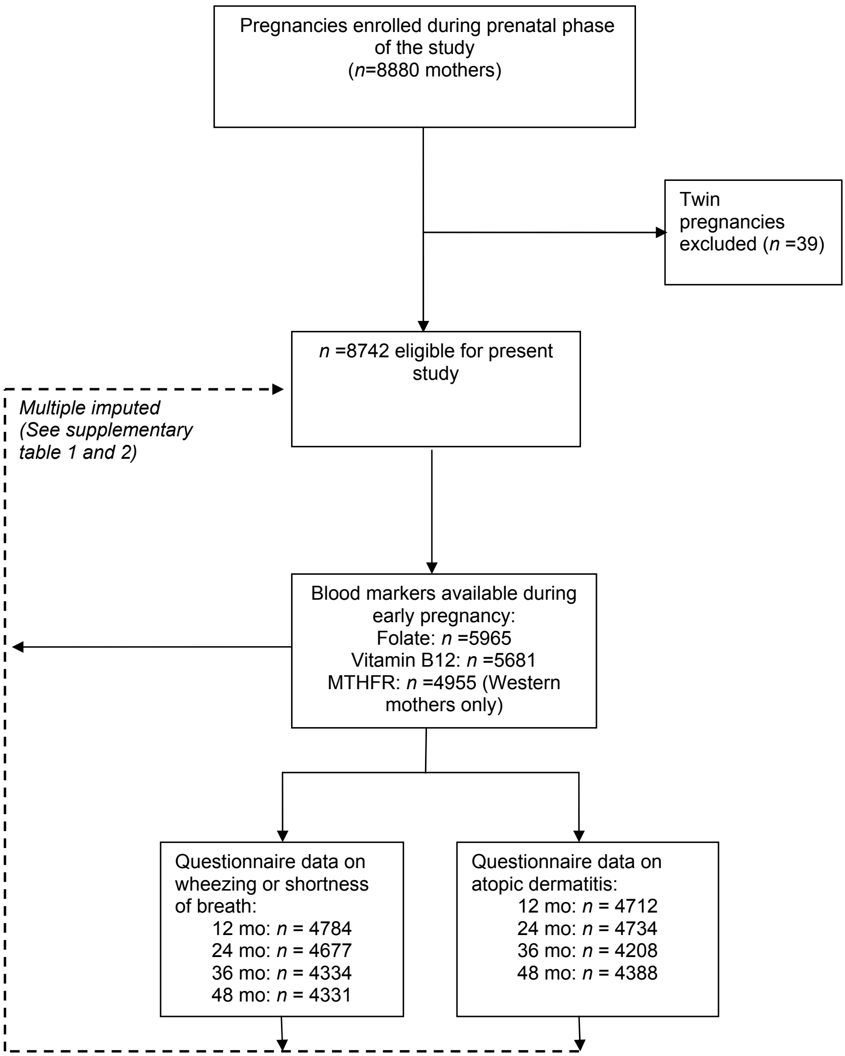

Figure S4.1.1: Flow chart of the total study group

Table S4.1.1: Details of the multiple imputation modelling

	Multiple imputation procedure
Sofware used:	SPSS 17.1 for windows.
Imputation method and keysettings:	Fully conditional specification (Markov chain Monte Carlo method); Maximum iterations: 10.
No of imputed data sets created:	5
Variable included in the imputation procedure and used in main analyses: (imputed and used as predictors of missing data)	Maternal MTHFR genotype, maternal folate concentration, maternal vitamin B12 concentation, maternal folic acid supplementation, maternal age, maternal BMI, parity, gestational age of the child, birthweight of the child, gender of the child, breast-feeding duration of the child, wheezing of the child at 12, 24, 36, and 48 months, atopic dermatitis of the child at 12, 24, 36, and 48 months, parental history of asthma, eczema, hay fever and allergy to housedust, maternal educational level, maternal smoking, maternal ethnicity, child ethnicity, maternal alcohol use during pregnancy, any creche attendance of the child in first two years of life, any history of fever accompanied with respiratory symptoms of the child at 12, 24, 36, and 48 months, symptoms of shortness of breath of the child at 12, 24, 36, and 48 mo.
Variables not used in main analyses but used as predictors of missing data to increase plausibility of missing at random assumption:	Maternal homocystein concentration, maternal history of hypertension, pre-eclampsia and diabetes gravidarum during pregnancy, marital status, any history of respiratory infections of the child at 12, 24, 36, and 48 months, any symptoms of dry cough of the child at 12, 24, 36, and 48 months, parental report of any history of food allergy in first 12 months of the child’s life.
Treatment of nonormally distributed variables	Log-transformed
Treatment of binary/categorical variables	Logistic regression and multinomial models

Table S4.1.2: Comparison in characteristics between original dataset and after multiple imputation procedure.

		Original dataset Valid %	After multiple imputation procedure
Child characteristics			
Gender, % boy		50%	50%
Missing		2%	-
Birth weight, grams (mean±SD)		3409±564	3406±564
Missing		2%	-
Gestational age, weeks (mean±SD)		39.8±1.9	39.8±1.9
Missing		1%	-
Any daycare in first 24 months of life, %		94%	92%
Missing		50%	-
Parental atopic constitution, %		42%	42%
Missing		9%	-
Breast-feeding, %			
	No breast-feeding	10%	14%
	Partial until 4 months	66%	59%
	Exclusive until 4 months	24%	27%
	Missing	44%	-
Mother characteristics			
Western ethnicity, %		58%	57%
Missing		8%	-
Maternal age, years (mean±SD)		30±5	30±5
Missing		-	-
Educational level, %			
	Low	12%	13%
	Mid	46%	47%
	High	42%	40%
	Missing	9%	-
Nullipara, %		56%	55%
Missing		1%	-
Body Mass Index, kg/m^2 (mean±SD)		25±5	25±5
Missing		1%	-
Maternal smoking during pregnancy, %		27%	29%
Missing		12%	-
Maternal alcohol use during pregnancy, %		39%	40%
Missing		11%	-
Plasma folate, nmol/L (mean±SD)		17.6±9.1	17.2±9.1
Missing		32%	-
Plasma vitamin B12, pmol/L (mean±SD)		188±93	187±89
Missing		35%	-
Folic acid use during pregnancy, %			
	No	29%	31%
	Start within 10th week after conception	31%	31%
	Start periconceptional	40%	38%
	Missing	26%	-

Table S4.1.3: Distribution of covariates among children with and without atopic dermatitis between 12 and 48 months

	Atopic dermatitis in the offspring							
	12 months		24 months		36 months		48 months	
	No *n* =6295	Yes *n* =2447	No *n* =6132	Yes *n* =2610	No *n* =7014	Yes *n* =1728	No *n* =6581	Yes *n* =2161
Child characteristics								
Gender, % boy	50%	51%	50%	50%	50%	51%	51%	50%
Birth weight, grams (mean±SD)	3409±571	3396±582	3407±579	3404±563	3400±577	3428±566	3406±574	3407±576
Gestational age, weeks (mean±SD)	39.8±1.9	39.8±1.9	39.8±1.9	39.8±1.8	39.8±1.9	39.9±1.9	39.8±1.9	39.8±1.9
Any daycare in first 24 months of life, %	92%	91%	91%	92%	91%	93%	92%	92%
Parental atopic constitution, %	40%	47%*	40%	47%*	40%	49%*	41%	46%*
Breast-feeding, %								
No breast-feeding	14%	13%	14%	13%*	14%	12%	14%	13%
Partial until 4 months	58%	60%	59%	58%*	59%	59%	59%	58%
Exclusive until 4 months	28%	26%	27%	29%*	27%	29%	27%	29%
Mother characteristics								
Western ethnicity, %	57%	55%	57%	57%	57%	57%	58%	53%*
Maternal age, years (mean±SD)	30±5	30±5	30±5	30±5	30±5	30±5	30±5	29±5
Educational level, %								
Low	13%	13%	13%	11%*	14%	10%*	13%	11%
Mid	47%	47%	47%	46%*	47%	45%*	46%	49%
High	40%	41%	40%	43%*	39%	45%*	40%	40%
Nullipara, %	55%	56%	54%	58%*	55%	57%	54%	58%*
Body Mass Index, kg/m^2 (mean±SD)	25±5	25±4	25±5	25±4	25±5	25±4	25±5	25±5
Maternal smoking during pregnancy, %	29%	29%	24%	28%	24%	27%	28%	30%
Maternal alcohol use during pregnancy, %	39%	40%	38%	41%	39%	42%	39%	40%

*p<0.05 relative to no symptoms.

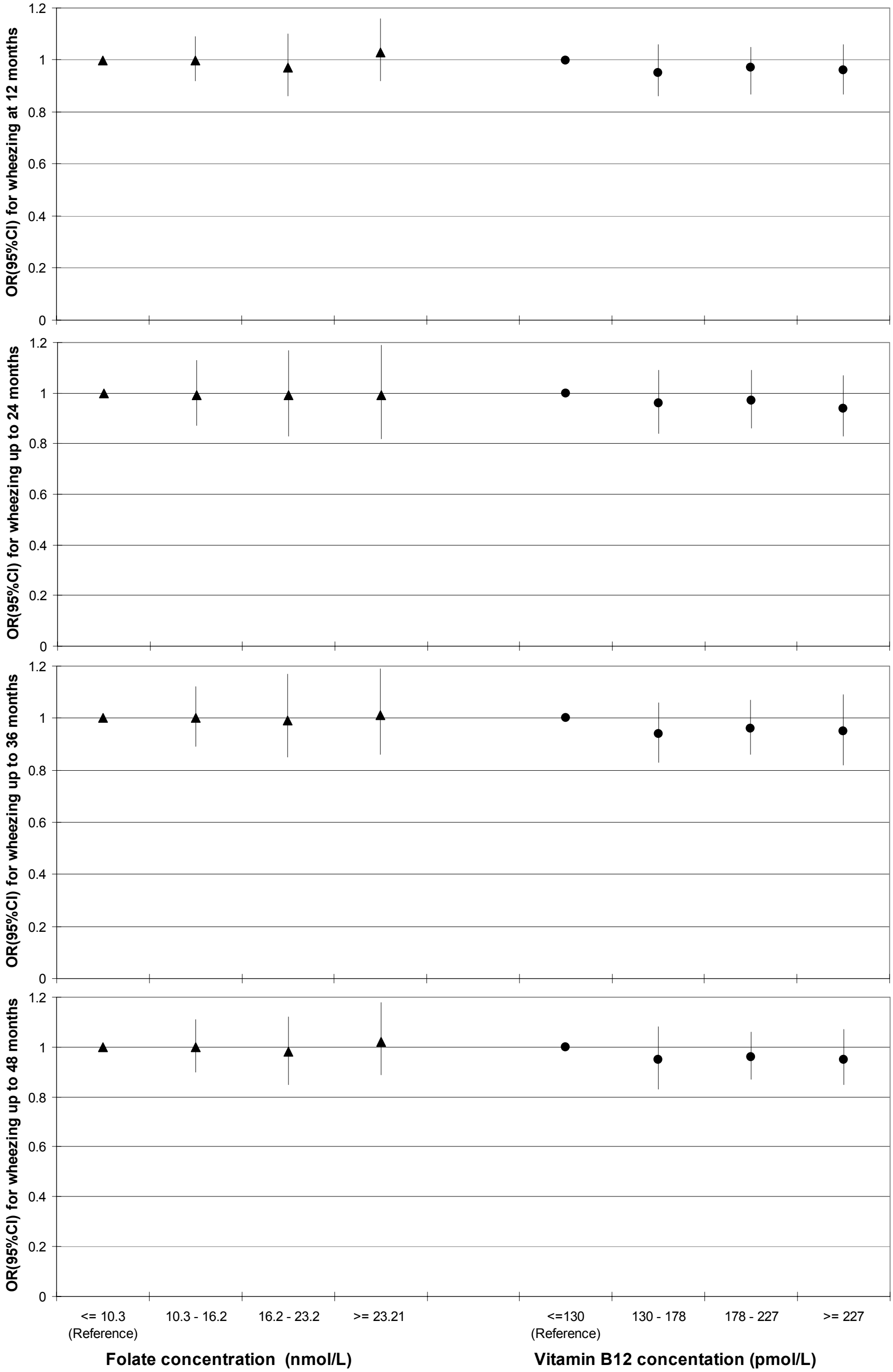

Figure S4.1.2: Time specific effects of folate and vitamin B12 on wheezing up to 48 months of life (▲ Folate concentration; ● Vitamin B12 concentration; figures present adjusted OR (95%CI).

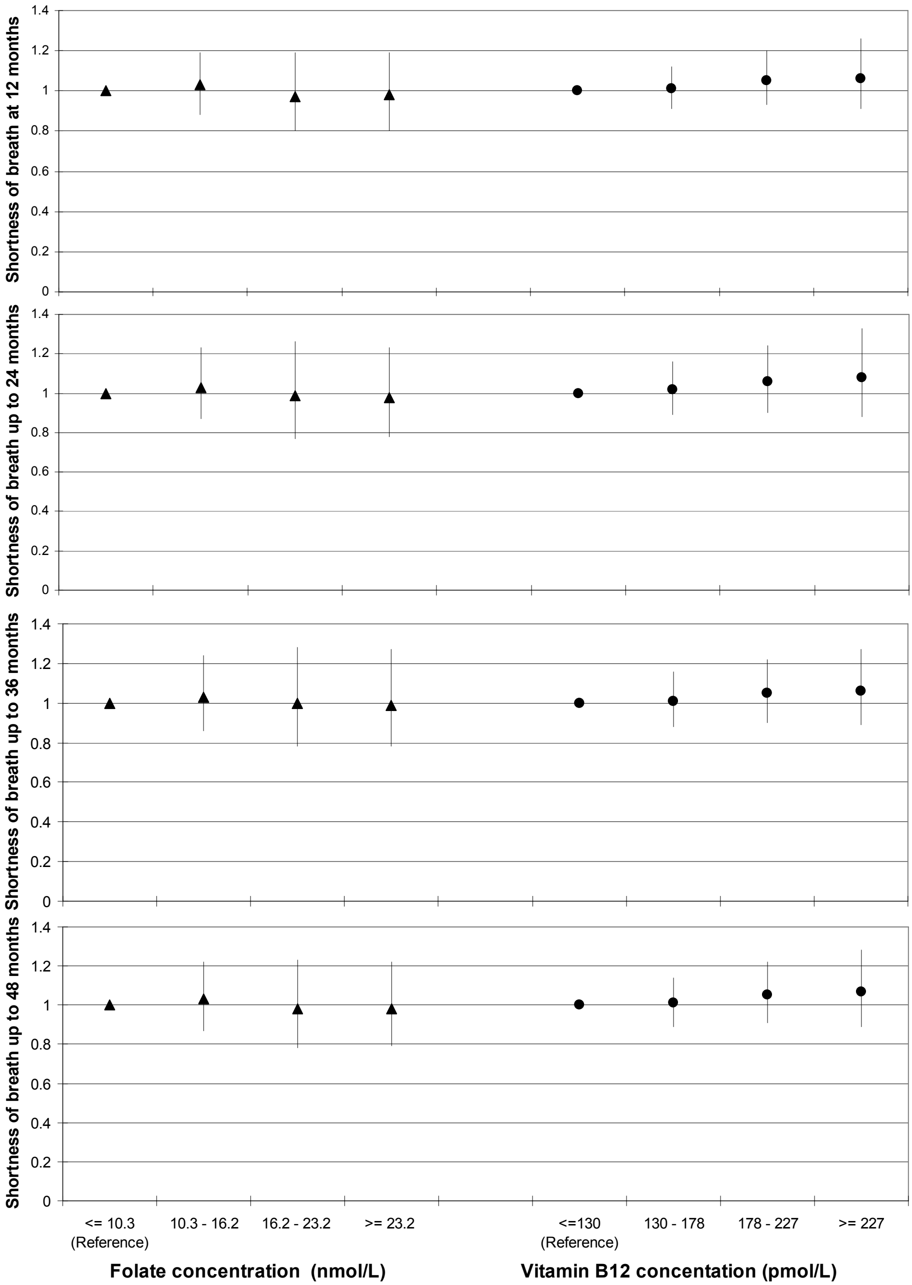

Figure S4.1.3: Longitudinal effects of folate and vitamin B12 on shortness of breath up to 48 months of life (▲ Folate concentration; ●Vitamin B12 concentration; figures present adjusted OR (95%CI).

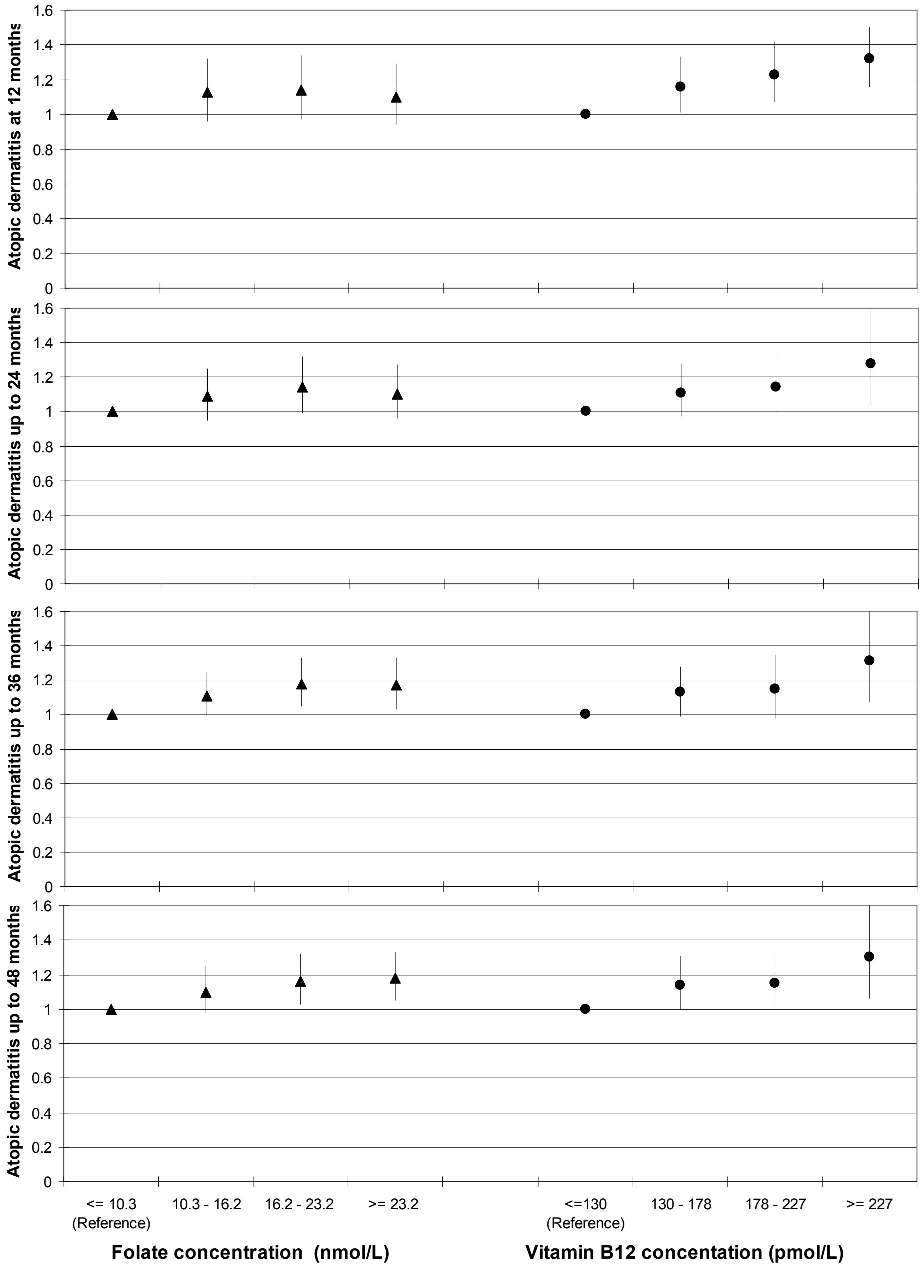

Figure S4.1.4: **Time specific effects of folate and vitamin B12 on atopic dermatitis during the first 48 months of life (▲ Folate concentration; ●Vitamin B12 concentration; figures present adjusted OR (95%CI).**

Chapter 4.2

Timing of introduction of food allergens and the development of wheezing and atopic dermatitis in childhood

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Arch Pediatr Adolesc Med. 2011 Oct;165(10):933-8.

ABSTRACT

The objective of this study was to examine whether the timing of introduction of the allergenic foods cow's milk, hen's egg, peanut, tree nuts, soy and gluten is associated with atopic dermatitis and wheezing up to 4 years of age.

This study was a population-based prospective cohort study (the Generation R Study) from fetal life until young adulthood. In Rotterdam, The Netherlands between April 2002 and January 2006. Participants included pre-school children participating in the Generation R study (*n*= 6905).

Main Exposure was timing of introduction of cow's milk, hen's egg, peanut, tree nuts, soy and gluten collected by questionnaires at 6 and 12 months of age. Information on the outcomes atopic dermatitis and wheezing were obtained by questions from the age adapted version of the "International Study of Asthma and Allergies in Childhood" core questionnaire and questionnaire data on parentally-reported doctor diagnosis for atopic dermatitis. Out of 6905 children, 31% had reported wheezing at age 2 and 14% at age 3 and 4 years. Atopic dermatitis was reported in 38%, 20% and 18% of children at the age of 2, 3 and 4 years respectively. The introduction of cow's milk, hen's egg, peanut, tree nuts, soy and gluten before the age of 6 months was not significantly associated with atopic dermatitis or wheezing at any age after adjustment for potential confounders. The results did not alter after stratification according to infant history of cow's milk allergy and parental history of atopy.

This study does not support the recommendation for delayed introduction of allergenic foods after 6 months for the prevention of atopic dermatitis and wheezing.

INTRODUCTION

The prevalence of atopic diseases in children has been increasing over the past few decades[1] and varies throughout the world.[2] Atopic diseases, including atopic eczema, asthma, allergic rhinitis and food allergy are common in childhood and cause very significant burden.[3, 4] Atopic diseases are complex and multifactorial involving genetic and environmental factors.[5] An important environmental factor that may influence the development of atopic diseases is early infant nutrition. The first year of life includes many transitions in food consumption.[6] Introduction of complementary feeding is essential for both developmental and nutritional concerns. The timing of complementary feeding is particularly important given the maturation of the gastrointestinal and renal systems.[7] Health risks that have been suggested to be associated with early complementary feeding include excessive infant weight gain,[8] increased body mass index,[9] respiratory illness during childhood,[10] and auto-immune diseases as type 1 diabetes[11] and celiac disease[12].

Recommendations for the timing of complementary feeding vary. The European Academy of Allergology and Clinical Immunology (EAACI),[13] The European Society for Paediatic Gastroenterology, Hepatology and Nutrition (ESPGHAN),[14] and the American Association of Pediatrics (APP)[15] recommend delayed introduction of solid foods for 4-6 months of age. However, the ESPGHAN recommends introduction of complementary feeding not to be delayed beyond the age of 6.5 months.[14] There is no current convincing evidence that delayed complementary feeding beyond the age of 4-6 months is protective for the development of atopic diseases.[13,15-18] In addition, few studies found early complementary feeding to be associated with an increased risk of atopic disorders such as atopic dermatitis.[16,19,20] Some studies even found an increased risk for atopic disease in relation to delayed food introduction.[21,22]

It has been suggested that a family history of atopic disease is associated with a significantly increased risk for development of atopic disease in childhood.[23] However, avoidance or delayed introduction of potentially allergenic foods has not been convincingly shown to reduce allergies, either in infants considered at risk for the development of allergy or in those not considered to be at risk[14]. Muraro et al found the majority of children who develop atopic disease, particularly recurrent wheezing and asthma, during early childhood not to belong to high risk groups for development of atopic disease.[23]

Whether delayed introduction of allergenic foods could decrease the risk of atopic diseases is controversial. Therefore, the aim of this study was to examine whether the timing of introduction of the following allergenic foods: cow's milk, hen's egg, peanut, tree nuts, soy and gluten is associated with atopic dermatitis and wheezing up to 4 years of age. Additionally, we aimed to assess whether the association differs between infants with and without a history of cow's milk allergy in the first year of life and those with and without a parental history of atopy.

METHODS

Participants and study design

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood and has been described in detail previously.[24, 25] Consent for postnatal follow-up was provided by a total of 7893 mothers with a delivery date between April 2002 and January 2006. The study was approved by the medical ethical review board of the Erasmus Medical Center, Rotterdam, The Netherlands.

Atopic dermatitis and wheezing

At the age of 2, 3 and 4 years, questions from the age adapted version of the "International Study of Asthma and Allergies in Childhood" (ISAAC) core questionnaires on asthma and atopic dermatitis were used. These questions were made suitable for younger children and have been used in several papers from this cohort.[26] Information was also available from questionnaire data on parentally-reported doctor diagnosis for atopic dermatitis. Questionnaire response rates were 69%, 64% and 63% at the age of 2, 3 and 4 years respectively. Atopic dermatitis and wheezing were defined as present or absent in the infant's 2nd, 3rd and 4th year of life.

Introduction of allergenic foods and gluten in the first year of life

At the child's age of 6 and 12 months parents were asked by questionnaire about the age of first time introduction of cow's milk (n = 4855), hen's egg (n = 4505), peanut (n = 4478), tree nuts (n = 4431), soy (n = 4658) and gluten (n = 4734) in their infant's diet. At the same age parents were also asked to complete a short food frequency questionnaire consisting of food-products frequently consumed according to a Dutch food consumption survey in infants.[27] Subsequently, the reported food products introduced were cross-checked with the short food-frequency questionnaires. For example, if at the age of 12 months the parents indicated to have never introduced peanuts in their child's diet although at the age of 6 months the parents filled in that their child had consumed peanut butter more than once, the introduction of this allergen was considered to be before or equal to 6 months of age. Additionally, first time introduction of cow's milk and soy was also cross checked with the type of bottle feeding (soy based or based on fully hydrolyzed whey protein or not) used at the age of 6 and 12 months.

Covariates

Variables possibly related to atopic dermatitis and wheezing such as gender, gestational age and birth weight, were obtained from obstetric records assessed in mid-wife practices and hospital registries.[25] Additionally, potential confounders and mediators were assessed by a combination of prenatal and postnatal questionnaires completed by both parents and included information on ethnicity, maternal social economic background, maternal smoking, parity and family history of asthma, eczema, hay fever or allergic rhinitis, and allergy to housedust. Ethnicity of the child was defined as follows: if both parents were born in The Netherlands, the ethnicity of the child was defined as Dutch; if one of the parents was born in another country than The Netherlands, that country applied; if parents were born in different countries other than The Netherlands, the country of the mother applied.[28] Maternal social economic background was defined according to educational level as follows; low: no education, primary school or less than 3 years of secondary school, mid: more than 3 years of secondary school, higher vocational training or bachelor's degree, and high: academic education.[29] Postnatal questionnaires completed by the mothers at 6, 12 and 24 months included information on the general health of the child (i.e. medication use, co- morbidity), day-care attendance, and the consumption of food products. Data on breast-feeding were collected by delivery reports and postnatal questionnaires at the age of 2, 6 and 12 months. Breast-feeding was defined as follows: I) never, II) exclusively for 6 months, III) exclusively for 4 months, partially at 6 months, IV) exclusively for 4 months, no breast-feeding at 6 months, V) partially for 6 months, VI) partially for 4 months, no breast-feeding at 6 months. In addition, the presence of cow's milk allergy was obtained by questionnaire at the age of 6 and 12 month by asking parents whether their child had attended a doctor because of cow's milk allergy. At the age of 24 months, parents were asked about the number of doctor-attended respiratory tract infections acquired by the child and use of antibiotics during the last 12 months. Data on gastroenteritis was obtained by asking parents about their child's bowel movement and defined as any episode of diarrhea accompanied by fever. Body Mass index (BMI) at 24 months was calculated from the child's weight and height available from child health centers. Being overweight was defined according to age and gender dependent BMI-thresholds for young children from Cole et al (2000).[30]

Population for analyses

To avoid the influence of metabolic disorders and clustering, the following children were excluded in the analyses for this study: twinborn (*n*= 238), siblings within the Generation R cohort (*n* = 343), presence of a congenital heart condition (*n*= 47), anemia between 12 and 24 months (*n* = 58) and growth retardation defined as height for age < -2SD based on the Netherlands growth curves of 12-24 months children (*n* =

163).[31] The presence of a congenital heart condition and anemia were defined according to parentally-reported doctor diagnosis obtained by questionnaire. Children whose parents did not provide informed consent for the use of questionnaire data were also excluded (n = 135). To prevent bias associated with missing data, variables with missing values were multiple imputed (n = 5 imputations) based on the correlation between the variable with missing values with other patient characteristics[32,33] according to the Markov Chain Monte Carlo (MCMC) method. Consequently n = 6905 were available after multiple imputation for statistical analyses.

Statistical analysis

Logistic regression analyses were performed with atopic dermatitis and wheezing at 2, 3 and 4 years separately as dependent variables. Introduction of allergenic foods in the first year of life were analyzed as independent variables and adjusted for potential confounders and mediators (i.e. gender, maternal social economic background, ethnicity, maternal smoking, gestational age, birth weight, parity, breast-feeding, use of any antibiotics, day-care attendance, gastroenteritis, number of respiratory tract infections, overweight, parental history of atopy). The selection of potential confounders and mediators was carried out by the alteration in odds ratio (OR). The potential confounder or mediator was kept in the multivariate model in case of an alteration of ≥ 10% in OR. To assess whether the association between the timing of allergenic food introduction and wheezing and atopic dermatitis was different among children with and without history of cow's milk allergy and parental history of atopy, statistical interaction was evaluated by adding the product term of the independent variable and subgroup (independent variable x subgroup) as covariate to the univariate model. Stratified analyses by history of cow's milk allergy or parental history of atopy were performed when the statistical interaction was significant (Table S4.2.1-S4.2.4). The pooled results of the 5 imputed datasets were reported in this paper as odds ratio's (OR's) and 95% confidence interval (95% CI). A p- value < 0.05 was considered as statistically significant. The statistical analyses were carried out by using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Study population

Maternal and child characteristics of the study population are presented in table 4.2.1. Out of 6905 children, 31% had reported wheezing at age 2 and 14% at 3 and 4 years of age. Atopic dermatitis was reported in 38%, 20% and 18% of children at the age of 2, 3 and 4 years respectively.

Table 4.2.1: Maternal and child characteristics (*n* = 6905)

Characteristics		*n*	(%)
Mother			
SES			
	Low	766	11 %
	Mid	4746	69 %
	High	1393	20 %
Ever smoked during pregnancy			
	No	5064	73 %
	Yes	1841	27 %
Parents with a history of atopy		3274	47 %
Parity		2899	42%
Child			
Male		3496	51 %
Ethnicity			
	Dutch/Western	4380	63 %
	Non- Western	2525	37 %
Gestational age at delivery (months; mean±SD)		39.9	±1.6
Birth weight (g; mean±SD)		3431	±540
History of cow's milk allergy in first year of life		846	12%
Breast-feeding			
	Never	781	11%
	6 months exclusive	345	5%
	4 months exclusive, partially at 6 months	1296	19%
	4 months exclusive, no breast-feeding at 6 months	357	5%
	6 months partially	795	12%
	4 months partially, no breast-feeding at 6 months	3331	48%
Use of any antibiotics *		2839	41 %
Overweight *		1755	25 %
Number of respiratory tract infections *			
	Never	3322	48 %
	1 - 3 times	2369	34 %
	> 4 times	1213	18 %
Day- care attendance *		5137	74 %
Gastroenteritis *		4599	67 %

* Between 12-24 months of age

CHAPTER 4.2

Introduction of allergenic foods and gluten

The introduction of tree nut before the age of 6 months was significantly associated with wheezing at 2 years of age (OR: 2.69; 95% CI: 1.25, 5.73). However, this association was explained by gender, maternal social economic background, ethnicity, maternal smoking, gestational age, birth weight, parity, breast-feeding, use of any antibiotics, day-care attendance, gastroenteritis, number of respiratory tract infections, overweight and parental history of atopy (Table 4.2.2). No significant association was found between early tree nut introduction and wheezing at age 3 (OR: 1.24; 95% CI: 0.70,

Table 4.2.2 Association between the introduction of allergenic foods and wheezing at age 2, 3 and 4 years (*n* = 6905)

Wheezing		Age 2		Age 3		Age 4	
Introduction ≤ 6 months	*n* %	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*
Cow's milk	4757 (69%)	0.83 (0.43, 1.57)	0.80 (0.44, 1.43)	1.02 (0.84, 1.23)	1.02 (0.85, 1.22)	0.97 (0.77, 1.21)	0.96 (0.77, 1.19)
Hen's egg	1466 (21%)	1.83 (0.95, 3.51)	1.39 (0.84, 2.28)	1.26 (0.83, 1.91)	1.13 (0.84, 1.51)	1.30 (0.99, 1.70)	1.11 (0.91, 1.34)
Peanut	1069 (15%)	2.16 (0.75, 6.13)	1.71 (0.60, 4.83)	1.25 (0.81, 1.93)	1.14 (0.77, 1.66)	1.25 (0.79, 1.97)	1.05 (0.69, 1.61)
Tree nut	875 (13%)	2.69 (1.25, 5.73)**	2.41 (0.83, 7.00)	1.24 (0.70, 2.20)	1.11 (0.68, 1.79)	1.30 (0.79, 2.13)	1.12 (0.75, 1.67)
Soy	2002 (29%)	1.73 (0.99, 3.01)	1.54 (0.96,2.46)	1.11 (0.80, 1.53)	1.05 (0.80, 1.38)	1.16 (0.87, 1.55)	1.06 (0.84, 1.35)
Gluten	3203 (46%)	1.30 (0.94, 1.79)	1.17 (0.86, 1.60)	1.07 (0.86, 1.31)	1.02 (0.83, 1.26)	1.10 (0.94, 1.28)	1.03 (0.87, 1.20)

OR: odds ratio; 95% confidence interval. OR's are compared to introduction > 6 months of age. *Adjusted for gender, SES mother, ethnicity, ever smoked during pregnancy, gestational age, birth weight, parity, breast-feeding, use of any antibiotics between 12 and 24 months, day-care attendance between 12 and 24 months, gastroenteritis between 12 and 24 months, number of respiratory tract infections between 12 and 24 months, overweight between 12 and 24 months, parental history of atopy. ***p* = 0.058 after adjustment for unequal variances.

Table 4.2.3 Association between the introduction of allergenic foods and atopic dermatitis at age 2, 3 and 4 years (*n* = 6905)

Atopic dermatitis		Age 2		Age 3		Age 4	
Introduction ≤ 6 months	*n* %	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*	Univariate model OR (95 % CI)	Multivariate model OR (95 % CI)*
Cow's milk	4757 (69%)	0.92 (0.68, 1.23)	0.91 (0.67, 1.23)	0.88 (0.75, 1.03)	0.88 (0.74, 1.03)	0.95 (0.77, 1.17)	0.95 (0.77, 1.15)
Hen's egg	1466 (21%)	1.27 (0.52, 3.10)	1.10 (0.51, 2.32)	0.84 (0.65, 1.09)	0.87 (0.69, 1.10)	1.11 (0.88, 1.39)	1.05 (0.81, 1.35)
Peanut	1069 (15%)	1.36 (0.48, 3.87)	1.11 (0.34, 3.61)	0.95 (0.72, 1.26)	0.99 (0.72, 1.36)	0.94 (0.70, 1.26)	0.87 (0.65, 1.16)
Tree nut	875 (13%)	1.64 (0.46, 5.85)	1.54 (0.35, 6.69)	1.09 (0.72, 1.65)	1.16 (0.76, 1.76)	1.12 (0.79, 1.60)	1.06 (0.72, 1.56)
Soy	2002 (29%)	1.47 (0.74, 2.92)	1.33 (0.72, 2.44)	0.92 (0.75, 1.14)	0.95 (0.75, 1.19)	1.01 (0.82, 1.23)	0.97 (0.80, 1.17)
Gluten	3203 (46%)	0.94 (0.69, 1.28)	0.90 (0.71, 1.14)	0.88 (0.76, 1.02)	0.90 (0.76, 1.06)	1.05 (0.85, 1.29)	1.02 (0.81, 1.27)

OR: odds ratio; 95% confidence interval. OR's are compared to introduction > 6 months of age. *Adjusted for gender, SES mother, ethnicity, ever smoked during pregnancy, gestational age, birth weight, parity, breast-feeding, use of any antibiotics between 12 and 24 months, day-care attendance between 12 and 24 months, gastroenteritis between 12 and 24 months, number of respiratory tract infections between 12 and 24 months, overweight between 12 and 24 months, parental history of atopy.

2.20) or 4 years (OR: 1.30; 95% CI: 0.79, 2.13) (Table 4.2.2). In addition no significant association was found between early tree nut introduction and atopic dermatitis up to age 4 years (Table 4.2.3). The introduction of cow's milk, hen's egg, peanut, soy and gluten before the age of 6 months in the infant's diet was not significantly associated with wheezing (Table 4.2.2) or atopic dermatitis (Table 4.2.3) at any age. These results were independent of gender, maternal social economic background, ethnicity, maternal smoking, gestational age, birth weight, parity, breast-feeding, use of any antibiotics, day-care attendance, gastroenteritis, number of respiratory tract infections, overweight and parental history of atopy. Additional adjustment for potential mediator history of cow's milk allergy did not alter the results for wheezing or atopic dermatitis (data not shown).

Parental history of atopy and infant's history of cow's milk allergy

A history of cow's milk allergy in the first year of life was more frequently found in children with reported wheezing and atopic dermatitis than in children without reported wheezing and atopic dermatitis ($p \leq 0.05$ for difference in history of cow's milk allergy at the age of 2, 3 and 4). A parental history of atopy was also more frequently found in children with reported wheezing (p=0.24, p<0.01, p=0.11 for difference in parental history at the age of 2, 3 and 4 respectively) and atopic dermatitis ($p < 0.05$ for difference in parental history at the age of 2, 3 and 4) (Table S4.2.1). Although a significant interaction was found with a history of cow's milk allergy for the introduction of peanut and gluten (Table S4.2.2), no significant association was found after stratification by history of cow's milk allergy (Table S4.2.3). No interaction was found between the timing of introduction of food allergens and parental history of atopy (Table S4.2.4).

DISCUSSION

This population-based prospective birth cohort study failed to demonstrate that the timing of introduction of allergenic foods such as cow's milk, hen's egg, peanut, tree nuts, soy and gluten was associated with atopic dermatitis and wheezing up to 4 years of age. The results did not alter after stratification for history of cow's milk allergy or parental history of atopy.

Various current feeding guidelines recommend complementary feeding to be introduced beyond 4-6 months of age.[13-15] However, there is no current convincing evidence that delayed complementary feeding beyond the age of 4-6 months is protective for the development of atopic disease.[13, 15-18] Few previous studies found earlier complementary feeding before 4 months of age to be positively associated with atopic diseases as eczema and wheezing.[10,19,34] A birth cohort in the Christchurch Child Development

Study in New Zealand found eczema rates to be significantly higher in infants who were introduced to solid foods before 4 months of age.[19,34] A cohort of children in Dundee found solid feeding before 15 weeks to be associated with an increased probability of wheeze during childhood.[10] However, these studies did not assess whether a longer delay of complementary feeding after the age of 6 months had a additional protective effect on atopic dermatitis and wheezing.

The results of this study are in agreement with the findings of other birth cohort studies. The LISA birth cohort study found no evidence supporting a delayed introduction of solid foods beyond 6 months of age for the prevention of eczema at the age of 2 years[35] and no evidence supporting a delayed introduction beyond 4 or 6 months for the prevention of asthma at age 6[17]. Filipiak et al also did not find evidence supporting delayed introduction of solid foods beyond 4 months of age or delayed introduction of the most potentially allergenic solids beyond 6 months for the prevention of eczema.[18] In addition, a birth cohort study in the United Kingdom found no evidence for a protective effect of late introduction for the development of eczema or wheezing at age 5-5 ½.[22] Conversely, this last study found a significant increased risk of eczema in relation to late introduction of the allergenic foods egg and milk. The KOALA birth cohort study found that a delayed introduction of cow's milk was associated with a higher risk of eczema in the first 2 years of life.[21] The latter association could possibly be explained by reverse causation since parents with a family history of atopy or infants with early symptoms of allergy may delay complementary feeding. Possible distortion by reverse causality has been suggested previously.[35]

An important strength of this study is the large study population drawn from the general population. Several other studies selected children of atopic parents who are at higher risk of developing atopic diseases which might lead to selection bias since atopic parents are more likely to introduce allergenic foods later in the infant's diet. An additional strength is the use of multiple imputation for missing data. Consequently, attrition bias was of minimum concern.[32, 33]

Some limitations of the study have to be considered in the interpretation of the results. Information on the timing of allergenic food introduction was asked retrospectively at 6 and 12 months of age, therefore minor misclassification because of recall bias cannot be excluded.

However, this would have only influenced our results if parents of children with wheezing or atopic dermatitis tended to misclassify having introduced allergenic foods after 6 months of age instead of before 6 months of age. Atopic dermatitis and wheezing were diagnosed on the basis of parent-reported questionnaires. This could have led to misclassification of the outcome since doctor diagnosis provides more accurate outcome diagnosis. Yet, we do not expect this misclassification to have influenced the effect of timing of food allergen introduction in particular, given that the outcome was measured after the introduction period. Another limitation of this study was that our study cannot examine the effect of allergenic food introduction before the age of 4 months in relation to atopic dermatitis and wheezing. Thus our study precludes conclusions on the effect of very early introduction of allergenic foods. However, Zutavern et al found no evidence supporting a delayed introduction of solids beyond 4 or 6 months of age for the prevention of asthma at the age of 6 years. For eczema any effect of a delayed introduction of solids could not be excluded.

Asthma assessment among young children is based on asthma-like symptoms, such as wheezing, often reported by parents through self-administered written questionnaires. Early wheezing in infancy is however not a very strong and independent predictor of childhood asthma. Diagnosis of asthma is difficult in young children, due to the non-specificity of the symptoms and the fact that conventional lung function tests cannot be carried out at such a young age.[36] Therefore, our results do not allow for conclusions regarding the introduction of allergenic foods and later development of asthma. However, previous studies found that the infant's diet had a greater effect on short-term outcomes rather than on long term-outcomes of atopic diseases.[7] Therefore, we do not expect the effect of the introduction of allergenic foods to influence the results for atopic dermatitis and wheezing differently beyond 4 years of age.

We considered confounding and reverse causality in our analysis by adjusting for potential confounders and by evaluating statistical interaction for history of cow's milk allergy and parental history of atopy. However, residual confounding and residual reverse causality cannot be fully excluded. Reverse causation may occur if a delayed introduction of allergenic foods is truly protective for wheezing and atopic dermatitis and parents of high risk infants were more likely to delay the introduction of allergenic foods after 6 months of age which may cancel out the effect.

Conclusion

In conclusion, the results presented in this study do not support a delayed introduction of allergenic foods beyond the age of 6 months for the prevention of atopic diseases and atopic dermatitis and wheezing. Further studies in our cohort should focus on asthma and atopic dermatitis at later ages in order to elucidate whether late introduction of food allergens delays the onset of atopic disease.

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SUPPLEMENTARY MATERIAL

Table S4.2.1: History of cow's milk allergy and parental history of atopy in children with wheezing and eczema.

	Children with wheezing	Children without wheezing	*p* value	Children with atopic dermatitis	Children without atopic dermatitis	*p*- value
History of cow's milk allergy						
Year 2	16%	11%	<0.01	17%	10%	<0.01
Year 3	15%	12%	0.05	19%	11%	<0.01
Year 4	17%	12%	<0.01	18%	11%	<0.01
Parental history of atopy						
Year 2	49%	47%	0.24	49%	46%	<0.05
Year 3	52%	47%	<0.01	53%	46%	<0.01
Year 4	50%	47%	0.11	51%	47%	<0.05

Table S4.2.2: Statistical interaction between the introduction of allergenic foods and history of cow's milk allergy.

	Atopic dermatitis			**Wheezing**		
	Year 2	Year 3	Year 4	Year 2	Year 3	Year 4
Introduction ≤ 6 months	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
Cow's milk	0.60	0.33	0.28	0.70	0.73	0.34
Henn's egg	0.80	0.99	0.91	0.48	0.46	0.42
Peanut	0.24	0.63	0.53	0.03	0.49	0.38
Tree nut	0.27	0.43	0.13	0.99	0.61	0.50
Soy	0.09	0.31	0.21	0.54	0.33	0.39
Gluten	0.36	0.10	0.13	0.91	0.12	0.04

Table S4.2.3: Association between the introduction of allergenic foods and wheezing stratified by history of cow's milk allergy

		No history of cow's milk allergy			**History of cow's milk allergy**		
	Introduction ≤ 6 months	***n*** **%**	**Univariate model** OR (95 % CI)	**Multivariate model** OR (95 % CI)*	***n*** **%**	**Univariate model** OR (95 % CI)	**Multivariate model** OR (95 % CI)*
Year 2	Peanut	826 (77%)	2.17 (0.77, 6.05)	1.65 (0.60, 4.55)	244 (23%)	1.62 (0.28, 9.29)	1.52 (0.23, 9.87)
Year 4	Gluten	2827 (88%)	1.15 (0.96, 1.38)	1.07 (0.88, 1.30)	376 (12%)	0.88 (0.57, 1.35)	0.84 (0.53, 1.35)

OR: odds ratio; 95% confidence interval. OR's are compared to introduction > 6 months of age.
*Adjusted for gender, SES mother, ethnicity, ever smoked during pregnancy, gestational age, birth weight, parity, breast-feeding, use of any antibiotics between 12 and 24 months, day-care attendance between 12 and 24 months, gastroenteritis between 12 and 24 months, number of respiratory tract infections between 12 and 24 months, overweight between 12 and 24 months, parental history of atopy.

Table S4.2.4: Statistical interaction between the introduction of allergenic foods and parental history of atopy.

	Eczema			Wheezing		
	Year 2	Year 3	Year 4	Year 2	Year 3	Year 4
Introduction ≤ 6 months	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
Cow's milk	0.87	1.00	0.81	0.63	0.74	0.14
Henn's egg	0.79	0.85	0.85	0.72	0.81	0.98
Peanut	0.25	0.85	0.71	0.98	0.78	0.75
Tree nut	0.87	1.00	0.09	0.99	0.70	0.46
Soy	0.73	0.93	0.24	0.84	0.87	0.53
Gluten	0.29	0.64	0.70	0.72	0.25	0.30

Chapter 4.3

Infant dietary patterns and respiratory outcomes

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Eur Respir J. 2012 Feb 23.

ABSTRACT

Overall diet in early childhood may affect the development of respiratory symptoms. This study examined whether childhood dietary patterns are associated with respiratory symptoms in Dutch pre-school children, and whether this association could be explained by energy intake.

A prospective cohort study was performed in 2173 children of 4 years and younger. Data on asthma-related symptoms were obtained by questions from the age adapted version of the "International Study of Asthma and Allergies in Childhood" questionnaires. Data on respiratory tract infections, defined as episodes of physician attended fever with respiratory symptoms, was obtained by questionnaire. Principal components analysis was used to develop dietary patterns.

Relative to low adherence, high adherence to the "Western" dietary pattern was significantly associated with frequent wheeze at 3 years of age (aRR: 1.39; 95% CI: 1.02, 1.89) and frequent shortness of breath (aRR: 1.44; 95% CI: 1.03, 2.01) and respiratory tract infections (aRR: 1.54; 95% CI: 1.08, 2.19) at 4 years of age. However, this association was partially explained by energy intake.

A "Western" diet may increase the risk of frequent respiratory symptoms at 3 and 4 years of age. In some measure, this association was explained by energy intake.

INTRODUCTION

Atopic diseases are common in children and have been increasing in prevalence. Asthma is the most common chronic disease in childhood.[1] Infectious diseases, particularly of the respiratory tract are a leading cause of morbidity and hospitalization in infants and children in industrialized countries.[2] Both asthma and infectious disease cause significant burden of disease in childhood[3], and identifying risk factors for the development of these diseases is of interest. From fetal life until childhood the immune system undergoes substantial maturation. The adequacy of this maturation process depends on environmental factors of which nutrition is suggested to play a role[4]. Diet during pregnancy has been suggested to be associated with asthma-related symptoms in the offspring[5, 6]. Duration and exclusiveness of breast-feeding has been found to be related to asthma and respiratory tract infections in infancy and childhood[2,7,8]. In addition, nutrition beyond the weaning period may also be of importance[4]. Relatively little attention has been paid to the influence of diet beyond the weaning period on respiratory symptoms. Traditional analyses in nutritional epidemiology often examine disease in relation to specific nutrients or foods[9]. Intake of specific nutrients and foods during childhood has a relation with the development of asthma and respiratory tract infections[10,11]. In addition, intake of calorie rich foods has been associated with a higher prevalence of asthma[12]. However, children eat a variety of foods with complex combinations of nutrients that are likely to be interactive or synergistic[9]. Therefore, dietary pattern analysis examining the association of overall diet may be more predictive of disease risk. So far, studies examining the effect of overall diet in childhood mainly focused on a traditional Mediterranean diet. These studies found a Mediterranean diet in early life to be associated with the development of asthma-related symptoms in childhood[13,14]. No studies examined the effect of a Western diet in childhood on the development of respiratory symptoms in children. However, a Western diet has been found to be associated with an increased risk of frequent asthma attacks in adult females[15]. The aim of this study was to examine whether different childhood dietary patterns are associated with respiratory symptoms in Dutch children up to 4 years of age. A second aim was to examine whether this association could be explained by total energy intake.

METHODS

Participants and study design

This study was embedded in the Generation R study, a population-based prospective cohort study from fetal life until young adulthood and has been described in detail previously[16]. In total, 9778 mothers with a delivery date from April 2002 through January

2006 enrolled in the study. Consent for postnatal follow-up was provided by 7893 participants. Data collection on nutritional intake of the child was implemented in the study from 2003 onwards. In total, 5088 mothers received a food frequency questionnaire (FFQ) for their child at the age of 14 months (Figure 4.3.1). The study was approved by the medical ethical review board of the Erasmus Medical Center, Rotterdam, the Netherlands.

Respiratory symptoms

Asthma-related symptoms

Data on asthma-related symptoms were obtained by questions adapted from the "International Study of Asthma and Allergies in Childhood" (ISAAC) core questionnaires on asthma at the age of 2, 3 and 4 years. These questions were made suitable for younger children and have been previously used in other studies[17]. Asthma symptoms, including wheezing and shortness of breath, were categorized according to frequency as follows: never, 1-3 times and ≥ 4 times[17]. Questionnaire response rates were 69%, 64% and 63% at the age of 2, 3 and 4 years, respectively.

Respiratory tract infections

Data on respiratory tract infections were obtained by parent-reported questionnaires at the age of 2, 3 and 4 years. Respiratory tract infections were defined by the number of parent-reported physician visit(s) for fever with respiratory symptoms; coughing, runny or blocked nose, or earache. Subsequently, respiratory tract infections were categorized according to frequency as follows: never, 1-2 times and ≥ 3 times[17].

Dietary patterns

At the child's age of 14 months (± 2 months) parents were asked to complete a FFQ. The FFQ was developed in cooperation with the division of Human Nutrition of Wageningen University and based on an existing validated food questionnaire described in detail previously[18] and adapted with food products frequently consumed according to a Dutch food consumption survey in infants[19]. The FFQ was validated against three day 24 hour recalls in Dutch children at the age of 14 months. The intra-class correlation coefficients for macronutrients were as follows: total energy: 0.4, total protein: 0.7, total fat: 0.4, carbohydrates: 0.4, and dietary fiber: 0.7. The frequency of consumption of a food item was to be recorded per day, per week, or per month over the past 4 weeks. Subjects were asked to report their regular portion size relative to the standard portion size according to the Dutch table of regular portion sizes and household units[20]. Total nutrient content was calculated per item according to the Dutch Nutrient Composition table[22]. Response rate was 72% (*n*=3643) (Figure 4.3.1).

Covariates

Variables possibly related to the outcomes as gender, gestational age and birth weight, were obtained from obstetric records assessed in mid-wife practices and hospital registries[16]. Additional potential confounders were assessed by a combination of prenatal and postnatal questionnaires completed by both parents. The questionnaires included information on maternal age, maternal socioeconomic status (SES), maternal smoking during pregnancy, multiple parities and parental history of atopy. Maternal SES was defined according to educational level as follows; low: no education, primary school or less than 3 years of secondary school, mid: more than 3 years of secondary school, higher vocational training or bachelor's degree, and high: academic education. Data on breast-feeding were collected by a combination of delivery reports and postnatal questionnaires at the age of 2, 6 and 12 months. Breast-feeding was classified as (I) never, (II) exclusively for 6 months, (III) exclusively for 4 months and partially at 6 months, (IV) exclusively for 4 months, with no breast-feeding at 6 months, (V) partially for 6 months, (VI) partially for 4 months, with no breast-feeding at 6 months. Cow's milk allergy in the first year of life was assessed by questionnaire at the age of 6 and 12 month by asking parents whether their child had attended a doctor for cow's milk allergy. At the age of 12 months parents were asked about vitamin D supplementation during the past 6 months. Postnatal questionnaires completed by the mother at age 12 and 24 months included information on day-care attendance.

Population for analyses

To avoid the influence of culture differences in the definition of the dietary patterns, only children of Dutch origin were included in the analyses (*n*=2443). Siblings within the Generation R cohort were randomly selected and excluded (*n* =270). To prevent bias associated with missing data, variables with missing values were multiple imputed (5 imputations) based on the correlation between the variable with missing values with other patient characteristics[21]. Consequently *n*=2173 were available after multiple imputation for statistical analyses (Figure 4.3.1).

Statistical analysis

The FFQ included 211 food items which were grouped into 21 different food groups. The scree plot from the PCA showed a clear break in the curve after the second component revealing the presence of two dietary patterns with Eigen values of 3.4 and 1.7. The percentage of variance explained by the dietary patterns was 16.3% and 8.2%. In the PCA, varimax rotation was used to obtain uncorrelated factors. For reasons of interpretability the population of analysis was categorized into tertiles according to their score for the dietary pattern as follows; low, moderate and high (using "low" score as reference category).

Log-binomial regression analyses were performed with the primary outcome variables; wheezing, shortness of breath and respiratory tract infections separately at the age of 2, 3 and 4 years. The defined dietary patterns at the age of 14 months were analyzed as primary exposure and adjusted for potential confounders. The selection of potential confounders was performed by the alteration in relative risks (RR's) and kept in the multivariate model in case of an alteration of ≥ 10% (multivariate model 1). To asses whether the association between the dietary patterns and respiratory symptoms was explained by total energy intake additional analysis were performed with adjustment for total energy intake resulting in a separate multivariate model (multivariate model 2). The pooled results of the 5 imputed datasets were reported in this paper as relative risks (RR's) and 95% confidence intervals (95% CIs). A *p*- value < 0 .05 was considered as

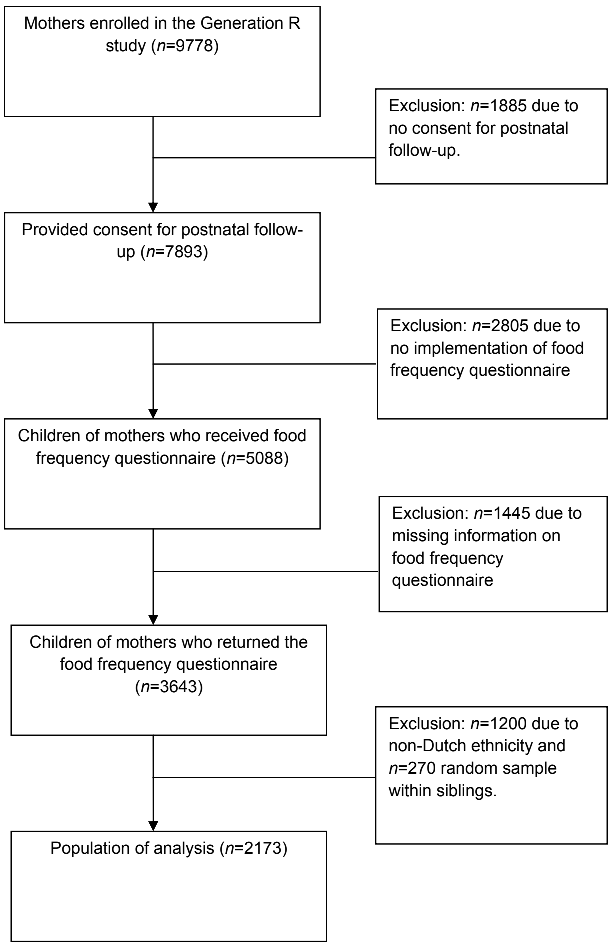

Figure 4.3.1: Flowchart of the participants within the Generation R Study

Table 4.3.1: Maternal and child characteristics (*n*=2173)

Characteristics		*n (%)*		*n (%)* After multiple imputation procedure	
Mother					
Maternal age, Mean±SD		31.8	±4.25	31.8	±4.25
Missing		0	0	-	-
SES (%)					
	Low	230	10	232	11
	Mid	1151	53	1157	53
	High	777	36	784	36
	Missing	15	1	-	-
Maternal smoking during pregnancy (%)		385	18	461	21
	Missing	371	17	-	-
Multiple parities (%)		732	34	760	35
	Missing	54	3	-	-
Parental history of atopy (%)		1121	52	1141	53
	Missing	36	2	-	-
Child					
Male (%)		1082	50	1082	50
	Missing	0	0	-	-
birth weight standard deviation score, Mean±SD		0.06	±1.01	0.06	±1.03
	Missing	178	8	-	-
Breast-feeding (%)					
	Never	213	10	229	11
	6 months exclusive	33	2	49	2
	4 months exclusive, partially at 6 months	371	17	387	18
	4 months exclusive, no breast-feeding at 6 months	153	7	161	7
	6 months partially	172	8	185	9
	4 months partially, no breast-feeding at 6 months	1008	46	1161	53
	Missing	223	10	-	-
History of cow's milk allergy first year (%)		124	6	237	11
	Missing	444	20	-	-
Vitamin D supplement use 6-12 months (%)		750	35	801	37
	Missing	127	6	-	-
Day care attendance first 2 years (%)		1493	69	1807	83
	Missing	575	27	-	-
Total energy intake at 14 months, Mean±SD		1276	±351	1276	±351
	Missing	0	0	-	-

statistically significant. The statistical analyses were performed using Stata Statistical Software for Windows, release 11 (Stata Corporation. Stata statistical software; college station, TX,USA).

RESULTS

Study population

Maternal and child characteristics of the study population are presented in Table 4.3.1 and the prevalence of the outcomes of interest in table S4.3.1.

Dietary patterns

The factor loadings of the food groups in the two dietary patterns present are shown in table S4.3.2. The individual factor loading scores for the food groups are correlation coefficients between the food products and the specific dietary pattern. Dietary pattern 1 was associated with starchy foods, fruit, vegetables, potatoes, vegetable oils, fish, legumes and meat. This pattern is referred to here as the "Health conscious" dietary pattern. Dietary pattern 2 was associated with refined grains, soups and sauces, savory and snacks, other fats, sugar containing beverages and meat. This pattern will be referred to as the "Western" dietary pattern.

Dietary patterns and respiratory symptoms

High adherence to the "Western" dietary pattern was significantly associated with frequent shortness of breath (≥ 4) (RR: 1.43; 95% CI: 1.01, 2.03) at age 2 years (Table 4.3.2). High adherence to the "Western" dietary pattern was also significantly associated with frequent wheeze (≥ 4) (RR: 1.39; 95% CI: 1.02, 1.89) and frequent shortness of breath (≥ 4) (RR: 1.66; 95% CI: 1.24, 2.21) at age 3 years (Table 4.3.2 & Table 4.3.3). However, the association between the "Western" dietary pattern and frequent shortness of breath (≥ 4) at the age of 2 and 3 years was mainly explained by maternal age, maternal SES, maternal smoking during pregnancy, parental history of atopy, multiple parities, SDS birth weight, gender, breast-feeding, vitamin D supplementation, day-care attendance and history of cow's milk allergy (Table 4.3.2). High adherence to the "Western" dietary pattern was also significantly associated with frequent wheeze (≥ 4) (RR: 1.70; 95% CI: 1.22, 2.36) and shortness of breath (≥ 4) (RR: 1.44; 95% CI: 1.03, 2.01) at age 4 years (Table 4.3.2 & Table 4.3.3). However, the association between the "Western" dietary pattern and frequent wheeze (≥ 4) at age 4 years was mainly explained by the variables mentioned previously (Table 4.3.3). Adherence to the "Western" dietary pattern was not significantly associated with frequent wheeze at age 2 years, or short-term wheeze (1-3 times) or shortness of breath (1-3 times) up to 4 years of age. High adherence to

Table 4.3.2: Association between the "Health conscious" versus "Western" dietary pattern and shortness of breath

	Shortness of breath					
	1-3 times			≥ 4 times		
Adherence score to dietary pattern (*n*= 2173)	Univariate model RR (95 % CI)	Multivariate model 1 RR (95 % CI)*	Multivariate model 2 RR (95 % CI)**	Univariate model RR (95 % CI)	Multivariate model 1 RR (95 % CI)*	Multivariate model 2 RR (95 % CI)**
Year 2						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.96 (0.71,1.30)	0.93 (0.69,1.27)	0.95 (0.70,1.28)	0.46 (0.55,1.31)	0.84 (0.56,1.27)	0.85 (0.58,1.26)
High	1.07 (0.81,1.43)	1.05 (0.78,1.41)	1.09 (0.79,1.51)	0.83 (0.47,1.91)	0.93 (0.46,1.91)	0.94 (0.55,1.60)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.95 (0.68,1.32)	0.88 (0.63,1.22)	0.89 (0.65,1.23)	1.27 (0.73,2.18)	1.14 (0.67,1.95)	1.18 (0.72,1.92)
High	1.15 (0.84,1.58)	1.02 (0.73,1.42)	1.08 (0.77,1.52)	1.43*** (1.01,2.03)	1.22 (0.79,1.90)	1.27 (0.87,1.86)
Year 3						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.99 (0.68,1.45)	0.96 (0.65,1.41)	0.97 (0.66,1.43)	1.11 (0.67,1.20)	0.90 (0.67,1.21)	0.89 (0.66,1.20)
High	0.88 (0.78,1.65)	1.07 (0.74,1.56)	1.09 (0.73,1.61)	1.01 (0.76,1.35)	0.98 (0.73,1.32)	0.95 (0.70,1.29)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.85 (0.58,1.23)	0.82 (0.56,1.19)	0.83 (0.57,1.21)	0.88 (0.64,1.19)	0.81 (0.58,1.12)	0.82 (0.58,1.14)
High	1.13 (0.79,1.62)	1.01 (0.70,1.48)	1.03 (0.68,1.54)	1.66*** (1.24,2.21)	1.31 (0.95,1.80)	1.32 (0.93,1.88)
Year 4						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.90 (0.60,1.37)	0.85 (0.56,1.29)	0.91 (0.59,1.39)	0.89 (0.65,1.22)	0.87 (0.63,1.21)	0.84 (0.61,1.17)
High	0.87 (0.58,1.31)	0.79 (0.52,1.20)	0.92 (0.58,1.44)	1.16 (0.85,1.57)	1.08 (0.78,1.49)	0.99 (0.71,1.40)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	1.12 (0.76,1.67)	1.08 (0.73,1.62)	1.15 (0.76,1.73)	1.09 (0.79,1.51)	1.03 (0.74,1.44)	1.01 (0.72,1.41)
High	0.85 (0.56,1.30)	0.68 (0.44,1.07)	0.81 (0.50,1.32)	1.84*** (1.35,2.51)	1.44*** (1.03,2.01)	1.36 (0.95,1.96)

RR: relative risk; 95% confidence interval. RR's are compared to low adherence to the dietary pattern. *Adjusted for maternal age, maternal SES, smoking during pregnancy, parental history of atopy, multiple parities, birth weight, gender, breast-feeding, vitamin D supplementation at 6-12 months, day-care attendance in the first two years of life, and history of cow's milk allergy in the first year. **Adjusted for multivariate model 1 + total energy intake. ***$p<0.05$

Table 4.3.3: Association between the "Health conscious" versus "Western" dietary pattern and wheeze

Wheeze						
	1-3 times			≥ 4 times		
Adherence score to dietary pattern (*n*= 2173)	Univariate model RR (95% CI)	Multivariate model 1 RR (95% CI) *	Multivariate model 2 RR (95% CI) **	Univariate model RR (95% CI)	Multivariate model 1 RR (95% CI) *	Multivariate model 2 RR (95% CI) **
Year 2						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	1.21 (0.89,1.65)	1.20 (0.88,1.65)	1.19 (0.86,1.64)	0.92 (0.56,1.52)	0.89 (0.53,1.52)	0.87 (0.50,1.51)
High	1.20 (0.89,1.62)	1.19 (0.87,1.62)	1.16 (0.84,1.62)	1.14 (0.62,2.11)	1.08 (0.59,2.00)	1.02 (0.54,1.91)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.88 (0.65,1.18)	0.86 (0.64,1.16)	0.84(0.62,1.14)	0.87 (0.56,1.36)	0.82 (0.53,1.27)	0.80 (0.51,1.23)
High	0.97 (0.73,1.30)	0.94 (0.68,1.29)	0.89 (0.63,1.25)	1.14(0.74,1.74)	0.98 (0.59,1.63)	0.90 (0.50,1.63)
Year 3						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.97 (0.66,1.43)	0.96 (0.65,1.41)	0.97 (0.65,1.43)	0.93 (0.69,1.25)	0.93 (0.69,1.26)	0.93 (0.68,1.27)
High	1.08 (1.73,1.61)	1.04 (0.70,1.55)	1.04 (0.69,1.58)	1.01 (0.75,1.37)	0.97 (0.71,1.34)	0.96 (0.69,1.34)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.93 (0.63,1.39)	0.92 (0.62,1.38)	0.94 (0.62,1.40)	0.90 (0.64,1.26)	0.84 (0.59,1.19)	0.86 (0.60,1.22)
High	1.15 (0.77,1.73)	1.13 (0.75,1.71)	1.15 (0.74,1.78)	1.75*** (1.31,2.34)	1.39*** (1.02,1.89)	1.47*** (1.04,2.07)
Year 4						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.81 (0.56,1.19)	0.78 (0.53,1.16)	0.79 (0.53,1.17)	0.98 (0.69,1.39)	0.96 (0.67,1.37)	0.93 (0.65,1.32)
High	0.98 (0.67,1.44)	0.94 (0.64,1.39)	0.94 (0.63,1.42)	1.25 (0.92,1.70)	1.19 (0.87,1.64)	1.09 (0.78,1.52)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.99 (0.68,1.45)	0.95 (0.65,1.39)	0.95 (0.64,1.40)	1.04 (0.74,1.47)	0.98 (0.69,1.40)	0.95 (0.67, 1.36)
High	1.04 (0.71,1.53)	0.86 (0.57,1.29)	0.84 (0.55,1.29)	1.70*** (1.22,2.36)	1.39 (0.99,1.94)	1.28 (0.89,1.83)

RR: relative risk; 95% confidence interval. RR's are compared to low adherence to the dietary pattern. *Adjusted for maternal age, maternal SES, smoking during pregnancy, parental history of atopy, multiple parities, birth weight, gender, breast-feeding, vitamin D supplementation at 6-12 months, day-care attendance in the first two years of life, and history of cow's milk allergy in the first year. **Adjusted for multivariate model 1 + total energy intake. ***p-value <0.05.

Table 4.3.4: Association between the "Health conscious" versus "Western" dietary pattern and respiratory tract infections

	Respiratory tract infections					
	1-3 times			≥ 4 times		
Dietary pattern (n= 2173)	Univariate model RR (95 % CI)	Multivariate model 1 RR (95 % CI)*	Multivariate model 2 RR (95 % CI)**	Univariate model RR (95 % CI)	Multivariate model 1 RR (95 % CI)*	Multivariate model 2 RR (95 % CI)**
Year 2						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.87 (0.66,1.15)	0.87 (0.66,1.15)	0.85 (0.64,1.13)	0.71 (0.75,1.25)	0.95 (0.73,1.23)	0.92 (0.71,1.19)
High	1.03 (0.77,1.37)	1.00 (0.75,1.34)	0.97 (0.71,1.32)	0.90 (0.66,1.23)	0.86 (0.62,1.19)	0.78 (0.56,1.09)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.98 (0.74,1.29)	0.99 (0.75,1.30)	0.97 (0.73,1.28)	0.92 (0.71,1.19)	0.89 (0.69,1.16)	0.87 (0.67,1.13)
High	1.04 (0.79,1.37)	1.04 (0.78,1.38)	0.99 (0.73,1.36)	1.11 (0.86,1.43)	1.01 (0.78,1.32)	0.93 (0.70,1.24)
Year 3						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	1.17 (0.87,1.58)	1.16 (0.86,1.57)	1.17 (0.87,1.58)	1.07 (0.79,1.46)	1.08 (0.79,1.48)	1.08 (0.79,1.48)
High	1.22 (0.89,1.66)	1.18 (0.85,1.65)	1.22 (0.87,1.69)	1.14 (0.81,1.61)	1.13 (0.80,1.60)	1.12 (0.78,1.61)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	1.03 (0.78,1.37)	1.02 (0.77,1.35)	1.02 (0.77,1.36)	0.94 (0.69,1.29)	0.91 (0.67,1.25)	0.91 (0.66,1.26)
High	1.05 (0.66,1.67)	1.01 (0.69,1.49)	1.03 (0.66,1.60)	1.29 (0.92,1.79)	1.12 (0.79,1.59)	1.12 (0.79,1.59)
Year 4						
Health conscious dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	0.91 (0.68,1.21)	0.90 (0.67,1.21)	0.88 (0.65,1.17)	1.08 (0.75,1.55)	1.07 (0.72,1.59)	1.03 (0.69,1.55)
High	0.85 (0.62,1.17)	0.83 (0.60,1.13)	0.77 (0.55,1.06)	1.09 (0.76,1.57)	1.02 (0.70,1.50)	0.92 (0.61,1.39)
Western dietary pattern						
Low	Reference	Reference	Reference	Reference	Reference	Reference
Moderate	1.12 (0.84,1.50)	1.13 (0.85,1.50)	1.12 (0.84,1.49)	1.01 (0.70,1.46)	1.01 (0.69,1.47)	1.00 (0.68,1.45)
High	1.36 (0.85,2.17)	1.35 (0.90,2.03)	1.32 (0.86,2.03)	1.83*** (1.28,2.61)	1.54*** (1.08,2.19)	1.46*** (1.00,2.13)

RR: relative risk; 95% confidence interval. RR's are compared to low adherence to the dietary pattern. *Adjusted for maternal age, for maternal SES, smoking during pregnancy, parental history of atopy, multiple parities, birth weight, gender, breast-feeding, vitamin D supplementation at 6-12 months, day-care attendance in the first two years of life, and history of cow's milk allergy in the first year. **Adjusted for multivariate model 1 + total energy intake. ****p*-value <0.05

the "Western" diet was significantly associated with frequent respiratory tract infections (≥ 3) (RR: 1.54; 95% CI: 1.08, 2.19) at 4 years of age (Table 4.3.4). Adherence to the "Western" diet was not significantly associated with respiratory tract infections at 2 and 3 years of age or short-term respiratory tract infections (1-2 times) at 4 years of age. Adherence to the "Health conscious" diet was not significantly associated with respiratory symptoms up to 4 years of age (Table 4.3.2, Table 4.3.3 & Table 4.3.4).

Influence of total energy intake

After adjustment for total energy intake, high adherence to the "Western" dietary pattern remained significantly associated with frequent wheeze (≥ 4) (aRR: 1.47, 95% CI: 1.04, 2.07) at 3 years of age and frequent respiratory tract infections (≥ 3) (aRR: 1.46, 95% CI: 1.00, 2.13) at 4 years of age (Table 4.3.3 & Table 4.3.4). After additional adjustment for total energy intake adherence to the "Western" dietary pattern was no longer significantly associated with shortness of breath up to 4 years of age (Table 4.3.2). Adherence to the "Western" dietary pattern remained not significantly associated with any respiratory symptom at age 2 years or short-term respiratory symptom up to 4 years of age. Adherence to the "Health conscious" dietary pattern remained not significantly associated with any respiratory symptom up to 4 years of age (Table 4.3.2, Table 4.3.3 & Table 4.3.4).

DISCUSSION

In this population-based prospective birth cohort study we observed a higher risk of frequent respiratory symptoms among children at the age of 3 and 4 years who had a greater adherence to a "Western" diet in early childhood. However, this association was partially explained by total energy intake. No association was found between a "Health conscious" diet and respiratory symptoms up to 4 years of age.

Comparison with other studies of childhood dietary patterns and respiratory outcomes is difficult as most studies did not use PCA to obtain dietary patterns. However, our "Health conscious" dietary pattern (including starchy foods, fruit, vegetables, potatoes, vegetable oils, fish, legumes and meat) has some similarities with a Mediterranean diet. A study in Mexico found a Mediterranean dietary pattern to have a protective effect on asthma and asthma-related symptoms in children aged 6-7 years[23]. A Greece study found adherence to a Mediterranean diet to be modest protective for wheezing symptoms in children aged 7-18 years[24]. Two Spanish studies additionally found a Mediterranean diet in childhood to be protective for symptoms of asthma in children aged 4 and 6-7 years[25, 26]. However, these studies were of cross-sectional design and recall bias and reverse causality might be a serious concern. To our knowledge only one prospective cohort study examined the association between a dietary pattern in childhood and atopic disease, and also found childhood adherence to a Mediterranean diet not to be significantly associated with asthma-related symptoms[14].

It has been suggested that the increase in prevalence of asthma is related to adoption of a Western lifestyle including a Western diet[27]. The "Western" diet in this study was characterized by high intake of refined grains, soups and sauces, savory and snacks, other fats, sugar containing beverages and meat. Although no other studies examined the overall effect of a "Western-like" diet in childhood on the development of asthma-related symptoms in children, a French study did find a "Western" dietary pattern to be associated with an increased risk of frequent asthma attacks in adult females[15].

Individual food components of a "Western" diet in childhood have been found to be associated with asthma symptoms in children. Sugar consumption during the perinatal period was associated with severe asthma symptoms in 6 and 7 year old children[28]. An increased intake of saturated fatty acids was also found to be related to the risk of asthma in children[29].

Adjustment for energy intake is a standard procedure in nutritional epidemiology for standardizing food and nutrient intake according to total food intake. However, most studies on dietary patterns and respiratory symptoms do not adjust for energy intake and found a Mediterranean diet to be protective for asthma-related symptoms[23,25]. Chatzi et al did adjust for total energy intake and found childhood adherence to a Mediterranean diet not to be significantly associated with asthma-related symptoms[14]. An association found between a dietary pattern consisting of high energy foods and a disease outcome may not be the effect of the foods themselves, but the effect of high calorie intake. It has been suggested that high calorie foods are associated with asthma[12]. Nevertheless, a Cochrane review showed only a small effect of calorie reduction on asthma[30]. Indeed, after adjustment for total energy intake the "Western" dietary pattern remained significantly associated with wheeze and respiratory tract infections. Therefore, the association between the "Western" dietary pattern and respiratory symptoms was only partially explained by total energy intake and needs further elucidation.

Our large study population drawn from the general population is an important strength of this study. An additional strength is the use of dietary patterns instead of single or few nutrients or food items. The effect of single nutrients may be too small to detect as the cumulative effects of multiple nutrients in a dietary pattern may be sufficiently large. Dietary patterns identified by PCA has the advantage of reducing large number of correlated dietary measurements down to a small number of overall dimensions of diet which are uncorrelated[9].

The time window of exposure is becoming a key aspect in the study of diseases involving systems as the immunological and respiratory systems[13]. Atopic disease often becomes manifest in early life, and it could be that the processes leading to atopic disease are initiated early in the immune development. This study assessed the effect of diet at the early age of 14 months whereas most studies examined the effect of diet at school age. An additional strength of this study is that it examined the effect of dietary patters prior to the outcome of disease contrary to other studies examining the effect of diet and outcome during the same time period which may lead to recall bias and reverse causality in these studies.

Some limitations have to be considered in the interpretation of the results. Information on the outcomes was obtained by parent-reported questionnaires. This could have led to misclassification of the outcome since physician diagnosis provides more accurate outcome diagnosis. However, we do not expect this misclassification to have influenced the effect of adherence to the dietary patterns, given that the outcome was measured after the food-frequency questionnaire was filled out and thus it will be unlikely that the misclassification was related to the child's diet. Several arbitrary decisions are involved in identifying dietary patterns by PCA. Decisions on combining food items into food groups, the number of factors to extract, the method of rotation, and the labelling of the components may influence the reproducibility of the findings[9]. Although we adjusted for potential confounders residual confounding cannot be fully excluded thereby precluding final conclusions regarding the causality of the study results.

Diagnosis of asthma is difficult in young children, due to the non-specificity of the symptoms and the fact that conventional lung function tests cannot be carried out at pre-school age[17]. Therefore, asthma assessment among young children is still mainly based on asthma-related symptoms. Since the diagnosis of asthma is difficult in preschool children our results precludes us from conclusions regarding the presence of asthma later in life. However, Caudri et al found wheezing and serious respiratory tract infections to be predictive for the development of asthma, in particular frequent wheezing (≥ 4 times per year) and frequent respiratory tract infections (≥ 3 times per year) were predictive of asthma at the age of 7 to 8 years[17].

Conclusion

In conclusion, our findings suggest that a "Western" diet may increases the risk of respiratory symptoms at the age of 3 and 4 years. But this association was moderately explained by total energy intake. This study does not support a protective effect of a "Health conscious" diet on respiratory outcomes in children younger than 4 years of age. Further studies on respiratory outcomes to determine the association between diet and respiratory symptoms and the influence of total energy intake are worthwhile.

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SUPPLEMENTARY MATERIAL

Table S4.3.1: Prevalence of outcomes

	Age 2		Age 3		Age 4	
	n	(%)	*n*	(%)	*n*	(%)
Wheeze						
Never	1654	76	1660	76	1689	78
1- 3 times	346	16	184	9	199	9
≥ 4 times	174	8	329	15	286	13
Shortness of breath						
Never	1414	65	1618	75	1705	78
1- 3 times	369	17	198	9	164	8
≥ 4 times	390	18	357	16	304	14
Respiratory tract infections						
Never	1097	50	1443	66	1409	65
1-2 times	491	23	388	18	396	18
≥ 3 times	586	27	342	16	367	17

Table S4.3.2: Factor loadings of the food items in the "Health conscious" and "Western" dietary pattern in children aged 14 months (r >0.2)

		Factor loading	
		Health conscious dietary pattern	Western dietary pattern
Food group	**Mean intake g/d**	**Factor loading**	**Factor loading**
Refined grains	14.9	-	0.57
Whole grains	61.9	-	-
Starchy foods	22.9	0.62	-
Dairy	626.0	-	-
Fruit	162.2	0.32	-
Soy substitutes	4.4	-	-
Vegetables	51.8	0.74	-
Potatoes	34.0	0.61	-
Soups and sauces	9.4	-	0.23
Savory and snacks	3.9	-	0.59
Confectionery	27.8	-	0.72
Vegetable oils	0.56	0.50	-
Other fats	10.6	-	0.58
Fish	8.2	0.22	-
Shellfish	0.30	-	-
Meat	25.5	0.21	0.27
Eggs	1.9	-	-
Legumes	4.0	0.59	-
Sugar containing beverages	197.5	-	0.59
Non-sugar containing beverages	56.4	-	-
Composite dishes	102.3	-	-
		Pearson's correlation coefficient	**Pearson's correlation coefficient**
Eigen value*		3.4	1.7
Variance explained (%)		16.3	8.2
Total energy intake		0.36	0.54

PCA was used as an extraction method in which the Pearson's correlation coefficients represent the relative contribution of that food group to the identified dietary pattern. * The Eigenvalue was used as indicator of the amount of variation explained by each dietary pattern

Chapter 4.4

Fish consumption in infancy and asthma-like symptoms at pre-school age

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ABSTRACT

The aim of this study was to assess whether timing of introduction of fish and the amount of fish consumption in infancy were associated with asthma-like symptoms at pre-school age.

This study was embedded in the Generation R study (a population-based birth cohort in Rotterdam, the Netherlands). At the age of 12 and 14 months, timing of introduction of fish into the infant's diet was assessed. The amount of fish consumption at 14 months was assessed by a semi-quantitative food frequency questionnaire. Presence of asthma-like symptoms in the past year was assessed at the child's age of 36 and 48 months.

Relative to no introduction in the first year of life, introduction between the age of 6 and 12 months was significantly associated with a lower risk of wheezing at 48 months (aOR: 0.64; 95%CI: 0.43, 0.94). When compared to introduction between 6 and 12 months, both no introduction in the first year and introduction between 0 and 6 months were associated with an increased risk of wheezing at 48 months (aOR: 1.57; 95%CI: 1.07, 2.31 and aOR: 1.53; 95%CI: 1.07, 2.19 respectively). The amount of fish at the age of 14 months was not significantly associated with asthma-like symptoms ($p>0.15$).

In conclusion, introduction of fish between 6 and 12 months but not fish consumption afterwards is associated with a lower prevalence of wheezing. A window of exposure between the age of 6 and 12 months might exist in which fish might be associated with a reduced risk of asthma.

INTRODUCTION

The prevalence of asthma in Westernized countries is increasing[1]. It has been suggested that adoption of a Westernized diet can be one of the reasons related to the increase of asthma[2]. Indeed, we have previously found that a 'Western-like' dietary pattern in toddlers was associated with asthma-like symptoms at pre-school age[3]. In contrast, we did not find any association with a dietary pattern characterized by high intake of fish, vegetables, and fruit[3] whereas adherence to this diet during pregnancy or at school-age has been noted to protect against asthma in prior studies[4-8]. Since we did not find a clear association between a dietary pattern including fish and asthma-like symptoms[3], we hypothesize that any potential beneficial effect of fish consumption in toddlers may be diluted in our previous study as a result of the dietary pattern approach and deserves, therefore, further study. Different studies have suggested that early life exposure of n-3 polyunsaturated fatty acids (n-3 PUFA), a major component in fatty fish, protects against the development of asthma[2, 9-10]. In line with this, a recent study reported a beneficial effect of introduction of fish before the age of 9 months on the development of wheezing at the age of 4.5 years[11], whereas another study reported no association between introduction of fish and asthma[12]. However, since fish can also be highly allergenic[13], the optimal timing of introduction of fish in the infant's diet and the adequate amount remains unclear. We aimed in present study to assess whether timing of introduction of fish in the first year of life and fish consumption afterwards were associated with the development of asthma-like symptoms at pre-school age.

METHODS

The present study was performed within the Generation R Study, a population-based multi-ethnic prospective birth cohort in Rotterdam, the Netherlands[14]. In total, 7210 children born between April 2002 and January 2006 whom parents had provided postnatal consent were included (Supplemental figure S4.4.1). The Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam, approved the study (MEC 198.782/2001/31). Written informed consent was obtained from all participants.

Timing of the introduction of fish and the amount of fish consumption in infancy

From 2003 onwards, nutritional data of the child was collected at the age of 14 months (mean±SD: 14±2 months) by using a semi-quantitative food frequently questionnaire (FFQ) which was validated in Dutch children and has been described in detail previously[15]. Questions from the FFQ on the consumption fish are found in Supplemental

CHAPTER 4.4

table S4.4.1. We divided the fish products into two groups on the basis of their fat content: I) Fatty fish defined as >10 g fat/100g: herring, mackerel, eel and salmon II) Other fish defined as 0-10 g fat/100g: white fish, fish fingers, squid, flounder, cod, pollack, haddock, tilapia, sole, tuna, whiting, trout, gurnard, perch, plaice, wolf fish, and swordfish[16]. Consumption of fish was categorized into: 'No consumption', 'Less than ½ serving per week', and 'At least ½ serving per week'. In addition, intake of 120g raw fish was counted as one serving of fish[17]. Both at the child's age of 12 and 14 months, parents were asked the following question: "Please indicate how old your child was when you gave it fish for the first time". The observed agreement between measurement at 12 and 14 months was 85% with a Cohen's kappa (*k*) of 0.31. Answers categories included "never given in first year of life', '0-3 months', '3-6 months', 'after 6 months and onwards'. Due to small numbers in the group of introduction between 0 and 3 months (*n*=7), this was combined with the group '3-6 months' into a new category: '0-6 months'.

Asthma-like symptoms

Information regarding the presence of wheezing and shortness of breath in the past year was obtained using an age-adapted version of the validated "International Study of Asthma and Allergies in Childhood"[18] questionnaire at the age of 36, and 48 months. The observed agreement for wheezing and shortness of breath between measurement by questionnaire and by physician interview in our cohort was 75% (*k*=0.30) and 81% (*k*=0.39) respectively[19]. To establish potential reverse causality, sensitivity analyses were performed with and without exclusion of any asthma-like symptoms at the age of 12 and 24 months. However, since effect-estimates were similar, final analyses were performed in all children.

Covariates

Variables possibly related to food consumption and wheezing were considered as potential confounders in this study and selected based on previous knowledge and literature[15,20]. Infant's gender, gestational age and birth weight were obtained from obstetric records assessed in mid-wife practices and hospital registries[14]. Information about maternal age, maternal BMI, parity, educational level, ethnicity, marital status, household income, and any family history of asthma, eczema, allergy to house dust, or hay fever was obtained by questionnaire at enrolment. Maternal smoking and alcohol habits were assessed by questionnaire in each trimester of pregnancy. We assessed maternal dietary fish intake at enrolment using a modified version of the validated semi quantitative FFQ described previously[21-22]. Mother's intake of folic acid was assessed by questionnaire at enrollment of the study as described earlier[23]. Data on breast-feeding was collected by a combination of delivery reports and postnatal questionnaires at the age of 2, 6 and 12 months. Breast-feeding was defined in this study as follows: I) never, II) partially through 4 months, III) exclusively through 4 months[24]. Postnatal questionnaires completed by the mother at the age 6 and 12 months included information on any day-care attendance (yes vs. no), any vitamin D supplementation (yes vs no), and any history of doctor-attended food allergy and eczema. Weight and height were measured at the child health centers during routine visits around the age of 48 months. The definition of overweight was established according to international cut-off points for children[25].

Statistical analysis

Univariate associations between maternal and child characteristics and timing of introduction of fish were performed with one-way ANOVA (continuous variables) and Chi_2-test (categorical variables). Logistic regression analyses were used to assess the association between timing of introduction of fish and amount fish consumption and asthma-like symptoms. A crude model was computed followed by a multivariate model adjusting for potential confounders. All analyses on the amount of fish consumption at 14 months were adjusted for total energy intake[26]. To assess whether the association between fish and asthma-like symptoms was different by strata of e.g. ethnicity, history of food allergy, and parental history of atopy; stratified analyses were performed by these groups and statistical interaction was tested by adding the product term of the fish variable multiplied by the stratum (e.g. fish consumption x food allergy) as an independent variable to the crude models.

A multiple imputation procedure (n= 5 imputations) was performed on all variables to reduce potential bias associated with missing data, (Supplemental figure S4.4.1, supplemental table S4.4.2)[27]. Analyses were then performed in each of the 5 imputed datasets separately and the final results were pooled and presented as Odds Ratios (OR) and 95% Confidence Intervals (95%CI). Statistical analyses were performed with PASW Statistics 17.0.

RESULTS

Characteristics of the study population and prevalence of wheezing according to timing of introduction of fish are shown in table 4.4.1 and table 4.4.2. A fish-free dietary regime at the age of 14 months was reported in only 0.1% of the children. Fish consumption of the children at the age of 14 months was higher in children introduced to fish in the first year of life relative to those without fish introduction in the first year of life (Figure 4.4.1a-b). Timing of introduction of fish showed no similarities with timing of introduction of other foods in the first year of life ($k \leq 0.15$, Supplemental table S4.4.4).

Table 4.4.1: Characteristics of the study population according to timing of introduction of fish (*n*=7210).

	Timing of introduction of fish into the infant's diet			
	Between 0 and 6 months *n* = 1281	Between 6 and 12 months *n* = 5498	Never given in first year *n* = 431	*p-value*
Mother				
Maternal age at intake; years (mean±SD)	29±5	31±5	31±5	<0.01
Maternal BMI at intake; kg/m² (mean±SD)	25.2±4.3	24.6±4.2	24.8±4.2	<0.01
Marital status (*n*, %)				<0.01
Married/living together	963 (75%)	4835 (88%)	372 (86%)	
Living alone	318 (25%)	663 (12%)	59 (14%)	
Household income (*n*, %)				<0.01
<2000 euro/month	816 (64%)	2044 (37%)	214 (50%)	
≥2000 euro/month	465 (36%)	3453 (63%)	217 (50%)	
Maternal educational level (*n*, %)				<0.01
Low	265 (21%)	511 (9%)	67 (16%)	
Mid	683 (53%)	2334 (42%)	202 (47%)	
High	333 (26%)	2653 (48%)	162 (36%)	
Family history of atopic disease (*n*, %)	563 (44%)	2634 (48%)	185 (43%)	0.05
Smoking during pregnancy (*n*, %)	421 (33%)	1397 (25%)	115 (27%)	<0.01
Alcohol use during pregnancy (*n*, %)	418 (33%)	2420 (44%)	151 (35%)	<0.01
Fish intake in pregnancy; serving/week (median, IQ range)	0.6 (0.2 – 1.2)	0.7 (0.4 -1.1)	0.4 (0 – 0.9)	<0.01
Fatty fish	0.2 (0 – 0.5)	0.3 (0 – 0.5)	0.2 (0 – 0.4)	<0.01
Lean fish	0.4 (0 – 0.7)	0.4 (0.1 – 0.6)	0.2 (0 – 0.5)	<0.01
Perinatal folic acid supplementation (*n*, %)	701 (55%)	4151 (76%)	287 (67%)	<0.01
Nulliparous (*n*, %)	572 (45%)	3164 (57%)	224 (52%)	<0.01
Child				
Male gender *n*, %)	648 (51%)	2763 (50%)	236 (55%)	0.26
Birth weight; grams (mean±SD)	3333±553	3420±562	3446±557	<0.01
Gestational age; weeks (mean±SD)	39.7±1.7	39.8±1.8	39.9±1.7	0.15
Ethnicity (*n*, %)				<0.01
Dutch or other Western	588 (46%)	3709 (67%)	256 (59%)	
Cape Verdian	74 (6%)	142 (3%)	17 (4%)	
Moroccan	151 (12%)	310 (6%)	41 (10%)	
Antillean or Surinamese	202 (16%)	518 (9%)	43 (10%)	
Turkish	137 (11%)	395 (7%)	42 (10%)	
African	52 (4%)	144 (3%)	10 (2%)	
Other	77 (6%)	279 (5%)	23 (5%)	
Early day care attendance (*n*, %)	711 (56%)	3501 (64%)	250 (58%)	0.13
Any vitamin D supplementation in first year of life (*n*, %)	794 (62%)	3122 (57%)	245 (57%)	0.08
Any history of food allergy (*n*, %)	334 (26%)	632 (11%)	153 (35%)	<0.01
Breast-feeding (*n*, %)				<0.01
Never	355 (28%)	965 (18%)	118 (28%)	
Partial up to 4 months of age	641 (50%)	3083 (56%)	204 (47%)	
Exclusive up to 4 months of age	285 (22%)	1450 (26%)	109 (25%)	
BMI for age *z*-score at 48 months (mean±SD)	0.1±0.9	0.1±1.0	0.1±0.9	0.85
Overweight at 48 months (*n*, %)	233 (18%)	1012 (18%)	71 (16%)	0.31

Table represents the pooled results after the multiple imputation procedure.

Table 4.4.2: Prevalence rates of wheezing according to weekly intake of fish and timing of introduction of fish in the infant's diet.

			Number of children with wheezing at 36 months *n*=2480	Number of children with wheezing at 36 months *n*=2439
Fatty fish consumption at 14 months	No fish consumption	4931 (68%)	1736 (35%)	1710 (35%)
	Less than 1/2 serving a week	1910 (27%)	622 ' (33%)	611 (32%)
	At least 1/2 serving a week	366 (5%)	121 (33%)	118 (32%)
Other fish consumption at 14 months	No fish consumption	2182 (30%)	745 (34%)	738 (34%)
	Less than 1/2 serving a week	2965 (41%)	980 (33%)	962 (32%)
	At least 1/2 serving a week	2063 (29%)	755 (37%)	740 (36%)
Timing of introduction of fish in first year of life	Never given in first year of life	431 (6%)	173 (40%)	192 (45%)
	0-6 months	1281 (18%)	667 (52%)	593 (46%)
	6-12 months	5498 (76%)	1639 (30%)	1654 (30%)

Table represents the pooled results after the multiple imputation procedure.

Table 4.4.3: Association between timing of introduction of fish and wheezing symptoms when compared to no introduction in first year of life

Timing of introduction of fish	**Univariate model OR (95%CI)**	***p*-value**	**Multivariate model* OR (95%CI)**	***p*-value**
		Wheezing at the age of 36 months		
Never given in first year of life	Reference		Reference	
0 - 6 months	1.58 (0.53, 4.75)	0.33	1.31 (0.53, 3.29)	0.48
6-12 months	0.65 (0.40, 1.07)	0.08	0.72 (0.51, 1.03)	0.07
		Wheezing at the age of 48 months		
Never given in first year of life	Reference		Reference	
0 - 6 months	1.25 (0.65,2.41)	0.44	0.98 (0.60, 1.59)	0.92
6-12 months	0.58 (0.33, 1.02)	0.06	0.64 (0.43, 0.94)	0.03

OR: odds ratios relative to 'never wheezing in the past year' derived from logistic regression with never given in first year of life' as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.**Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life.

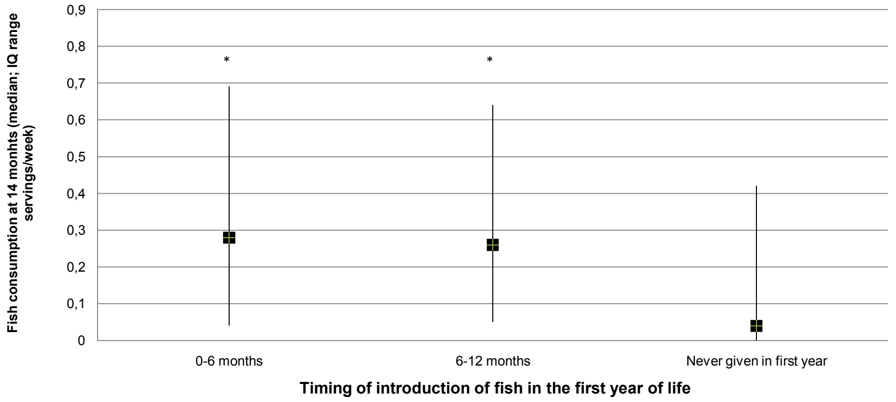

Figure 4.4.1a: Fish consumption at 14 months according to timing of introduction of fish in first year of life (*P*<0.05 when compared to never given in first year of life).

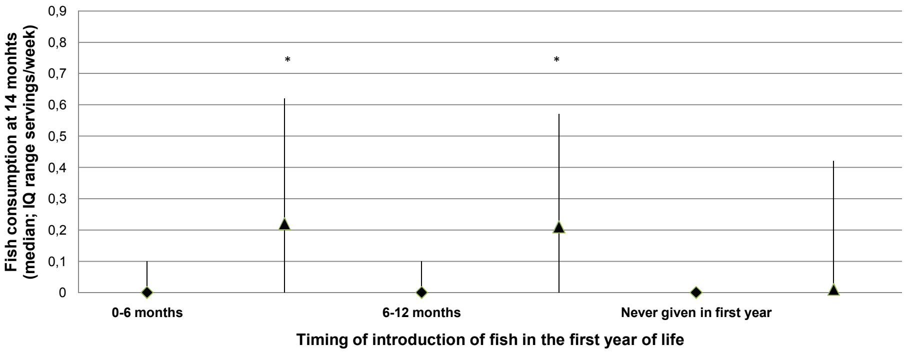

Figure 4.4.1b: Type of fish consumption at 14 months according to timing of introduction of fish in first year of life (*P*<0.05 when compared to never given in first year of life; ▲Total fish consumption; ⬩: Fatty fish consumption).

Table 4.4.4: Association between timing of introduction of fish and wheezing symptoms when compared to introduction between 6 and12 months.

Timing of introduction of fish	Univariate model OR (95%CI)	*p*-value	Multivariate model* OR (95%CI)	*p*-value
	Wheezing at the age of 36 months			
Never given in first year of life	1.53 (0.93, 2.53)	0.08	1.38 (0.97, 1.97)	0.07
0 - 6 months	2.43 (1.28, 4.60)	0.02	1.82 (1.00, 3.32)	0.05
6-12 months	Reference		Reference	
	Wheezing at the age of 48 months			
Never given in first year of life	1.72 (0.98, 3.03)	0.06	1.57 (1.07, 2.31)	0.03
0 - 6 months	2.16 (1.04, 4.48)	0.04	1.53 (1.07, 2.19)	0.03
6-12 months	Reference		Reference	

OR: odds ratios relative to 'never wheezing in the past year' derived from logistic regression with 'never given in first year of life' as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.*Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life.

Timing of introduction of fish and asthma-like symptoms

Children who were introduced to fish between 6 and 12 months had a significantly lower prevalence of wheezing at 48 months than children who were not introduced to fish in the first year of life (Table 4.4.3). Symptoms of shortness of breath were less prevalent in children introduced to fish between 6 and 12 months but this was not statistical significant (Supplemental table S4.4.5). Relative to introduction between 6 and 12 months, both fish introduction between 0 and 6 months and no introduction in the first year of life were associated with an increased prevalence of wheezing and shortness of breath at 48 months (Table 4.4.4, Supplemental table S4.4.6). The associations between timing of introduction of fish and asthma-like symptoms were not different within strata of ethnicity, family history of allergic disease, maternal fish consumption during pregnancy, any history of food allergy, eczema, breast-feeding duration, and type of fish consumption at 14 months of age (p-$_{interaction}$>0.15 for all comparisons).

Amount of fish servings and wheezing and shortness of breath

No association was found between the amount of fish servings and wheezing and shortness of breath at 36 and 48 months (Table 4.4.5 and Supplemental table S4.4.7). Fatty fish consumption of less than ½ serving per week was significantly associated with wheezing at 48 months but this association was mainly explained by confounding factors (Table 4.4.5).

Table 4.4.5: Association between infant fish consumption at 14 months and asthma-like symptoms

Fish consumption	Univariate model OR (95%CI)	*p*-value	Multivariate model* OR (95%CI)	*p*-value
		Wheezing at the age of 36 months		
Total fish consumption				
No fish consumption	Reference		Reference	
Less than ½ serving/week	1.05 (0.83, 1.34)	0.65	0.99 (0.77, 1.28)	0.95
At least ½ serving/ week	1.07 (0.85, 1.34)	0.53	0.99 (0.80, 1.24)	0.96
Fatty fish consumption				
No fatty fish consumption	Reference		Reference	
Less than ½ serving/week	0.89 (0.75, 1.06)	0.19	0.96 (0.81, 1.14)	0.62
At least ½ serving/ week	0.91 (0.66, 1.24)	0.54	1.04 (0.77, 1.40)	0.80
Other fish consumption				
No other fish consumption	Reference		Reference	
Less than ½ serving/ week	0.95 (0.83, 1.08)	0.41	0.90 (0.79, 1.04)	0.15
At least ½ serving/ week	1.10 (0.83, 1.46)	0.44	0.99 (0.77, 1.28)	0.95
		Wheezing at the age of 48 months		
Total fish consumption				
No fish consumption	Reference		Reference	
Less than ½ serving/week	1.01 (0.85, 1.20)	0.91	0.94 (0.78, 1.13)	0.48
At least ½ serving/ week	1.03 (0.81, 1.32)	0.77	0.94 (0.76, 1.18)	0.58
Fatty fish consumption				
No fatty fish consumption	Reference		Reference	
Less than ½ serving/week	0.88 (0.77, 0.99)	0.04	0.93 (0.82, 1.06)	0.26
At least ½ serving/ week	0.86 (0.53, 1.37)	0.48	0.97 (0.61, 1.55)	0.88
Other fish consumption				
No other fish consumption	Reference		Reference	
Less than ½ serving/ week	0.94 (0.78, 1.14)	0.51	0.89 (0.70, 1.13)	0.30
At least ½ serving/ week	1.08 (0.82, 1.42)	0.52	0.96 (0.73, 1.26)	0.74

OR: odds ratios relative to 'never wheezing in the past year' derived from logistic regression with no fish consumption as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.*Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life and total energy intake at 14 months.

Results did not differ within strata of ethnicity, maternal fish consumption during pregnancy, any family history of allergic disease, any history of food allergy, eczema, and breast-feeding duration (p-$_{interaction}$ >0.20 for all comparisons).

DISCUSSION

This study showed that introduction of fish between 6 and 12 months but not the amount of fish servings afterwards was associated with a lower prevalence of wheezing in pre-school children. However, no introduction in the first year of life or introduction between 0 and 6 months was associated with an increased prevalence of asthma-like symptoms.

Most studies on fish intake and asthma have assessed maternal fish intake during pregnancy and asthma in the offspring. Both epidemiological studies and randomized controlled trials showed protective effects of fish and fish fatty acids during pregnancy and asthma in children[28-29]. However, evidence from epidemiological studies assessing the effects of fish intake in young children and asthma is scarce and contradictory. Nafstad et al. found no significant association between introduction of fish in the first year and any asthma diagnosis[30]. On the other hand, Hesselmar et al. found a tendency between early fish introduction of fish and asthma (between group-difference of fish introduction: 9 vs 13 months)[12]. Another study by Kull et al. found that consumption of fish more than once a week in the first year of life was associated with a lower prevalence of asthma[31]. Arvaniti et al.[6] and Tabak et al.[32] showed that fish intake was associated with a lower prevalence of asthma in older children (> 8 y). In contrary, Willers et al. showed no association between the amount of fish consumption at the age of 2-3y and 7-8y and asthma diagnosis at 8y[33]. Age differences and variation in measuring fish consumption make it difficult to compare our results with previous studies but there seems to be a tendency for a beneficial effect on asthma when fish exposure occurs in the first year of life. Our study suggests that appropriate timing of introduction of fish rather than the amount of fish servings after 12 months is important in the association with wheezing. Underlying mechanisms why fish may protect against asthma are speculative. Fish is a great source of n-3 PUFA's such as decosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Several studies suggest that DHA and EPA have immunoregulatory and/or anti-inflammatory properties[29, 34]. Lower levels of n-3 fatty acids have been found in asthmatics relative to controls[35] whereas others found no association between levels of n-3 fatty acids and asthma in schoolchildren aged 8-13y[36]. In addition, the optimal intake of n-3 fatty acids in young children still remains controversial. In 2002, the Institute of Medicine in Washington concluded that there is insufficient evidence to provide clear recommendation on n-3 PUFA intake in children[37] whereas the Technical Committee on Dietary Lipids of the International Life Sciences Institute of North America stated in 2009 that a daily EPA and DHA intake between 250 and 500 mg/day reduces the risk of coronary head disease later in life[38]. The European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies concluded in 2009 that in older infants, DHA intake of 50 to 100 mg per day can improve visual function but evidence does not permit to define an age specific dietary recommendation for EPA and DHA from 2 years onwards.[39] None of the recommendations on EPA and DHA were related to any influence of asthma. In line with this, the European Society for Pediatric Gastroenterology Hepatology and Nutrition commented last year on supplementation of n-3 fatty acids to diet of children from 2 years onwards and possible benefits on clinical outcomes[40]. The committee concluded that there is no convincing evidence that supplementation with n-3 fatty acids has beneficial effects on asthma in children[40]. In case that n-3 fatty acids are the components in fish that may protect against asthma-like symptoms, one would expected that not only timing of introduction but also the amount of fish consumption and fatty fish in particular determines the effect. The latter was not the case in our study which may be explained in several ways. First, timing of introduction of fish may act as a proxy of different lifestyle and behaviors, for instance, it may be associated with intake of other foods that may have an influence on asthma-like symptoms. Nonetheless, we have previously demonstrated that timing of introduction of food allergens and a dietary pattern including fish, vegetables, and fruit at 14 months of age were not significantly associated with asthma-like symptoms in our cohort[3, 41]. Also, the timing of introduction of fish showed no similarities with timing of introduction of other foods (Supplemental table S4.4.4). This raises questions whether confounding by other diet factors play a role. Second, to our knowledge, not many studies assessed the amount of fish consumption at such a young age and its effect on asthma-like symptoms. Most studies have been carried out in older children or assessed the timing of introduction of fish in the infant's diet rather than the amount of fish consumption afterwards. Yet, Goksor et al[11] showed that early fish introduction but not the frequency of fish consumption at 12 months was associated with recurrent wheeze, which is in line with our results. Similar results were also found in the Prevention of Allergy among Children in Trondheim study[42]. Assessing nutrient intake at very young age is challenging since variation in certain foods can be limited and the diet can be subject to major changes at this age[43]. The lack of significant results on the amount of fish consumption in infancy may be due to the relatively low fish consumption in this age group. Therefore, further studies on the effect of fish consumption at pre-school age are necessary. Third, contamination of fish by for example mercury and polychlorinated biphenyls (PCB's) might be an explanation for negative results on fish consumption. Although studies suggest that exposure to these components may have immunological effects early in life[44-45], the effect on the development of asthma is rather indistinct. Fish is the major contributor of mercury consumption[46], and no association between mercury exposure and wheezing symptoms have been found in pre-school children[47]. In addition, a report by the Dutch Institute for Food Safety showed that high mercury intake is not a major problem in Dutch infants[48]. Another study showed that PCB's exposure may both increase and decrease the risk of allergic disease in children[49]. Nonetheless, intake of PCB's and dioxins is due to contributions of many food items in which fish contributes for 16-26% to the total intake of dioxins and PCB's in the Dutch population[50]. Since particularly the intake of fish is relatively low in this population of infants, we think it is unlikely that the intake of mercury, PCB's and dioxins from fish may have markedly influenced our results.

Finally, given the results found with timing of introduction rather than quantity of fish, there might be a specific window of exposure between the age of 6 and 12 months in which fish may be protective for developing asthma. The idea that a specific window of opportunity exist in the first year of life in which some environmental factors may decrease or increase the risk of asthma has been proposed by others[51]. In addition, others studies suggest that early life exposure to n-3 PUFA's may play a role in the induction of oral tolerance[52].

We found an increased risk of wheezing when children were not introduced to fish in the first year of life or when they received any fish between 0 and 6 months of life. This may suggest that tolerance to fish is particularly induced with introduction between 6 and 12 months which may provide some room for beneficial effects of additional components of fish that individually or in combination might be associated with a decreased risk of asthma and asthma-like symptoms.

A limitation of this work is the lack of objective measurement on asthma. The main outcomes in this study were parental-reported asthma-like symptoms. In pre-school children a diagnosis of asthma is based on symptoms of wheezing or shortness of breath[53] since it is complicated to perform lung function measurements in young children. Moreover, the diagnosis of asthma as proposed by current guidelines relies on symptoms rather than measurements[54].

Although a reasonable level of agreement was seen between timing of introduction of fish assessed at 12 months and at 14 months, misclassification on the timing of fish may occur. If this misclassification would be also related to asthma-like symptoms, this would have influenced our results but this seems highly unlikely. Also, we did not have data on type of the amount of fish consumed at moment of first introduction. Nevertheless, nutritional assessment can be subject to measurement error and, therefore, repeated measures of n-3 fatty acids in blood during infancy can be useful to shed light on the association between fish consumption and asthma development, and whether any potential beneficial effect of timing of introduction of fish can be attributed to n-3 fatty acids.

Since this is an epidemiological study, this study does not permit any final conclusions with respect to the causality of the described associations since residual confounding by other lifestyle factors of the child may exist.

Conclusion

Introduction of fish between the age of 6 and 12 months but not dietary fish intake at 14 months was associated with a lower prevalence of wheezing at pre-school age. A specific window of opportunity between the age of 6 and 12 months might exist in which fish may protect against asthma.

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SUPPLEMENTARY MATERIAL

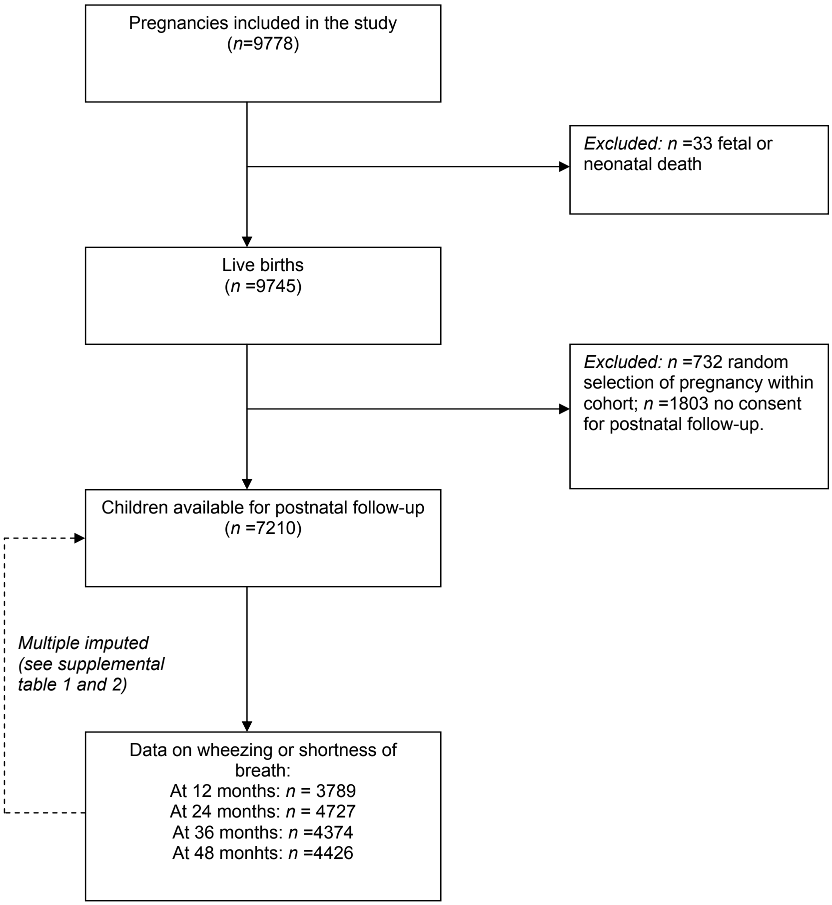


Figure S4.4.1: Flow chart of the study

Table S4.4. 1: Questions on fish consumption at the age of 14 months from the Food Frequency Questionnaire

a) How often did your child eat fish or shell-fish in the past month?	0= Not this month 1= 1 time a month 2= 2-3 times a month 3= 1 time a week 4= 2-3 times a week 5= 4-5 times a week 6= 6-7 times a week
b) How many portions of fish ate you child at such a day? (One portion of fish is for example one herring or one fish finger)	1= ¼ 2= ½ 3= 1 4= 1.5 5= 2 or more
c) Which types of fish or shell-fish did your child usually eat?	Ready to eat fried or baked seafood (deep fried white fish fillets) a) Always b) Often c) Sometimes d) Never Fish fingers: a) Always b) Often c) Sometimes d) Never Plaice, squid, flounder, cod, pollack, haddock, tilapia, sole, tuna, whiting: a) Always b) Often c) Sometimes d) Never Trout, gurnard, perch, plaice, wolf fish, swordfish: a) Always b) Often c) Sometimes d) Never Herring, mackerel, eel and salmon: a) Always b) Often c) Sometimes d) Never *Not included in calculating the amount of fish:* Shellfish scrimps, mussels, lobster: a) Always b) Often c) Sometimes d) Never
d) How often have you baked or fried the fish products yourself?	a) Always b) Often c) Sometimes d) Never

Table S4.4.2: Details of the multiple imputation modeling

	Multiple imputation procedure
Software used:	SPSS 17.1 for windows.
Imputation method and keysettings:	Fully conditional specification (Markov chain Monte Carlo method); Maximum iterations: 10.
No of imputed data sets created:	5
Variable included in the imputation procedure: (imputed or used as predictors of missing data)	Age filling in questionnaire at 6 months, Age filling in questionnaire at 12 months, Age filling in FFQ at 14 months, fish consumption (grams/day) at age 14 months, maternal age, marital status, gestational age at intake study, maternal BMI at intake, paternal BMI at intake, parity, gestational age at birth, gender, birthweight, Wheezing episodes at 12, 24, 36, 48 months, shortness of breath episodes at 12, 24, 36, and 48, child's ethnicity, household income, maternal educational level, exclusiveness of breast-feeding, folic acid supplementation during pregnancy, family history of asthma, eczema, hay fever or allergy to housedust, maternal energy intake, maternal fish intake, any daycare attendance at 6 and 12 months, timing of introduction of fish at 12, and 14 months, ever breast-feeding, any breast-feeding at 2 months, any bottle feeding at 2 months, any breast-feeding at 6 months, age stop breast-feeding, maternal smoking during pregnancy, maternal alcohol consumption during pregnancy.
Variables additionally added as predictors of missing data to increase plausibility of missing at random assumption:	Maternal BMI in first, second, and third trimester, fetal weight in first, second and third trimester, post maturity, maternal folate concentration during pregnancy, maternal homocystein concentration during pregnancy, maternal vitamin B12 concentration during pregnancy, history of upper and lower respiratory infections at 12, 24, 36, and 48 months, history of doctor attended cow's milk allergy in first year of life, history of eczema at 12, 24, 36, and 48 months, history of diabetes gravidarum, history of pregnancy induced hypertension, maternal protein intake, maternal fat intake, maternal carbohydrate intake, maternal dietary fiber intake, parenting stress score at 14 months.
Treatment of nonormally distributed variables	Predictive mean matching
Treatment of binary/categorical variables	Logistic regression models

Table S4.4.3: Comparison between study characteristics of original data and after the multiple imputation procedure.

	Original data *n*=7210 (valid %)	After multiple imputation procedure *n*=7210
Mother		
Maternal age at intake, years (mean±SD)	30.3±5.3	30.3±5.3
Missing	-	-
Maternal BMI at intake, kg/m^2, (mean±SD)	24.7±4.5	24.7±4.2
Missing	12%	-
Marital status (%)		
Married/living together	87%	86%
Living alone	13%	14%
Missing	11%	-
Household income (%)		
<2000 euro/month	37%	43%
≥2000 euro/month	63%	57%
Missing	28%	-
Maternal educational level (%)		
Low	10%	12%
Mid	44%	44%
High	46%	44%
Missing	12%	-
Family history of atopic disease (%)	48%	47%
Missing	11%	-
Smoking during pregnancy (%)	27%	27%
Missing	21%	-
Alcohol use during pregnancy (%)	42%	41%
Missing	20%	-
Fish intake in pregnancy, serving/week (median (IQ range))	0.8 (0.4, 1.1)	0.8 (0.5, 1.1)
Missing	34%	-
Perinatal folic acid supplementation (%)	73%	71%
Missing	33%	-
Nulliparous (%)	57%	55%
Missing	4%	-
Child		
Male gender (%)	51%	51%
Missing	-	-
Birth weight, grams (mean±SD)	3406±565	3406±565
Missing	0.1%	-
Gestational age, weeks (mean±SD)	39.8±1.8	39.8±1.8
Missing	1%	-
Ethnicity (%)		
Dutch or other Western	59%	63%
Cape Verdian	3%	3%
Moroccan	6%	7%
Antillean or Surinamese	9%	11%
Turkish	7%	8%
African	2%	3%
Other	5%	5%
Missing	9%	-
Early day care attendance (%)	77%	62%
Missing	58%	-

Table S4.4.3: Comparison between study characteristics of original data and after the multiple imputation procedure. *Continued*

	Original data *n*=7210 (valid %)	After multiple imputation procedure *n*=7210
Any vitamin D supplementation (%)	56%	58%
Missing	35%	-
Breast-feeding (%)		
Never	11%	20%
Partial up to 4 months of age	66%	54%
Exclusive up to 4 months of age	23%	26%
Missing	33%	-
Fish intake, serving/week (median (IQ range))	0.3 (0, 0.6)	0.3 (0, 0.6)
Missing***	54%	-
Fatty fish	0 (0, 0.1)	0 (0, 0.1)
Other fish	0.2 (0, 8.7)	0.2 (0, 0.6)
Timing of introduction of fish (%)		
Between 0 and 6 months	1%	18%
Between 6 and 12 months	96%	76%
Never given in first year	3%	6%
Missing	44%	-
Wheezing (%)		
36 months	13%	34%
Missing	40%	-
48 months	13%	34%
Missing	39%	-
Shortness of breath (%)		
36 months	11%	30%
Missing	40%	-
48 months	10%	41%
Missing	39%	-

*Data after the multiple imputation procedure represent the pooled results derived from the 5 imputed datasets. In case of ≥50% missing additional 10 imputations were performed but did not alter the results. ***Food frequency questionnaire at the age of 14 months was implemented at a later stage in the study.

Table S4.4.4: Degree of similarity between timing of introduction of fish and timing of introduction of other foods (Cohen's kappa [*k*]).

	k
Fish	1.00
Dairy	0.05
Grains	0.01
Meat	0.15
Egg	0.13
Vegetables	0.04
Fruit	0.01
Peanuts	0.02
Nuts	0.01
Soy	0.00

Table S4.4.5: Association between timing of introduction of fish and shortness-of breath when compared to no introduction in first year of life.

Timing of introduction of fish	Univariate model OR (95%CI)	*p*-value	Multivariate model* OR (95%CI)	*p*-value
	Shortness of breath at the age of 36 months			
Not introduced	Reference		Reference	
0 - 6 months	1.53 (0.57, 4.10)	0.33	1.26 (0.48,3.34)	0.57
6-12 months	0.65 (0.29, 1.48)	0.25	0.73 (0.36, 1.47)	0.32
	Shortness of breath at the age of 48 months			
Not introduced	Reference		Reference	
0 - 6 months	1.60 (0.78, 3.31)	0.16	1.21 (0.56, 2.65)	0.57
6-12 months	0.54 (0.19, 1.58)	0.20	0.61 (0.25, 1.47)	0.21

OR: odds ratios relative to 'never wheezing in the past year' derived from log-binomial regression with no introduction as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.*Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life.

Table S4.4.6: Association between timing of introduction of fish and shortness-of breath when compared to introduction between 6 and 12 months.

Timing of introduction of fish	Univariate model OR (95%CI)	*p*-value	Multivariate model* OR (95%CI)	*p*-value
	Shortness of breath at the age of 36 months			
Not introduced	1.53 (0.67, 3.47)	0.25	1.37 (0.68, 2.75)	0.32
0 - 6 months	2.33 (1.07, 5.11)	0.04	1.72 (0.84, 3.53)	0.11
6-12 months	Reference		Reference	
	Shortness of breath at the age of 48 months			
Not introduced	1.84 (0.63, 5.35)	0.20	1.64 (0.68, 3.97)	0.21
0 - 6 months	2.96 (1.56, 5.61)	0.01	1.99 (1.22, 3.27)	0.02
6-12 months	Reference		Reference	

OR: odds ratios relative to 'never wheezing in the past year' derived from log-binomial regression with no introduction as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.*Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life.

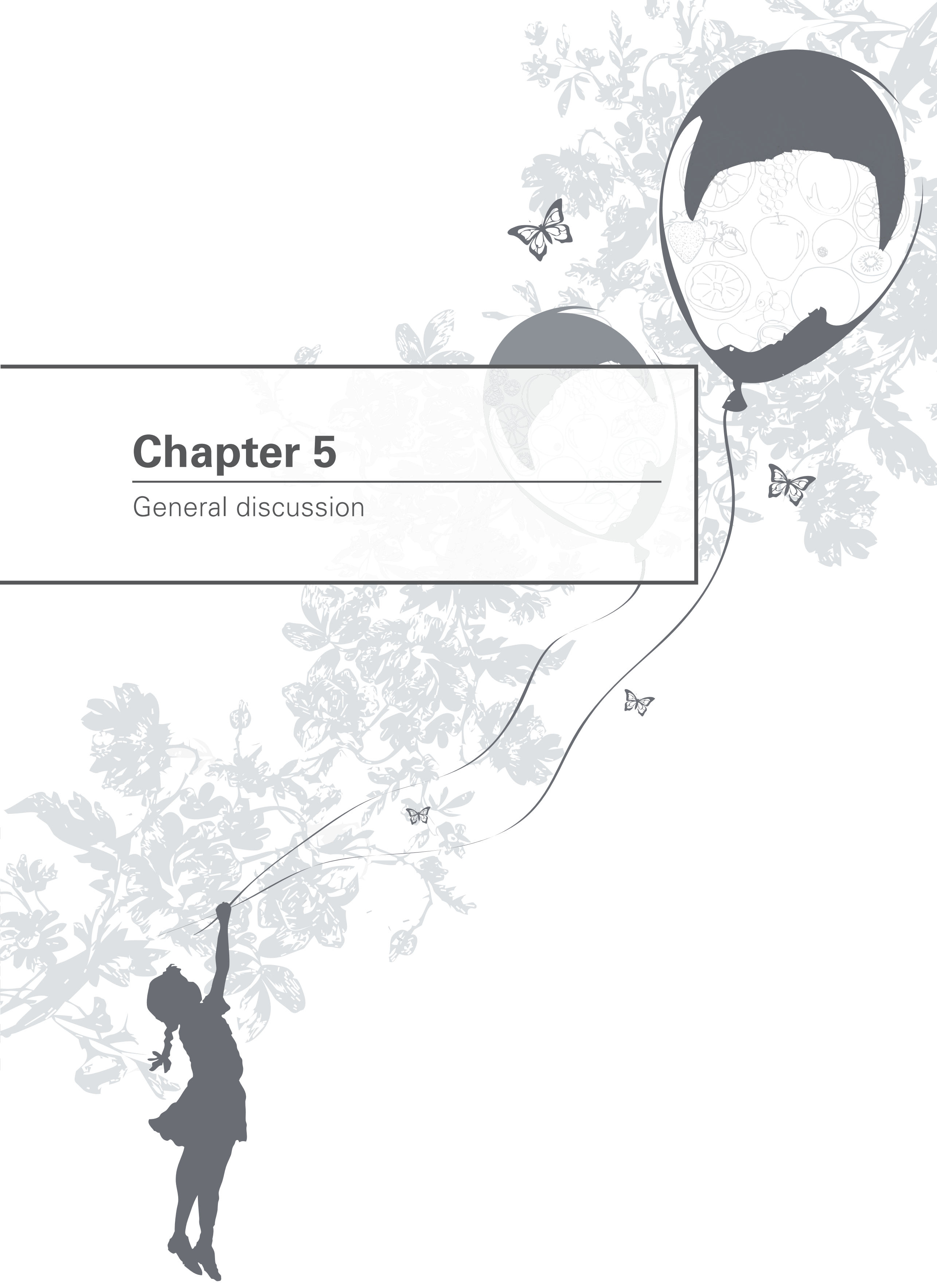
Table S4.4.7: Association fish consumption and shortness-of breath.

Fish consumption	Univariate model OR (95%CI)	*p*-value	Multivariate model* OR (95%CI)	*p*-value
Shortness of breath at the age of 36 months				
Total fish consumption				
No fish consumption	Reference		Reference	
Less than ½ serving/week	1.02 (0.85, 1.23)	0.80	0.97 (0.80, 1.17)	0.71
At least ½ serving/ week	1.07 (0.88, 1.32)	0.49	1.01 (0.82, 1.25)	0.91
Fatty fish consumption				
No fatty fish consumption	Reference		Reference	
Less than ½ serving/week	0.82 (0.63, 1.07)	0.13	0.88 (0.69, 1.12)	0.27
At least ½ serving/ week	0.79 (0.40, 1.58)	0.45	0.91 (0.48, 1.72)	0.74
Other fish consumption				
No other fish consumption	Reference		Reference	
Less than ½ serving/ week	0.95 (0.79, 1.15)	0.61	0.93 (0.74, 1.12)	0.38
At least ½ serving/ week	1.14 (0.93, 1.41)	0.19	1.05 (0.85, 1.30)	0.66
Shortness of breath at the age of 48 months				
Total fish consumption				
No fish consumption	Reference		Reference	
Less than ½ serving/week	1.09 (0.89, 1.34)	0.38	1.00 (0.79, 1.28)	0.99
At least ½ serving/ week	1.09 (0.88, 1.34)	0.42	0.97 (0.80, 1.18)	0.75
Fatty fish consumption				
No fatty fish consumption	Reference		Reference	
Less than ½ serving/week	0.86 (0.72, 1.03)	0.10	0.93 (0.76, 1.13)	0.45
At least ½ serving/ week	0.84 (0.52 – 1.38)	0.46	0.98 (0.57, 1.66)	0.92
Other fish consumption				
No other fish consumption	Reference		Reference	
Less than ½ serving/ week	0.99 (0.81, 1.22)	0.92	0.92 (0.69, 1.22)	0.52
At least ½ serving/ week	1.14 (0.93, 1.39)	0.19	0.98 (0.80, 1.20)	0.83

OR: odds ratios relative to 'never wheezing in the past year' derived from log-binomial regression with no introduction as reference category after the multiple imputation procedure; 95%CI: 95% confidence intervals.*Adjusted for: maternal age, maternal BMI, maternal alcohol and smoking during pregnancy, household income, maternal educational background, family history of asthma, eczema, hay fever or allergy to housedust, maternal fish consumption during pregnancy, folic acid supplementation during pregnancy, parity, birth weight, gestational age, infant's gender, infant's ethnicity, breast-feeding duration, early daycare attendance, any vitamin D supplementation in first year of life and total energy intake at 14 months.

Chapter 5

General discussion



The main goals of this thesis were to assess the determinants and consequences of dietary patterns and growth in the first two years of life; particularly to assess the effect of nutrition in early life regarding common childhood diseases such as constipation, asthma-like symptoms and atopic dermatitis in pre-school children (**Chapter 1**).

MAIN FINDINGS AND COMPARISONS WITH RECENT STUDIES

Early life nutrition

During the last few years, there is an increased awareness that nutritional exposure in early life can play an important role in the growth and development of children and in establishing disease susceptibility in this manner[1]. After birth, the optimal feeding for the child is exclusive breast-feeding and after 6 months complementary feeding is generally recommended.

Several studies have suggested a link between early introduction of complementary feeding and the development of overweight but these results are still inconsistent [2]. As a result, we aimed to assess the effect of timing of introduction of solids on weight development up to 4 years of age. We found that children who were introduced to solids between the ages of 3 and 6 months had an already higher weight gain before receiving solids. Shortly after the introduction of solids, a small decrease in weight was found in these children, whereas after the age of 12 months no major differences in weight between the different groups of age of solid introduction were found (**Chapter 2.1**). Our results imply that differences in growth may not necessarily be a cause of timing of introduction of solids. Grote et al. [3] demonstrated that the early solid introducers (before the age of 3 months) were heavier than all other children at 6 months of age. However, at 24 months of age no major differences remained in anthropometric measures according to the timing of introduction of solid foods. The authors reported that energy intake was positively associated with early solid introduction suggesting that when formula-fed children receive solids early, it leads to an increased energy supply in the first year of life. While we also found no long-term effect on growth according to timing of introduction of solids, the results of Grote et al. [3] are somewhat different when compared to our study. This discrepancy may be related to whether the child received predominantly breast-feeding or formula-feeding. Our study was comprised of children receiving both breast-feeding and formula-feeding in the first year of life whereas the study of Grote et al. [3] only included children that were formula-fed. Although we adjusted for the duration of breast-feeding in our study, the effect of complementary feeding may markedly differ among children with full breast-feeding than those fully fed with formula-feeding. It has been suggested that children are more likely to be fed on demand when given breast-feeding whereas formula-fed children may be more often fed according to a fixed schedule with a predetermined amount of volume that may increase the risk to overconsumption [4]. Hence, an increase in energy consumption as a result of early solid introduction may, therefore, be better compensated by the amount of breast-feeding that the child receives than when the child receives a fixed amount of formula-feeding leading to different effects of complementary feeding on growth.

In the KOALA birth cohort study, it was reported that obesity-prone behaviors cluster at the ages of 2 and 5 years [5-6]. We found comparable results in our study on determinants of dietary patterns of children aged 14 months (**Chapter 2.2**). We explored whether certain dietary patterns were already present in the second year of life, and which determinants play a role in adherence to these dietary patterns. We found that a 'Western-like' dietary pattern (characterized by intake of sugar-containing beverages, refined grains, confectionery and snacks) and a 'Health conscious' dietary pattern (characterized by fruit, vegetables, starchy foods, legumes and fish) can already be identified at the age of 14 months. We also showed that a 'Western-like' dietary pattern was associated with unfavorable lifestyle factors of mother and child such as maternal smoking during pregnancy, high maternal BMI, early solid introduction of the child and watching TV. Similarly, children with a low socioeconomic background were more likely to adhere to a 'Western-like' dietary pattern. Comparable results have been found recently by the ALSPAC study who assessed dietary patterns at the age of 6 and 15 months [7]. These findings advocate multivariate approaches in nutritional research with the dietary exposure variables being incorporated with other lifestyle factors when studying health outcomes. Although we did not find a direct association between a 'Western-like' dietary pattern and the child's weight in the second year of life, these study results imply that other known risk-factors for obesity (e.g. high maternal BMI, low socioeconomic background and sedentary behavior) already cluster with unhealthy eating patterns at a very young age. This might reflect a vulnerable group of children who are at high risk for developing obesity and metabolic diseases in later life.

Gastrointestinal outcomes

Constipation is a common symptom in the pediatric population (Figure 5.1). Although the transition from breast- to formula-feeding is often considered as a potential cause of constipation in infants [8], the influence of complementary feeding on constipation has not been sufficiently studied before. We explored whether timing of introduction of food allergens and gluten was associated with constipation since studies suggest that both celiac disease and food allergy may be a cause of constipation [9-10]. We found that early gluten introduction (i.e. before the age of 6 months) but not timing of introduction of other food allergens in the first year of life were associated with functional constipation (**Chapter 3.2**). The link between early gluten introduction and constipation could be explained by alteration in gut microbiota or the spectrum of celiac disease (Figure

5.2. Differences in gut microbiotia have been suggested to play a role in functional bowel disorders[11], allergies[12], and celiac disease[13]. Early life nutrition can also affect the gut microbial composition[12]. Recent studies highlighted that reactions to gluten may not be limited to the clinical spectrum of celiac disease, but may emerge to other (gastrointestinal) symptoms as well[14], in which interaction with the gut microbiota may also play a role [15]. Although these hypotheses cannot be tested in the studies we have performed, further research is worthwhile to examine underlying mechanisms of the effect of nutrition on the gastrointestinal tract in more detail.

In addition to the changing spectrum of celiac disease, we also studied whether the presence of several levels of celiac disease auto antibodies of the mother during pregnancy had an influence on fetal growth development (**Chapter 3.1**). Celiac disease can be characterized by major nutrient deficiencies due to malabsorption [14], and fetal demands which are not met by the maternal nutrient supply can impair fetal growth trajectories[1, 16]. We found that fetuses of mothers with intermediate or positive levels of celiac disease auto antibodies during pregnancy experienced different fetal growth trajectories. Lower fetal weight and birth weight were found in fetuses of mothers both with intermediate and positive levels of celiac disease auto antibodies. However, these results were not explained by indices of maternal nutritional status suggesting that the influence of celiac disease auto antibodies on fetal growth may have pathways beyond those related to nutritional deficiencies during pregnancy. Interestingly, the effect of intermediate anti-tTG on birth weight was predominantly present in those mothers carrying the HLA risk molecules for celiac disease (i.e. HLA-DQ2 and –DQ8). These results emphasize that even intermediate levels of celiac disease auto antibodies may have consequences on health and development. On the one hand, this may be a subclinical state related to the spectrum of celiac disease in which fetal growth impairment may be a first symptom, but it may also be a transient feature with no clear pathology despite the effect on impaired fetal growth. Therefore, further study is needed to establish the clinical value of intermediate anti-tTG in subjects carrying HLA-DQ2 or -DQ8.

Conflicting evidence exists regarding the function of dietary fiber in the etiology of childhood constipation. Several studies have indicated that children with constipation have a substantially lower fiber intake than healthy controls, but these results are not supported by others [17]. For this reason, we assessed the effect of whole dietary patterns at the age of 14 months on constipation, instead of the intake of dietary fiber in particular (**Chapter 3.3**). We found that adherence to a 'Western-like' dietary pattern was associated with a higher prevalence of constipation in the long run whereas a 'Health conscious' dietary pattern had only a short-term protective effect on constipation. These results were not explained by socio-demographic, lifestyle factors, or by feeding practices in the first year of life (i.e. breast-feeding and timing of introduction of solids). These time-specific effects of the dietary pattern can only be speculated upon,

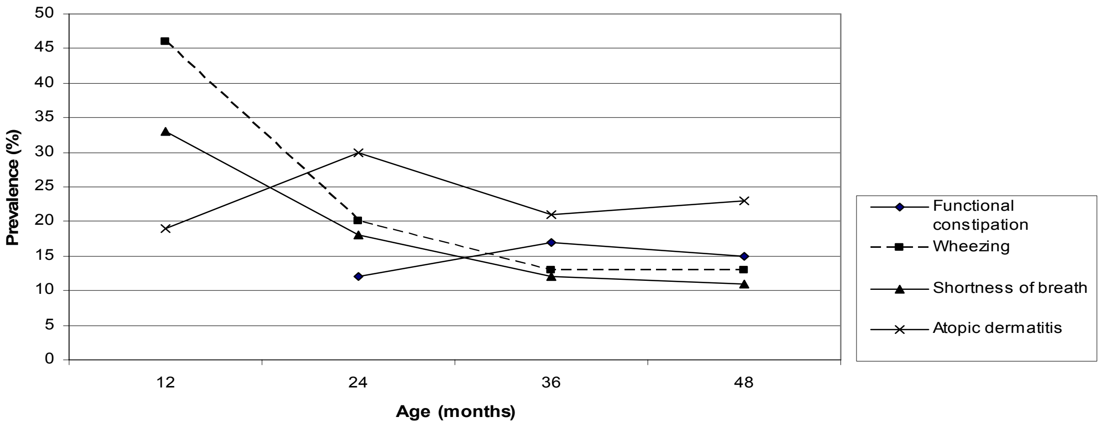


Figure 5.1: Prevalence of constipation, atopic dermatitis and respiratory symptoms within the Generation R Cohort

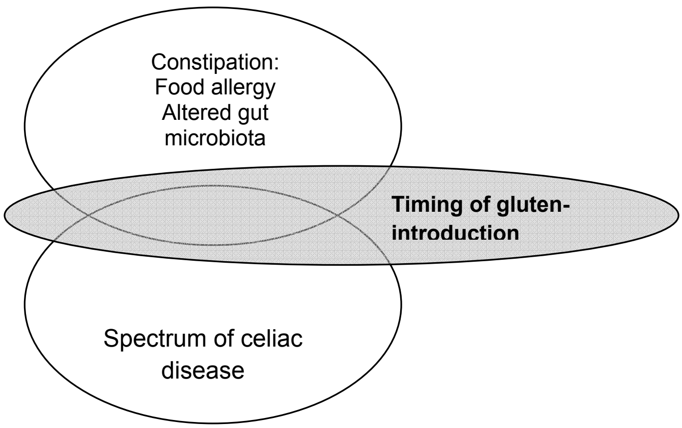


Figure 5.2 Proposed pathway in which timing of introduction may influence development of constipation

since the assessment of dietary patterns concerned a cross-sectional measurement. Nevertheless, the study highlights that certain combinations of food products instead of one particular nutrient in the child's diet may be important in the development of constipation. We also assessed in **chapter 3.3** whether sedentary behavior, overweight and obesity were associated with constipation in pre-school children. We did not find any link between overweight, sedentary behavior and constipation. Additional analyses demonstrated that the association between the dietary patterns and constipation were not explained by these variables. A recent meta-analysis among adults showed that some gastrointestinal symptoms, but not constipation, are more common in subjects with obesity [18]. It was expected that many obese individuals may not have regular physical activity or may have less healthy eating habits that may result in symptoms of constipation. Similar results were found in a study among adolescents [19]. In children, however, some studies have shown that symptoms of constipation are more common in children with obesity [20-21]. We were not able to confirm these findings. This may be explained by the fact that most other studies were carried out in a population of children with constipation or overweight selected from outpatient care which may comprise of more subjects with severe complaints related to overweight and constipation than in the general population.

We also studied whether cortisol had a role in constipation in childhood. Cortisol is a hormone that is secreted from the hypothalamic pituitary-adrenal (HPA) axis [22]. In several situations there appears to be a coordinated response of cortisol for energy homeostasis and support physical activity behaviors. Cortisol is known as a stress hormone since it can be excreted in response to psychological stressors[22] which may be a determinant of functional bowel disorders [23]. We illustrated that, in the second year of the child's life, cortisol response to stress, cortisol awakening response, and diurnal rhythm were not significantly associated with constipation and abdominal pain (**Chapter 3.4**). Studying cortisol at such a young age can be complicated by the fact that the HPA system is still under development in infancy and childhood [24]. However, an earlier study was able to demonstrate associations between cortisol levels and behavioral outcomes in pre-school children in this cohort [25] which may imply that cortisol as a marker of stress exposure just does not play an important role in constipation and abdominal pain in 2-year old Dutch infants.

Asthma-like symptoms and atopic dermatitis

Asthma-like symptoms and atopic dermatitis are very prevalent in young children (Figure 5.1). Generally weak evidence suggest that while there are links between diet and asthma, the nature of the associations, the timing and the therapeutic potentials are far from clear.

Studies, particularly in children, are required to establish if early life nutrition can be a potential target for intervention. We, therefore, assessed the influence of certain aspects of early life nutrition on asthma-like symptoms and atopic dermatitis in three life periods: during pregnancy, in the first year of life, and shortly after the period of introduction of complementary feeding.

In the past five years, papers regarding potential adverse effects of folic acid during pregnancy on asthma- and allergic outcomes have been emerging. Among other functions, folate provides, together with vitamin B12 as co-factor, methylgroups for the synthesis of methionine and its derivate S-adenosyl-methionin [26].

The latter is the most important methyldonor in the human body for DNA methylation (**Figure 5.3**). It is becoming more and more evident that DNA methylation plays a role in the etiology of diseases. The hypothesis that exposure to folic acid in pregnancy may increase the risk of allergic airway disease was first proposed by Hollingsworth et al. [27], who showed that feeding mice with folic acid and other B-vitamins increases the risk of respiratory disease caused by increased DNA methylation. According to this study and conflicting results by other subsequent epidemiological studies [28-32], we assessed the influence of folic acid supplementation, folate, and vitamin B12 levels during pregnancy on asthma-like symptoms and atopic dermatitis (**Chapter 4.1**). We found no significant association between *MTHFR* 677C>T polymorphism, folic acid supplementation, and folate and vitamin B12 levels and asthma-like symptoms. However, the offspring of mothers with high folate, and vitamin B12 levels during pregnancy had a higher prevalence of atopic dermatitis. These results support the hypothesis that methyl donors may have a function in potentially allergic diseases. When compared to other studies, different effects of folate seem to be dependent on the timing of folate exposure. Magedelijns et al. [32] showed that folate levels in particularly late pregnancy was associated with a lower risk of asthma whereas Haberg et al.[30] showed that high folate levels in the second trimester of pregnancy were associated with a higher risk of asthma in the offspring. Mainly protective effects of folate have been found when exposure was during child – or adulthood [33-34]. Hence, the timing of exposure to methyldonors (i.e. folate or vitamin B12) early in life may be of importance with regards to a possible risk of allergic disease.

Concerns are rising about current recommendations about delaying complementary feeding as an approach to prevent allergic disease since studies suggest that there might be a window of tolerance for introduction of complementary feeding between 3 and 6 months [35]. The term oral- or mucosal tolerance refers to the suppression of an immune response to an antigen by prior administration of the antigen by the oral route in a certain time period [36]. In **chapter 4.2** we have assessed the association between timing of introduction of allergenic foods and the prevalence of asthma-like symptoms and atopic dermatitis. We did not find any association between timing of introduction of the allergenic foods: cow's milk, hen's egg, peanut, tree nuts, soy and gluten before the age of 6 months and the prevalence of atopic dermatitis or asthma-like symptoms. These results do not support the recommendation to delay the introduction of allergenic foods to prevent allergic disease in childhood.

Recently, Sausenthaler et al. [37] reviewed the results of the LISaplus and GINIplus study in light of the general recommendations on complementary feeding by ESPHAN and the American Academy of Pediatrics. The authors conclude that introduction of solid foods before the age of 4 months may increase the risk of allergy but postponing introduction beyond 6 months had no benefit in allergy prevention [37]. Other available studies on the effect of timing of complementary feeding show no major differences in allergy prevalence as long as the age of introduction is between 4 and 6 months[35].

Interestingly, in the LISA study it was demonstrated that complementary feeding with a high diversity at the age of 4 months increases the risk of eczema [37]. Although we did not assess food diversity at moment of introduction of solid foods in our study, the results of the LISA study imply that high food diversity at a very young age may be an important risk factor for allergic disease in addition to timing of solid foods which needs further study.

Moderate evidence suggest that vitamin A, D, E, zinc, fruit and vegetables are associated with a lower risk of asthma in children but results are not consistent [38]. A 'single food/nutrient' approach may be insufficient for taking into account interactions among food components. The effect of a single nutrient or food may be too small to detect, but cumulative effects of multiple food components as included in a dietary pattern approach may be more adequate to be detectable [39]. For this reason, we assessed the effects of a 'Western-like' and 'Heath conscious' dietary pattern as defined in **chapter 2.2** of this thesis on respiratory symptoms. Relative to low adherence, high adherence to the "Western" dietary pattern was significantly associated with asthma-like symptoms. Another study showed comparable results on a diet with a high intake

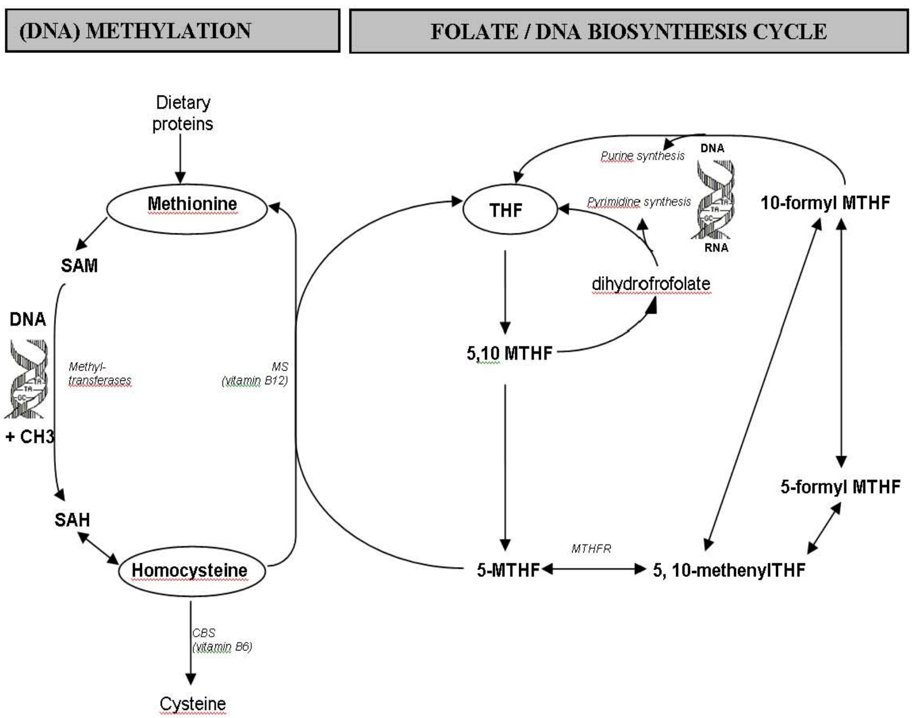


Figure 5.3: One-carbon metabolism. Methionine derived from the diet, protein breakdown or from remethylation of homocysteine forms *S*-adenosylmethionine (SAM). SAM can donate a methyl group by methyltransferases for DNA methylation.

of fat and simple sugars which was associated with an increased risk of asthma in Taiwanese schoolchildren [40]. These results may not be accurately comparable, since it concerns a very different study population. However, similar results have been found in another recent study which showed that unhealthy eating behavior was associated with increased asthma prevalence among school-children [41]. A 'Western-like' dietary pattern is generally high in saturated fat and low in anti-oxidant content. It has been hypothesized that regular high intake of saturated fat and low antioxidant intake may cause asthma by inducing chronic, low grade inflammation [42].

Although studies on dietary patterns in pre-school and asthma-like symptoms are scarce, existing studies found an association between a 'Mediterranean' diet (rich in plant foods, cereals, legumes and fish) and asthma in young children [38, 43]. Although a 'Mediterranean diet' has similarities with the 'Health conscious' dietary pattern that we studied in our study population, we found no significant association between this dietary pattern and asthma-like symptoms.

A 'Mediterranean-like dietary pattern' contains omega-3 fatty acids which are found in fish products and may have anti-inflammatory properties [44]. Therefore, we additionally explored the effect of fish consumption early in the infant's life and asthma-like symptoms (**chapter 4.4**). Strikingly, we found that not the amount of fish at 14 months of age was associated with the development of asthma-like symptoms, but particularly the timing of introduction of fish was associated with wheezing specifically. Introduction between the ages of 6 and 12 months was significantly associated with a lower prevalence of wheezing, but introduction between 0 and 6 months or no introduction in the first year were associated with an increased prevalence of asthma-like symptoms. We expected that omega-3 fatty acids were the components in fish, that protect against asthma-like symptoms; but since we only found an effect on timing of introduction of fish and not the amount, it is difficult to ascribe the effect of introduction of fish to omega-3 fatty acids only. A randomized controlled trial in the first 5 years of life also found no effect of increasing omega-3 fatty acid intake from birth onwards on asthma development [45]. We speculate that components in fish are particularly protective or harmful when exposure occurs in specific periods in the first year of life. This idea was generally applied to allergen exposure since very early exposure to allergens may induce allergic reactions because of insufficient development of the gastrointestinal and immunological system [46]. Fish is considered as a highly allergenic food product but fish allergy is not very common in Dutch children [47]. Therefore, it is unlikely that the association between timing of introduction of fish and wheezing is explained by whether the child had fish allergy. Nevertheless, it may be the case that a certain window of exposure exists (e.g. between 3 and 6 months) that induce mucosal tolerance for exposures that correlates with fish consumption of the child leading to a suppression of the immune system and thereby lower the risk of asthma-like symptoms.

METHODOLOGICAL CONSIDERATIONS

Specific strengths and limitations have been discussed for the studies described in chapter 2-4 of this thesis. In the following paragraphs come general methodological considerations will be described to appreciate the results. These considerations will be related to the study design, assessment of the disease outcomes, and assessment of food consumption data.

Study design

The studies described in this these were embedded in the Generation R Study, a population-based birth cohort study. Cohort studies are observational epidemiological studies comparing in a pre-defined population outcomes across groups with and without certain exposures (e.g. nutrition) after these are followed-up over time. Participation rates of large birth cohort studies are mostly around 30-40% [48]. Therefore, relative to other birth cohort studies the participation rate of the Generation R study was high (60-70%). However, selection bias may occur when the decision to participate may be related to socioeconomic and health conditions and these conditions are also related to the outcome of the study. These differences between participants and non-participants have implications for prevalence studies because the prevalence estimates may not be generalizable when there is selective participation [49]. However, the interpretation of our study results would only be altered when the association that is studied would be completely different among non-participants relative to those participated in the study. Several studies have shown that association measures are not markedly influenced by selective non-participation in cohort studies and thus reduced external validity may not be a major problem [48-50]. Accordingly, we assume that our results on the association between (nutritional) exposures and outcomes presented in this thesis are not influenced by selection bias.

Although this prospective cohort study enables to assess several exposures and outcomes longitudinally, longer duration of the study may lead to greater losses of follow-up and missing data. In the presence of missing data, the validity of the study results is dependent on the pattern of the missing data and the variables included in the analysis [51]. Several patterns of missing data can exist; missing can be "completely at random" (MCAR), meaning that missingness is unrelated to any subject characteristic, missing data can be "missing at random" (MAR) when it is related to subject characteristics that are measured in the study and included in the statistical models (e.g. maternal educational background), and missing can be "not at random" (MNAR) which means that the missingness is related to subject characteristics not measured in the study (e.g. subjects with a specific disease status may be more likely to drop out from the study) [51-52]. Indeed, non-response analysis in the studies described in this thesis showed that missing data was associated with lower socioeconomic background and unfavorable lifestyle factors such as maternal smoking, or lower folate and vitamin B12 levels during pregnancy.

A method to deal with missing data is multiple-imputation. Multiple imputations are increasingly recommended in epidemiology to adjust for the potential bias and loss of information that may occur in analyses restricted to subjects with complete data ('complete-case analyses'). Complete-case analysis is valid when data is MCAR, or when data is MAR but unrelated to the outcome that is studied. The ability of a multiple imputation procedure to reduce bias depends on the existence of variables that are associated with both missingness and the outcome variable [51]. Results of a multiple imputation procedure are valid under the assumption that data is MAR [51-52]. However, the distinction between data being MAR or being MNAR cannot be tested using the data in this thesis. Therefore, we assumed that missing data in this thesis was MAR and we applied a multiple imputation procedure in all studies presented in this thesis to reduce potential bias associated with attrition.

It should be noted that prospective cohort studies do no conclusively prove causal relationships. In **chapter 2.2** we showed that several lifestyle and socioeconomic factors are associated with dietary patterns of the children. Some of these factors are also associated with the outcome of constipation or asthma-like symptoms such as for example socioeconomic background. We intended to adjust our analyses for these potential confounders (i.e. the underlying factors that may affect both the exposure and outcome variable but are not intermediates in the pathway from exposure to outcome). However, it remains an actual possibility that the observed associations in this thesis are still a consequence of residual confounding by complex social and behavioral factors associated with the exposure and the outcomes which are not fully measured by the existing variables included in the statistical analyses, which may have led to an overestimation of the results.

Information bias related to the outcome assessment

Most of the information assessed in this thesis was derived from questionnaires filled out by the parents. This may have resulted in misclassification of the disease outcomes.

We defined constipation in our study by using the Rome II criteria (Table 1.1). These criteria have been developed in 1999 and attempted to provide a symptom-based definition of functional constipation in childhood [53]. However, later between 2004 and 2006, the Rome II criteria were revised since they were found to be too restrictive (Table 5.1) [54]. In this thesis, we were not able to define the outcome of constipation according to the most recent ROME III criteria. Nevertheless, a study by Baber et al. [55] demonstrated that recent changes in the ROME-criteria have resulted in more children being diagnosed with functional bowel disorders when they have unexplained abdominal pain.

Table 5.1 Rome III criteria for functional constipation in pre-school children

ROME III criteria for children under the age of 4 years (Rasquin 2006)
Two or more symptoms of the following:
· Two or fever defecation per week
· At least one episode of fecal incontinence per week
· Stool retentive posturing
· Painful or hard bowel movements
· Large diameter stools that could obstruct the toilet
· Presence of large fecal mass in the abdomen or rectum
· No objective evidence of an organic disease responsible for the symptoms.

Therefore, it may be likely that the prevalence of constipation in our cohort may be an underestimation rather than an overestimation of the 'true' prevalence of constipation in pre-school children when measured by the most recent criteria.

We used the questionnaire of the International Study of Asthma and Allergy in Childhood (ISAAC) questionnaire to assess the prevalence of asthma-like symptoms and atopic dermatitis [56]. This ISAAC questionnaire is developed for children aged 6-7 years and 13-14 years and is used worldwide but not validated for 1-4 years old children [56].

One of the difficulties in studying asthma is the lack of consistent diagnostic criteria. Especially at pre-school age, differentiation from other respiratory diseases is difficult [57]. However, regular tests to support on asthma diagnosis such as spirometry can only be performed in children older than 5 or 6 years [58]. Therefore, assessment of 'asthma-like symptoms' are often based on parent-reported symptoms through self-administered written questionnaires or through personal interviews by physicians. Mohangoo et al. [59] showed that the prevalence of wheezing and shortness of breath is higher when reported by the parents by questionnaire than when it is confirmed by physicians. Since there is no gold standard available for the diagnosis of asthma-like symptoms, it is difficult to judge whether self-reported asthma-like symptoms are an over-estimation of the 'true' prevalence or that these symptoms are under-diagnosed by physicians. However, a survey on respiratory symptoms in infants showed that parents often use the symptom of 'wheezing' inappropriately [60], which suggest that assessing 'asthma-like symptoms' by parental-derived questions may overestimate the 'true' prevalence of 'asthma-like symptoms'.

Most allergic responses are induced by immunoglobulin E (IgE) antibodies specific for a certain allergen. This allergen-specific IgE can be assessed by radioallergosorbent test (RAST) or skin tests which are often seen as a marker for atopic constitution in addition to clinical symptoms [61]. We did not have data on IgE sensitization therefore preclude final conclusion on whether asthma-like symptoms or atopic dermatitis may be linked to allergic disease.

Taking this into consideration, misclassification likely occurs when using questionnaire-based data on disease outcomes. Misclassification would only have influenced the results on the association of diet with the outcomes when the misclassification is related to nutritional exposure specifically. For example, potential food allergy may be important to many parents of children with symptoms of eczema or constipation. We had parental-reported data on doctor-attended food allergy instead of diagnosed by double-blind placebo-controlled food challenges (the gold standard)[62]. If parents of children with atopic dermatitis, asthma-like symptoms, or constipation believe that their child have food allergy, they might give their child a different diet. This strategy may influence our results on diet and disease outcomes. Nevertheless, we showed in **chapter 2.2** that children with a history of (self-report) food allergy had a tendency to adhere less to a 'Western-like' dietary pattern and thus may eat healthier than children without symptoms. This implies that it is more likely that our results on a ('Western-like') dietary pattern and disease outcomes are an under-estimation rather than an over-estimation of the 'true' effect of the dietary patterns on disease outcomes described in this thesis.

Information bias related to food consumption data

Estimating nutritional data in epidemiological studies can be prospective or retrospective. Prospective methods mainly include some form of record keeping at multiple time points. An advantage of this approach is that it concerns a direct estimation of current diet with daily variation taken into account. However, a major disadvantage of this approach is that it requires intensive labor for respondents and data processing. Moreover, it is very expensive particularly in large populations [63].

Most studies, therefore, rely on retrospective dietary assessment methods such as 24h dietary recalls, dietary history methods, or food frequency questionnaires (FFQ) [63]. The most common method used in epidemiological studies is a FFQ which we have used in studies described in this thesis. It evaluates a person's usual intake of type and amount of food over a defined period of time. It is relatively inexpensive and easy to administer. A disadvantage of using FFQ's is that it relies on memory and that the reported diet may be a distortion of usual diet [63]. Moreover, reported values from FFQ are subject to substantial measurement error that can affect the analyses and interpretation of the results[64].

In general, measurement error can be classified into two types: differential and non-differential [65]. Differential measurement error is related to the outcome of interest and can occur when cases recall their diet differently than subjects without the outcome of interest. This type of error is known as recall bias and occurs mainly when the outcome is measured before or at the same time when nutritional data is collected (e.g. in case-control studies). This type of measurement error is less likely to occur in the studies described in this thesis because diet was usually reported before assessment of the outcomes.

Non-differential measurement error is not particularly related to the outcome but the amount of over- or under-reporting may lead to attenuation in estimated effect size and loss of statistical power to detect significant diet–disease associations since confidence intervals will be wider [65].

A study by Lutomski et al.[66] showed that almost a third of participants in a nutritional study are under-reporters whereas only 12% were over-reporters of total energy intake [66]. Under-reporters of total energy intake were more likely to report lower energy intake from fat, fried food and snacks than accurate reporters. The opposite was true for the over-reporters who reported higher intake of unhealthy food products than the accurate reporters on total energy intake. Subjects of low-socioeconomic status were more likely to be over-reporters whereas subjects with overweight were more likely to be under-reporters. The consequences of this measurement error on study results are thus dependent on the direction of the misreporting and the subset of the population. For example, we found an association between a 'Western-like' dietary pattern and respiratory symptoms in children whereas we did not find an association between a 'Healthy-conscious dietary pattern' and respiratory symptoms. If parents of children with asthma-like symptoms were more likely to be under-reporters of total energy intake, this may lead to an underestimation of the association between both a 'Western-like dietary pattern' and a 'Health conscious' dietary pattern and asthma-like symptoms. On the other hand, if the degree of over- or under-reporting was unrelated to any subject characteristic (e.g. random), it is likely that the direction of the association would not be different but the measurement error may reduce power to reach a statistical significant results which influence the final conclusions of the study [65].

A method to deal with this dietary measurement error is adjustment for total energy intake [65] which we performed in our studies on diet and disease outcomes. Several methods to adjust for total energy-intake have been described by Willet et al[67] such a nutrient-density method or the residual method. Especially, when the effect of total energy intake can be large, these methods are preferred above only adjustment for total energy in the regression models. However, we assessed the effect of dietary patterns and food products on disease outcomes on which the nutrient-density or partition method cannot be applied. Using the residual-method as method for energy adjustment may remove variation in picking up certain dietary patterns in the population [68]. For that reason, adjustment for total energy intake was performed after defining the dietary patterns in our study.

Another more complicated method to overcome the problem of measurement-error is a regression calibration adjustment in which the effect-sizes are corrected by an attenuation-factor on the basis of a validation study. The reference instrument predominantly used in validation studies is a more detailed self-report, such as multiple 24-hour recalls which we used to validate the FFQ used in this thesis. Unfortunately, these self-report instruments do not meet the requirements for a proper reference instrument because their measurement errors, although less than when using FFQ's, are still substantial [64]. Reference instruments that can be used as gold standard to estimate 'true' dietary macronutrient intake is only available for total energy intake (doubly labeled water for assessment of energy expenditure) and total protein intake (24-hour urinary nitrogen excretion) which are expensive and difficult to use [69].

Accordingly, when we compare the results from the FFQ with results from 3 days 24-hour recalls, the intraclass-correlation coefficients for macronutrients ranged from 0.4-0.7 (**Table 5.2**) which is not optimal but comparable with other nutrition validation studies [70]. It shows that measurement errors are indeed present in the studies described in this thesis. However, if the numbers of under- and over-reporting described by Lutomski et al. [66] are generally true, it would be most likely that our study results on diet and disease outcomes are an under-estimation of the 'true' effect rather than an over-estimation as a result of measurement-error.

Implications for policy and practice

Some messages for public health implications according to the results of this thesis need to be emphasized.

First, the effects of timing of complementary feeding on child health seem to be dependent on the type of complementary feeding (e.g. gluten or fish). Accordingly, a 'one size fits all' recommendation on timing of introduction of complementary feeding does not take sufficient account for variety of health effects of different food products and needs further study before final recommendations can be made.

Second, since unhealthy eating patterns are already identifiable at 14 months of age and cluster with unfavorable lifestyle factors, interventions on improving healthy eating in children should be started before the age of 2 years. Detailed monitoring of dietary behavior even after period of complementary feeding is necessary since diet in infancy is a modifiable exposure variable with implications on child health. However, the majority of programs, which are mainly focused on the prevention of obesity, target children at school age (>5 years) [71]. We demonstrated that unhealthy eating behavior at 14 months may have consequences on the development of constipation and asthma-like symptoms. Promising findings have been found in 0-5 year olds to improve healthy eating behavior based on interventions at healthcare settings but it is still an understudied

Table 5.2 Intra-class correlation coefficient (ICC) for macronutrient intake measured by FFQ relative to 3 days 24-hour recalls in Dutch infants age 14 months.

Nutrient	ICC
Energy (kcal)	0.4
Total protein (grams)	0.7
Total fat (grams)	0.4
Carbohydrates (grams)	0.4
Dietary fiber (grams)	0.7

age group[71]. A Dutch example in order to improve healthy lifestyle of young children has been described in the 'Transitional plan for children with overweight' of youth health care in the Netherlands[72]. It describes methods for primary and secondary prevention of overweight in youth health care which includes recommendations to improve dietary behavior from birth onwards. Specifically, it comprises of the promotion of exclusive breast-feeding up to 6 months, reduction of sugar containing beverages, and stimulating cereal containing breakfast, and fruit and vegetable consumption before the age of 2 years [73]. This plan can form a basis for further extension of promoting healthy eating habits in children younger than 2 years of age. This should also include reduction of products rich in refined grains and saturated fat, confectionery, and salted snacks. It should be noted that interventions to improve healthy behavior at this very young age need to be embedded into ongoing practice and operating health care systems. Therefore, incorporating nutritional assessment tools in digital medical records in youth health care are necessary to monitor eating behavior of pre-school children more comprehensively and to provide targeted recommendations on food consumption. This monitoring strategy can be particularly performed in children at risk of unhealthy eating behavior such as for example in children with sedentary behavior, and of mothers with low-socioeconomic background, overweight, unfavorable lifestyle factors (e.g. smoking), and with multiple children (**Chapter 2.2**). Besides detailed dietary monitoring of children with a low-socioeconomic background, interventions to promote healthy behavior in young children should be suitable for socially disadvantaged families. We found that low household income was a significant determinant of an unhealthy dietary pattern of the child. A policy that is based on a tax on fatrich foods along with subsidy on fruit and vegetables has shown to be effective in moving diets in the direction of a recommended diet that may decrease the risk of diseases in future. However, this also may increase socioeconomic inequalities in eating behavior since products with the highest taxes are concentrated in socially deserving households [74-75]. Thus, strategies to improve healthy eating in families with low socioeconomic background should be combined with other interventions. The environment has been suggested to play a mediating role in removing negative effects of socio-economic circumstances. In addition, tailored (pre)school-based interventions in low socio-economic neighborhoods involving both the staff, parents and children can be promising to improve healthy eating habits in children with low socioeconomic background [76]. Finally, adaptation of compensation- and insurance-arrangements on dietary counseling specifically for families of low socioeconomic background can give a helping hand in supporting healthy eating in children of these families.

Third, mandated programs for fortification of food with folic acid have been implemented in several countries or are increasingly being considered by others. Although these folic acid fortification programs increases folic acid intake effectively and reduces the risk of neural tube defects (NTD's) substantially [77], concerns about potential risks of very high folic acid intake that may result in epigenetic changes are being debated. Since the neural tube is formed during the first trimester, folic acid supplementation after this period provides no further benefit in preventing NTD's and it has been suggested that folic acid supplementation after this period may promote adverse effects in susceptible individuals [77]. Currently, there is no need for changing the recommendation on folic acid supplementation for women planning a pregnancy since studies on risks of folic acid are still inconsistent. Nevertheless, careful monitoring of existing and proposed mass interventions on food fortification with folic acid on potential risks on allergic disease in the general population is needed.

Fourth, positive and intermediate celiac disease auto-antibodies levels were found in 0.5% and 4.4% of the mothers of the Generation R cohort respectively. The majority of the mothers were not aware of the fact that they had this feature which stresses that celiac disease is still under-diagnosed in the general population. Although these mothers may not have obvious clinical symptoms, we found that elevated celiac disease auto antibodies have consequences on fetal growth. No population screening for celiac disease is currently available since celiac disease does not meet the general criteria for screening by Wilson & Jungner (Table 5.3) [78]. Screening of celiac disease is controversial since it can be discussed if truly symptom-free patients will benefit from screening when considering the social impact of a gluten-free diet. Also, it is currently unclear how to treat subject with latent and potential celiac disease since the health consequences of these subtypes are still indistinct[79]. However, when there is a true causal effect of both intermediate and positive celiac disease auto antibody levels on fetal growth and these levels can be reduced by treatment with a gluten-free diet, considering screening for celiac disease auto antibodies in women of childbearing age may be justified in the long term.

Table 5.3 Wilson-Jungner criteria for screening programs[78]

1.	The condition being screened for should be an important health problem
2.	The natural history of the condition should be well understood
3.	There should be a detectable early stage
4.	Treatment at an early stage should be of more benefit than at a later stage
5.	A suitable test should be devised for the early stage
6.	The test should be acceptable
7.	Intervals for repeating the test should be determined
8.	Adequate health service provision should be made for the extra clinical workload resulting from screening
9.	The risks, both physical and psychological, should be less than the benefits
10.	The costs should be balanced against the benefits

IMPLICATIONS FOR FUTURE RESEARCH

Early life nutrition

To improve understanding of longitudinal relationships between diet and child health, regular dietary assessments during childhood are needed in order to track and trace dietary patterns and to assess the effects of diet and health outcomes accurately. Dietary habits may change during childhood and adolescence due to physiological development, changes in parental influence and social and environmental changes. Dietary habits have mainly been tracked in adults or between adolescence and adulthood, while just a few studies have tracked diet during childhood [80]. For that reason, further study on tracking of dietary patterns in young children is needed to establish whether certain eating habits in early childhood influence eating behavior in later life. Although dietary pattern analyses are helpful in order to provide public health messages on adequate nutrition, it does not go into depth about potential mechanisms on which nutrition acts in the etiology of asthma-like symptoms and constipation. Therefore, further research on the influence of specific nutrients such as for example folate, vitamin B12, vitamin D and omega-3 fatty acids are still necessary. Moreover, to improve the quality of studies assessing the effects of nutrition on health, multiple indices of nutritional intake and nutritional status are needed. In addition to an FFQ, this can include digital food records along with biomarkers such as blood concentration of methyl donors, vitamin D, and fatty acids which can be a promising approach in order to adequately measure the effect of nutrition and especially understand the functional components of diet on disease outcomes.

Further study on the appropriate timing of introduction of complementary feeding should focus on the type of complementary feeding and whether the effects are modified when the complementary feeding is introduced during period of full formula- or breast-feeding. Also, the diversity and the amount of the complementary feeding should be taken into account when assessing potential risks or benefits. Although epidemiological studies can provide useful insight in potential etiological pathways, results on the effect of complementary feeding are still far from clear. Randomized clinical trials provide the highest degree of evidence and, within this respect, it may be time for randomized controlled trials examining functional effects of age at introduction and composition of complementary feeding. These studies should point toward potential effects on growth, and risk of disease later in life which enables health care workers and nutritionists to provide clear and targeted recommendations on appropriate timing of introduction of solid foods. Results of a multicenter trial ‘PREVENTCD’ will be published shortly about the effect of early and gradual introduction of gluten during breast-feeding [81] on the development of celiac disease and the Prevention of

Overweight in Infancy (POI.nz) study is ongoing which will focus on the promotion of breast-feeding and delaying solid foods and the effect on growth development [82].

Finally, more evidence is needed to determine effective interventions to improve healthy lifestyles in young children, particularly those aged 0-5 years. These interventions may include activities to support staff in youth health care to implement health promotion strategies and support parents to encourage children to eat healthy foods and spend less time watching television. It may also include the creation of an environment and culture, such as at daycare or schools encourage children to eat healthy foods and increase physical activity.

Allergic and gastrointestinal outcomes

Specific IgE measurements can provide insight in whether the observed associations between diet and wheezing, and atopic dermatitis are truly linked to allergic disease. Also, measurements of exhaled nitric oxide (FEno) have gained interest as a marker of eosinophilic airway inflammation which has been found to correlate with asthma symptoms, lung function and atopy [83]. The latter measurement has been performed in 5-years olds in Generation R and is currently being processed. Reproducing the results on diet and asthma-like symptoms at a later age and assessing the associations with FEno can shed light whether diet early in life has long-term effects on asthma-like symptoms and whether this can be explained by airway inflammation and atopic constitution.

Since potential adverse effect of folic acid exposure during pregnancy on allergic outcomes have been proposed to be explained by epigenetic mechanisms [84], further study on methylation of genes involved in asthma and allergy along with FEno and lung-function measurements are needed. Additionally, assessment of folate and vitamin B12 along with co-factors that are involved in methylation pathway such as L-methionin, and vitamin B6 during several periods in pregnancy may gain insight whether the adverse effect of folic acid during pregnancy can be attributed to altered methylation status.

In view of the fact that the spectrum of celiac disease is changing and subtypes of gluten-sensitivity may exist, identifying characteristics of adults and children with even intermediate celiac disease auto antibodies are needed to provide a better definition of gluten reactivity and the burden of gluten-related disorders in the population [85]. Also, replication of the results on timing of gluten introduction and constipation by assessing celiac disease auto antibodies in these children will be performed to clarify whether the association between timing of gluten introduction and constipation is explained by the spectrum of celiac disease.

Although we did not find an association between cortisol and constipation and abdominal pain, it does not imply that stress does play a role in these outcomes. Further study on other indicators of stress, such as for example within family settings, in relation to gastrointestinal outcomes are meaningful in order to establish whether these can be potential targets for treatment and counseling in children with constipation.

Finally, to better understand how nutrition has an influence on gastrointestinal- and allergic disease, studying the gut microbiota can be very promising since several studies hypothesize that the gut microbiota play a role in the development of celiac disease [13], allergies [12], and functional bowel disorders [11]. Several relevant technologies and databases are emerging in order to study the gut microbiome to a great extent [86]. Beyond providing the global view of the human gut microbiome, these methods enable to study associations between the microbial genes of the gut and disease outcomes, and environmental factors including diet.

CONCLUSION

To conclude, the effects of timing of complementary feeding on child health seem to be dependent on the type of complementary feeding. Accordingly, a 'one size fits all' recommendation on timing of introduction of complementary feeding cannot be given and should be further evaluated in randomized controlled trials. Even at a very young age (i.e. before 2 years of age), unhealthy eating patterns can already be identified in young children which clusters with unfavorable lifestyle factors and low socioeconomic background. Interventions to improve dietary behavior should, therefore, be started early in life and be suitable for socially disadvantaged families.

We speculate that the increased prevalence of constipation and respiratory symptoms in children can be partly explained by the adoption of a Westernized diet early in life. Also, evidence was found for an association between high folate and vitamin B 12 during pregnancy and the development of atopic dermatitis in the offspring. Although there is no need for changing current recommendations on folic acid supplementation, potential harmful effects of folic acid should be evaluated in mass intervention programs.

Subtypes of gluten-sensitivity may exist which may explain a part of the spectrum of constipation in childhood and evidence suggests that increased celiac disease autoantibodies in pregnant women have consequences on fetal growth which needs further study.

Targeted interventions in order to improve early life nutrition and further clarifying the etiology of gastrointestinal and allergic diseases are points of attention when improving and maintaining child health.

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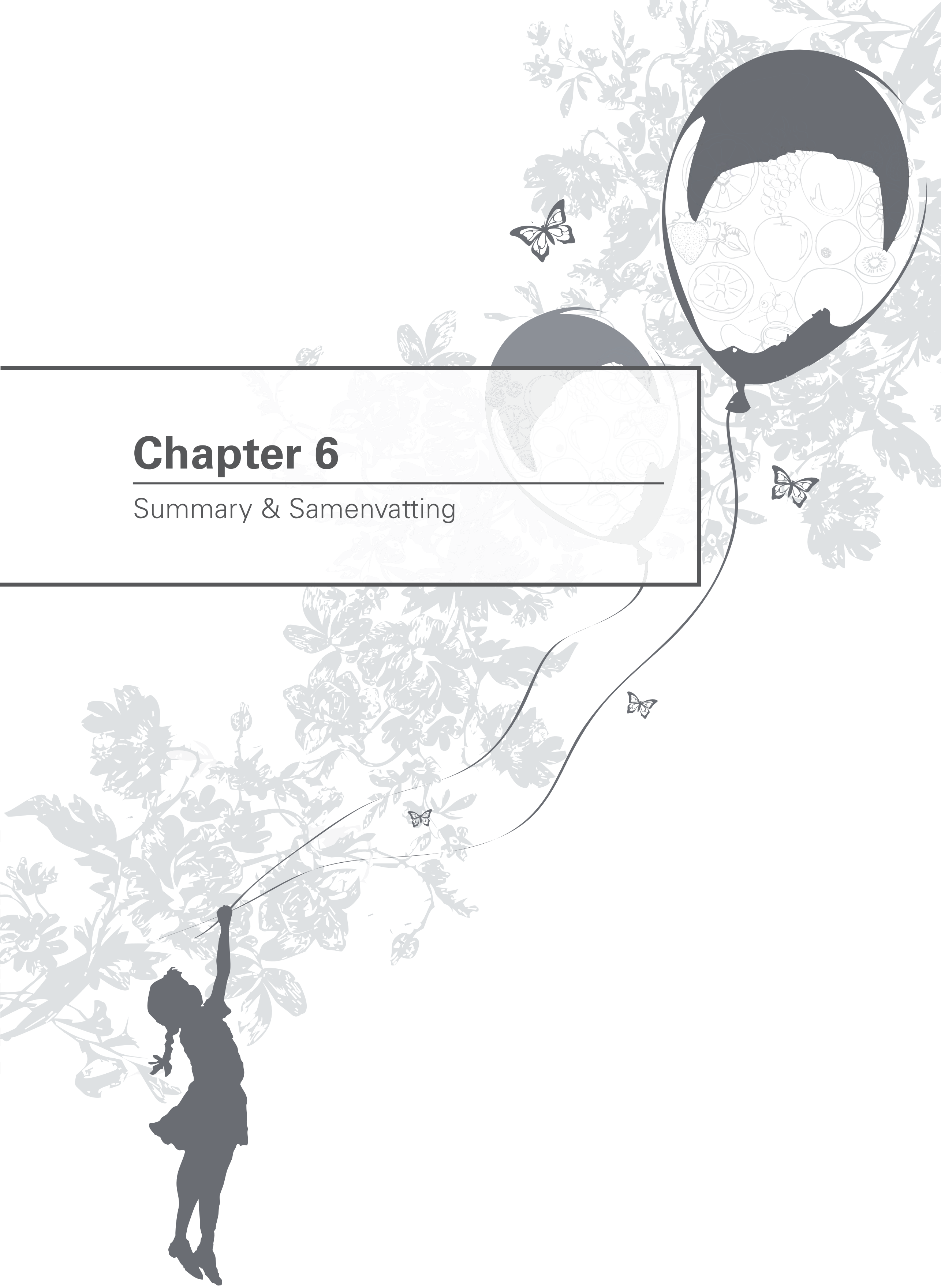
Chapter 6

Summary & Samenvatting



SUMMARY

Chapter 1 provided a description of the background and aims of the studies in this thesis. It described previously published studies on early life nutrition, gastrointestinal and allergic outcomes. Several studies have assessed effects of timing of introduction of complementary feeding but since the results are still inconsistent, the optimal age of introduction of complementary feeding has been debated widely. Also, the effect of nutrition shortly beyond the period of complementary feeding has been an area that is understudied. Although considerable knowledge have been gained with studies focusing on single nutrients or food groups, interest of nutritional research has shifted to the analysis of whole diets or dietary patterns. The associations between dietary patterns and diseases have been reviewed for adults. However, less is known about effects of dietary patterns of children at pre-school age. This thesis particularly focused on the outcomes of constipation, celiac disease auto antibodies, asthma-like symptoms, and atopic dermatitis, which are all prevalent in the general population. In line with this, the main goals of this thesis were to assess:

- *Infant nutrition:*
  - Consequences of timing of complementary feeding
  - Determinants of dietary patterns in toddlers
- *Gastrointestinal outcomes:*
  - Consequences of celiac disease autoantibodies during pregnancy
  - Nutritional and endocrinological determinants of functional constipation in childhood
- *Asthma-like symptoms and atopic dermatitis:*
  - Nutritional determinants of asthma-like symptoms and atopic dermatitis during the pre- and postnatal phase.

These aims were explored within the framework of the Generation R Study, a population-based prospective multi-ethnic cohort study from fetal life onwards. The studies in this thesis particularly referred to the pre-school period of the child (i.e. from birth to 4 years of age).

In **chapter 2.1** the association between the timing of introduction of solids (0-3 months, 3-6 months, or after 6 months) and weight-for-height change between birth and 48 months was studied. This study demonstrated that before solids were introduced, weight-for-height gain was higher in children introduced to solids between 3 and 6 months than in children introduced to solids between 0 and 3 months and between 6 and 12 months. Shortly after the introduction of solids, children introduced to solids before 6 months showed a relative decrease in weight-for-height whereas weight-for-height change did not differ between the different ages of solid introduction from 12 months onwards. We concluded that differences in weight-for-height in childhood are not caused by early introduction to solids in the first year of life.

The aim of the study described in **chapter 2.2** was to identify common dietary patterns in toddlers and to explore parental and child indicators of these dietary patterns. This study showed that a 'Health conscious' dietary pattern characterized by pasta, fruits, vegetables, oils, legumes and fish, and a 'Western-like' dietary pattern characterized by snacks, other fats, confectionery and sugar-containing beverages can already be identified in Dutch children aged 14 months. Low socioeconomic background, low parental age, parental smoking, multiparity, high maternal BMI, maternal nutritional intake during pregnancy, female gender, early solid introduction, and television-watching of the child were determinants of a 'Western-like' dietary pattern. Adherence to a 'Health conscious dietary pattern' of the children was inversely associated with maternal co-morbidity, alcohol consumption during pregnancy, and female gender while single parenthood, folic acid use, and dietary fiber intake during pregnancy were determinants of a 'Health conscious' dietary pattern. In this study we emphasized that particularly adherence to a 'Western-like' diet at a young age is associated with unfavorable lifestyle factors of both the parents and child, and low socioeconomic background.

In **chapter 3.1** we assessed different antibody levels against tissue transglutaminase (anti-tTG) of the mother during pregnancy and the relation with fetal growth and birth outcomes. Out of 7046 mothers, 0.5% had positive anti-tTG levels (> 6 U/ml) during pregnancy and 4.4% had intermediate levels of anti-tTG (0.8 - 6 U/ml). Relative to mothers with negative anti-tTG, positive anti-tTG during pregnancy was associated with a lower estimated fetal weight in second and third trimester. Both intermediate and positive maternal anti-tTG levels were significantly associated with lower birth weight. The effect of intermediate anti-tTG on birth weight was predominantly present in mothers carrying the HLA risk molecules for celiac disease. These results were not explained by indices of maternal nutritional status and suggest that the relationship between anti-tTG and fetal growth have pathways beyond those related to impaired nutrition status during pregnancy.

Chapter 3.2 described the association between the timing of introduction of food allergens and gluten early in life and functional constipation in childhood. At the age of 24 months, 12% of the children had functional constipation. Introduction of gluten before or at the age of 6 months was significantly associated with functional constipation. No association was found between timing of introduction of cow's milk, hen's egg, soy, peanuts, and tree nuts with functional constipation. These results suggest that early gluten introduction in the first year of life provide a trigger for functional constipation in a subset of children which may be explained by the spectrum of celiac disease or by specific effects of gluten on the gut microbiota.

The influence of the dietary patterns described in chapter 2.2 on the development of constipation in childhood was reported in **chapter 3.3**. In this study, we found that adherence to a 'Western-like' dietary pattern was associated with a higher prevalence of constipation up to 48 months. Adherence to a ‘Health Conscious’ dietary pattern was only associated at short-term, with a lower prevalence of constipation at 24 months. No association between overweight, sedentary behavior and constipation was found, which also did not explain the association between the dietary patterns and constipation. We concluded that specific dietary patterns in early childhood could be associated with higher or lower risks for constipation but these effects may be time-dependent. In the general population of Dutch children, overweight and sedentary behavior had no major role in the development of childhood constipation.

In a subgroup of the Generation R cohort, we assessed whether diurnal cortisol rhythm and cortisol stress reactivity were associated with functional constipation and abdominal pain in infancy (**chapter 3.4**). In this subgroup, 13% of the infants had functional constipation and 17% had abdominal pain at 24 months. Diurnal cortisol rhythm did not differ significantly between children with and children without functional constipation and abdominal pain. Although cortisol reactivity after a stressful situation procedure was slightly higher in infants with abdominal pain, the association was not statistically significant. Also, no association was found between the cortisol stress reactivity and functional constipation which imply that cortisol as a marker for stress does not play an important role in functional constipation or abdominal pain in Dutch children aged 24 months of age.

The study described in **chapter 4.1** determined whether serum folate and vitamin B12 concentration, folic acid supplementation, and *MTHFR* C677T polymorphism during pregnancy were associated with wheezing, shortness of breath, and atopic dermatitis in children up to 48 months of age. We found that maternal folate of at least 16.2 nmol/L and vitamin B12 of at least 178 pmol/L were positively associated with the development of atopic dermatitis but not with wheezing and shortness of breath. Maternal *MTHFR* C677T polymorphism and folic acid supplementation were not associated with wheezing, shortness of breath, and atopic dermatitis. These results suggest that high folate and vitamin B12 levels during pregnancy may increase the risk of atopic dermatitis in the offspring which may have consequences on evaluating mandatory fortification programs.

In **chapter 4.2** we examined whether the timing of introduction of the allergenic foods such as cow’s milk, hen’s egg, peanuts, tree nuts, soy, and gluten was associated with atopic dermatitis and wheezing in children 48 months of age. We found that the timing of introduction of cow’s milk, hen’s egg, peanuts, tree nuts, soy, and gluten before the age of 6 months did not appear to be significantly associated with the development of atopic dermatitis or wheezing in the pre-school period. The effects estimates were also not different between groups of children with and without a history of cow’s milk allergy or parental history of atopic disease. We have concluded that these results do not support the recommendation for delayed introduction of allergenic foods after age

6 months in order to prevent the development of atopic dermatitis and wheezing in pre-school children.

We have also assessed whether the dietary patterns which we have identified in chapter 2.2, were associated with respiratory symptoms in Dutch pre-school children, and whether this association was explained by total energy intake (**chapter 4.3**). We found no association between adherence to a 'Health conscious' dietary pattern and respiratory symptoms. However, relative to low adherence, high adherence to a 'Western-like' dietary pattern was significantly associated with frequent symptoms of wheezing, shortness of breath and respiratory tract infections. This association was only partially explained by total energy intake suggesting that there still might be an 'independent' effect of a 'Western-like' dietary pattern on respiratory outcomes beyond an increased energy supply that is associated with this dietary pattern.

To complement the results described in chapter 4.2 and chapter 4.3, we additionally assessed the association between fish consumption and asthma-like symptoms taking account for both the timing of introduction of fish and the amount of fish consumption in infancy in **chapter 4.4**. We found that introduction of fish into the infant's diet predominantly occurred between 6 and 12 months (76%). Introduction between the age of 6 and 12 months was significantly associated with a lower prevalence of wheezing at the age of 48 months whereas no introduction in the first year or introduction between 0 and 6 months were associated with an increased prevalence of wheezing at 48 months. The amount of both fatty fish and lean fish servings at the age of 14 months was not significantly associated with asthma-like symptoms. These results suggest that a window of exposure between the age of 6 and 12 months might exist in which introduction of fish may be associated with a reduced risk of asthma-like symptoms.

Finally, in **Chapter 5** a general discussion regarding the results of this thesis has been described related to recent published studies. It also provided recommendations for policy and practice and future research. Within this respect, we highlighted that a 'one size fits all' recommendation on timing of complementary feeding cannot be given according to the variety in results on complementary feeding in this thesis. We recommended to monitor the diet after the period of complementary feeding in young children more comprehensively in order to start interventions to improve dietary behavior early in child's life. Within this respect, special attention should be paid to children of socially disadvantaged families.

We also stressed not to change current recommendations on folic acid supplementation for women planning pregnancy since the evidence of potential harmful effects of folic acid supplementation is still insufficient. However, potential risks of allergic disease should be evaluated in mass intervention programs on mandated folic acid fortification. Although screening for celiac disease is still controversial, when there is a true causal effect of both intermediate and positive celiac disease auto antibody levels on fetal growth, screening for celiac disease auto antibodies in women of childbearing age may be justified in the long term.

Further follow-up of this cohort is recommended with special focus on endpoints of asthma including measurements of exhaled nitric oxide, IgE, and celiac disease auto-antibodies in children. Also, multiple indices of dietary intake which includes the longitudinal assessment of nutritional biomarkers and exploring the gut microbiota are worthwhile to gain insight in the influence of nutrition on gastrointestinal and allergic outcomes.

SAMENVATTING

Hoofdstuk 1 beschrijft de achtergrond en de doelstellingen van de studies beschreven in dit proefschrift. Het omvat eerder gepubliceerde studies over voeding in het vroege leven en de epidemiologie van veelvoorkomende gastrointestinale en allergische symptomen. Verscheidene studies hebben de effecten van de leeftijd van introductie van bijvoeding in het eerste levensjaar onderzocht. Aangezien de resultaten niet eenduidig zijn, is de optimale leeftijd van het introduceren van bijvoeding nog steeds een punt van discussie. Ook is de invloed van voeding kort na de periode van bijvoeding een gebied dat weinig is onderzocht. Hoewel veel kennis is opgedaan met onderzoek dat gericht is op individuele voedingsstoffen of groepen voedingsmiddelen, is de belangstelling van voedingsonderzoek de afgelopen jaren verschoven naar de analyse van gehele diëten of voedingspatronen. De associaties tussen specifieke voedingspatronen en ziekten is uitgebreid onderzocht onder volwassenen. Er is echter minder bekend over de effecten van voedingspatronen van jonge kinderen. Naast voeding in het vroege leven, is dit proefschrift in het bijzonder gericht op veelvoorkomende uitkomsten onder de algemene bevolking zoals obstipatie, coeliakie, astma-achtige klachten, en atopische dermatitis. De belangrijkste doelstellingen van dit proefschrift waren het in kaart brengen van:

- *Voeding in het vroege leven:*
  - Consequenties van leeftijd van introductie van bijvoeding
  - Determinanten van voedingspatronen onder jonge kinderen
- *Gastrointestinale uitkomsten:*
  - Consequenties van coeliakie antistoffen tijdens de zwangerschap
  - Voedings- en endocrinologische determinanten van functionele obstipatie bij kinderen.
- *Astma-achtige klachten en atopische dermatitis:*
  - Voedingsdeterminanten van astma-achtige klachten en atopische dermatitis in de pre- en postnatale fase.

De doelstellingen van dit proefschrift zijn onderzocht binnen de Generation R studie, een multi-etnisch prospectief bevolkingsonderzoek vanaf het foetale leven in Rotterdam. De studies beschreven in dit proefschrift richten zich voornamelijk op uitkomsten in de voorschoolse periode van het kind (vanaf geboorte tot de leeftijd van 4 jaar).

In **hoofdstuk 2.1** is de associatie tussen de leeftijd van introductie van bijvoeding (0-3 maanden, 3-6 maanden of na 6 maanden) en postnatale groei onderzocht. Deze studie liet zien dat kinderen die bijvoeding kregen tussen de leeftijd van 3 en 6 maanden al zwaarder waren voordat ze bijvoeding kregen vergeleken met de groep die tussen 0 en 3 en na 6 maanden bijvoeding geïntroduceerd kregen. Kort na de introductie van bijvoeding hadden kinderen die voor de leeftijd van 6 maanden bijvoeding kregen een lichte daling in gewicht maar na 12 maanden werd er geen verschil meer in groei gevonden

CHAPTER 6

tussen de verschillende groepen van kinderen die bijvoeding geïntroduceerd kregen. We concludeerden hieruit dat verschillen in groei bij kinderen niet veroorzaakt worden door vroege introductie van bijvoeding in het eerste levensjaar.

Het doel van de studie dat is beschreven in **hoofdstuk 2.2** was om voedingspatronen op de peuterleeftijd te identificeren. Deze studie liet zien dat een 'Gezondheidsbewust' eetpatroon, gekenmerkt door inname van deegwaren, fruit, groenten, plantaardige oliën, peulvruchten en vis, en een 'Westers' eetpatroon, gekenmerkt door snacks, dierlijke vetten, zoet en suikerwerk, en suikerrijke dranken, al te identificeren is bij kinderen op de leeftijd van 14 maanden. Lage sociaaleconomische status, jonge leeftijd en roken van de ouders, multipariteit, hoge BMI en voeding van de moeder, geslacht, vroege introductie van bijvoeding en TV kijken van 2 uur of meer per dag waren determinanten van een 'Westers' eetpatroon bij kinderen van 14 maanden. Het hebben van een 'Gezondheidsbewust' eetpatroon was negatief geassocieerd met co-morbiditeit van de moeder, alcoholconsumptie tijdens de zwangerschap en vrouwelijk geslacht terwijl eenouderschap, foliumzuurgebruik tijdens de zwangerschap en vezelinname tijdens de zwangerschap positief geassocieerd waren met dit 'Gezondheidsbewust' eetpatroon. Deze studie benadrukt dat specifiek een 'Westers' eetpatroon al op heel jonge leeftijd geassocieerd is met een laag sociaaleconomische klasse en ongezonde leefstijl van zowel ouders als het kind.

In **hoofdstuk 3.1** onderzochten we de relatie tussen verschillende antistoflevels van tissue transglutaminase (anti-tTG) van de moeder tijdens de zwangerschap en de relatie met foetale groei en geboorteuitkomsten. Een half procent van de 7046 moeders had positieve anti-tTG (>6 U/ml) tijdens de zwangerschap, terwijl 4.4% intermediaire anti-tTG (0.8 – 6 U/ml) had tijdens de zwangerschap. Positieve anti-tTG was geassocieerd met een lager foetaal gewicht in het tweede en derde trimester van de zwangerschap. Zowel intermediaire als positieve anti-tTG waren tevens geassocieerd met een lager geboortegewicht waarbij het effect van intermediaire anti-tTG met name aanwezig was bij moeders die dragers waren van een van de HLA risicomoleculen voor coeliakie. Deze resultaten werden niet verklaard door verminderde voedingsstatus van de moeder en suggereren dat de relatie tussen anti-tTG tijdens de zwangerschap en foetale groei bestaat uit pathofysiologische processen die niet zijn gerelateerd aan verminderde voedingsstatus tijdens de zwangerschap.

Hoofdstuk 3.2 beschrijft de associatie tussen de leeftijd van introductie van voedselallergenen en gluten in het eerste levensjaar en functionele obstipatie tijdens de peuterleeftijd. Op de leeftijd van 24 maanden had 12% van de kinderen symptomen van functionele obstipatie doorgemaakt. Het introduceren van gluten voor of op de leeftijd van 6 maanden was geassocieerd met een hogere prevalentie van functionele obstipatie op de peuterleeftijd. Er werd geen associatie met functionele obstipatie gevonden voor de leeftijd van introductie van andere voedselallergenen zoals koemelk, kippen-ei, soja, pinda's, en noten. De resultaten suggereren dat voor sommige kinderen vroege introductie van gluten in het eerste levensjaar een trigger kan vormen voor functionele obstipatie. Mogelijk is dit te verklaren door het spectrum van coeliakie of door specifieke effecten van gluten op de darmflora.

De invloed van de voedingspatronen beschreven in hoofdstuk 2.2 op het ontwikkelen van obstipatie is gerapporteerd in **hoofdstuk 3.3.** In deze studie vonden we dat het hebben van een 'Westers' eetpatroon geassocieerd was met een hogere prevalentie van obstipatie tot en met de leeftijd van 48 maanden. Een 'Gezondheidsbewust' eetpatroon was alleen op korte termijn geassocieerd met een lagere prevalentie van obstipatie. Er werd geen associatie gevonden tussen het hebben van overgewicht en obstipatie en sedentair gedrag en obstipatie. We concludeerden hieruit dat specifieke eetpatronen geassocieerd zijn met obstipatie maar dat deze effecten tijdsafhankelijk zijn. In de algemene populatie onder Nederlandse kinderen lijkt overgewicht en sedentair gedrag geen belangrijke rol te spelen bij het ontwikkelen van obstipatie.

In een subgroep van het Generation R cohort onderzochten we of dagritme van cortisol and cortisol respons na stress een rol speelden bij functionele obstipatie en buikpijn bij peuters (**hoofdstuk 3.4**). In deze subgroep had 13% van de kinderen klachten van obstipatie en 17% buikpijnklachten op de leeftijd van 24 maanden. Het cortisol dagritme was niet verschillend tussen kinderen met en zonder obstipatie of met en zonder buikpijn. Hoewel de cortisol stress respons lichtelijk verhoogd was bij kinderen met buikpijn, was dit niet significant verschillend vergeleken met kinderen zonder buikpijn. Ook werd er geen significante associatie gevonden tussen de cortisol stress respons en functionele obstipatie. Deze resultaten impliceren dat cortisol als een marker van stress geen belangrijke rol speelt bij functionele obstipatie en buikpijn bij Nederlandse kinderen van 24 maanden.

In **hoofdstuk 4.1** onderzochten we of folaat en vitamine B12 concentratie, foliumzuursuppletie, en *MTHFR* C677T polymorfisme van moeders tijdens de zwangerschap geassocieerd waren met 'wheezing', kortademigheid en atopische dermatitis tot en met de leeftijd van 48 maanden. We vonden dat maternale folaat levels van minstens 16.2 nmol/L en vitamine B12 levels van minstens 178 pmol/L geassocieerd waren met een verhoogde prevalentie van atopische dermatitis maar niet met 'wheezing' en kortademigheid. We vonden geen associatie tussen *MTHFR* C677T polymorfisme en foliumzuursuppletie, en de bovengenoemde uitkomsten. Deze resultaten suggereren dat hoge folaat en vitamine B12 levels tijdens de zwangerschap het risico op atopische dermatitis bij de kinderen zou kunnen verhogen. Dit kan mogelijk consequenties hebben voor de evaluatie van programma's op gebied van verplichte foliumzuurfortificatie van voedingsmiddelen.

In **hoofdstuk 4.2** onderzochten we of de leeftijd van introductie van allergene voedingsmiddelen zoals koemelk, kippen-ei, pinda's, noten, soja, en gluten geassocieerd waren met atopische dermatitis en ‘wheezing’ tot en met de leeftijd van 48 maanden. We vonden dat de leeftijd van introductie van deze voedingsmiddelen voor de leeftijd van 6 maanden niet significant geassocieerd was met het ontwikkelen van ‘wheezing’ en atopische dermatitis. De effecten waren ook niet verschillend tussen groepen kinderen met en zonder koemelkallergie of familiegeschiedenis voor atopie. We concludeerden hieruit dat de aanbeveling om allergene voedingsmiddelen na de leeftijd van 6 maanden te introduceren ter preventie van ‘wheezing’ of atopische dermatitis niet ondersteund kan worden met deze resultaten.

We hebben ook bekeken of de voedingspatronen die we vonden in hoofdstuk 2.2 geassocieerd waren met respiratoire symptomen en of deze resultaten te verklaren waren door totale energie inname (**hoofdstuk 4.3**). We vonden geen associatie tussen een ‘Gezondheidsbewust’ eetpatroon van de kinderen en respiratoire symptomen. Echter, het hebben van een meer ‘Westers’ eetpatroon was geassocieerd met een hogere prevalentie van frequente luchtweginfecties en ‘wheezing’. Deze associatie was slechts gedeeltelijk te verklaren door totale energie inname. Dit suggereert dat er nog steeds een ‘onafhankelijk’ effect van een ‘Westers’ eetpatroon op het ontwikkelen van respiratoire klachten bestaat buiten een verhoogde energie inname dat gerelateerd is aan dit eetpatroon.

In aanvulling op de resultaten beschreven in hoofdstuk 4.2 en hoofdstuk 4.3, hebben we de associatie tussen vis consumptie bij kinderen en astma-achtige klachten onderzocht. Hierin keken we zowel naar de leeftijd van introductie van vis als de hoeveelheid vis consumptie van de kinderen (**hoofdstuk 4.4**). We vonden dat vis voornamelijk tussen de leeftijd van 6 en 12 maanden werd geïntroduceerd (76%). Introductie tussen de 6 en 12 maanden was significant geassocieerd met een lagere prevalentie van ‘wheezing’ op de leeftijd van 48 maanden terwijl geen introductie van vis in het eerste levensjaar of introductie van vis tussen de leeftijd van 0 en 6 maanden geassocieerd was met een hogere prevalentie van ‘wheezing’ op de leeftijd van 48 maanden. De hoeveelheid porties van vette vis of magere vis op de leeftijd van 14 maanden was niet significant geassocieerd met astma-achtige klachten. Deze resultaten suggereren dat er mogelijk een ‘window of opportunity’ bestaat tussen de leeftijd van 6 en 12 maanden waarin vis het risico op astma-achtige klachten zou kunnen verlagen.

Tenslotte beschrijven we in **hoofdstuk 5** een algemene discussie over de resultaten beschreven in dit proefschrift in relatie tot andere studies die recent gepubliceerd zijn. Ook worden aanbevelingen gegeven voor praktische- en beleidsmaatregelen en verder vervolgonderzoek. In dit hoofdstuk benadrukken we dat een gestandaardiseerd advies over leeftijd van introductie van bijvoeding eigenlijk niet gegeven kan worden gezien het feit dat de resultaten over de gevolgen van vroege of late introductie van bijvoeding erg uiteenlopend zijn. Ook adviseren we om de voeding van jonge kinderen vlak na de periode van bijvoeding en lactatie meer in detail te monitoren. Op deze manier is het mogelijk om interventies ter verbetering van het voedingsgedrag van jonge kinderen vroeg in het leven te starten. Speciale aandacht hierin is echter vereist voor kinderen van families met een laag sociaaleconomische achtergrond.

We benadrukten ook dat het op dit moment nog niet nodig is om huidige aanbevelingen voor foliumzuur suppletie voor vrouwen met een zwangerschapswens te wijzigen omdat het huidige bewijs over potentiele schadelijke effecten van foliumzuur nog onvoldoende is. Echter, potentiële risico's op allergieën dienen wel geëvalueerd te worden in bestaande programma's voor foliumzuurfortificatie van voedingsmiddelen.

Ondanks dat screening van coeliakie in de algemene populatie op dit moment controversieel is, is deze screening voor vrouwen in de vruchtbare leeftijd op termijn mogelijk wel gerechtvaardigd wanneer er daadwerkelijk een causaal effect bestaat tussen intermediaire en positieve coeliakie antistoffen en verminderde foetale groei. Verder vervolg van dit cohort is aan te bevelen met specifieke focus op uitkomsten als astma (waaronder metingen van stikstof mono-oxide fracties in de uitademingslucht), IgE, en coeliakie antistoffen bij kinderen. Ook meerdere indicatoren van de voedingsinname zoals longitudinale metingen van biomarkers en het exploreren van de darmflora zijn waardevol om inzicht te krijgen in de invloed van voeding op gastrointestinale en allergische aandoeningen.

List of abbreviations

AAP	American Association of Pediatrics
ACTH	AdrenoCorticoThropic Hormone
AD	Atopic Dermatitis
ALSPAC	Avon Longitudinal Study of Parents and Children
anti-tTG	Tissue Transglutaminase Antibodies
AUC	Area Under the Curve
ß	Regressioncoefficient
BMI	Body Mass Index
CAR	Cortisol Awakening Response
CD	Celiac Disease
CI	Confidence Interval
CRH	Corticotropic-Releasing Hormone
CV	Coefficient of Variation
DHA	DocosaHexaenoic Acid
EAACI	European Academy of Allergy and Clinical Immunology.
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
EPA	EicosaPentaenoic Acid
FeNo	Fraction of Exhaled Nitric Oxide
FFQ	Food Frequency Questionnaire
GEE	Generalized Estimating Equations
HPA	Hypothalamic Pituitary Adrenal
IBS	Irritable Bowel Syndrome
ICC	Intra-class Correlation Coefficient
IgE	Immunoglobin- E
ISAAC	International Study of Asthma and Allergy in Childhood
MAR	Missing At Random
MCAR	Missing Completely At Random
MCMC	Markov Chain Monte Carlo
MNAR	Missing Not At Random
MTHFR	Methylenetetrahydrofolaat reductase
NOSIK	Nijmeegse Ouderlijke Stress Index / Parenting Stress Index
NTD	Neural Tube Defects
OR	Odds Ratio
PCA	Principal Component Analysis
PCB	Poly Chlorinated Biphenyls
PIAMA	Preventie en Incidentie van Astma en Mijt Allergie
PUFA	Polyunsaturated Fatty Acids
RR	Relative Risk

SAM	S-adenosylmethionine
SD	Standard Deviation
SES	Social Economic Status
SGA	Small for Gestational Age
SSP	Strange Situation Procedure
TV	Television
WFH	Weight-For-Height
WHO	World Health Organization

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Publication list

LIST OF PUBLICATIONS

1) Kiefte-de Jong JC, de Vries JH, Franco OH, Jaddoe VWV, Hofman A, de Jongste JC, Moll HA. Fish consumption in infancy and asthma-like symptoms: the Generation R Study. Pediatrics. 2012. Provisionally Accepted.
2) Nettleton JA, Hivert M, Lemaitre RN, McKeown NM, Mozaffarian D, Tanaka T, Wojczynski MK, Hruby A, Djousse L, Ngwa JS, Follis JL, Dimitriou M, Ganna A, Houston DK, Kanoni S, Mikkilä V, Manichaikul A, Ntalla I, Renstrom F, Sonestedt, van Rooij FJA, Bandinelli S, de Koning L, Ericson U, Hassanali N, Kiefte-de Jong JC et al. Meta-analyses including 15 cohorts show inverse associations between healthy diet and fasting glucose and insulin and no evidence of modification by multiple loci associated with glucose homeostasis. Am J Epidem. 2012. In Press.
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4) Kiefte-de Jong JC, de Vries JH, Bleeker SE, Jaddoe VW, Hofman A, Raat H, Moll HA. Socio-demographic and lifestyle determinants of 'Western-like' and 'Health conscious' dietary patterns in toddlers. Br J Nutr. 2012 Apr 5:1-11.
5) Ramdas WD, Wolfs RC, Kiefte-de Jong JC, Hofman A, de Jong PT, Vingerling JR, Jansonius NM. Nutrient intake and risk of open-angle glaucoma: the Rotterdam Study. Eur J Epidemiol. 2012 Mar 30.
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trial assessing a treatment strategy consisting of pravastatin, vitamin E, and homocysteine lowering on plasma asymmetric dimethylarginine concentration in mild to moderate CKD. Am J Kidney Dis. 2009 Jan;53(1):41-50.

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PhD portfolio

SUMMARY OF PHD TRAINING AND TEACHING ACTIVITIES

Name PhD student: J.C. Kiefte-de Jong Erasmus MC Department: Pediatrics Generation R	PhD period: 12-01-01-2009 until 12-09-2012 Promotor: Prof. H.A. Moll		
1. PhD training			
		Year	**Workload (ECTS)**
General academic skills			
-	Biomedical English Writing and Communication	2010	4 ECTS
-	Research Integrity	2010	0.5 ECTS
-	Workshop subsidieaanvragen NWO	2011	0.3 ECTS
-	Workshop subsidieaanvragen VENA	2011	0.3 ECTS
-	TULIPS Grant Writing Weekend	2012	0.6 ECTS
Research skills			
-	Advances in Genome Wide Association (NIHES)	2009	1.4 ECTS
-	Genome Wide Association Analysis (NIHES)	2010	2.8 ECTS
-	Modern Statistical Methods (NIHES)	2010	4.3 ECTS
-	Maternal and child Health (NIHES)	2011	0.9 ECTS
-	Masterclass: from problem to solution in public health (NIHES)	2012	1.1 ECTS
In-depth courses (e.g. Research school, Medical Training)			
-	International course on Nutritional Epidemiology, Imperial College, London (WCRF Fellowship).	2010	2.9 ECTS
Presentations (including presentations on international conferences)			
-	Presentation 'Dag voor Jonge Onderzoeker 2009', NVK	2009	1 ECTS
-	Presentation Researchmeeting Generation R, Erasmus MC, 'Infant Nutrition factors and constipation '.	2009	1 ECTS
-	Poster presentation on Annual Meeting of the European Society for Pediatric Research (ESPR), Hamburg, Germany	2009	1 ECTS
-	Presentation Grant Round, Sophia Children's Hospital	2010	1 ECTS
-	Oral and poster presentation on ESPGHAN 2010, Istanbul, Turkey	2010	2 ECTS
-	Oral poster presentation ESPGHAN 2011, Sorrento, Italy	2011	2 ECTS
-	Presentation Research Day Pediatrics, Erasmus MC	2011	1 ECTS
-	Presentation EUCONET International Workshop 'Nutrition resources', Bristol, UK.	2011	1 ECTS
-	Presentation Dag voor Jonge Onderzoeker, NVK 2011	2011	1 ECTS
-	Presentation Researchmeeting Generation R, Erasmus MC 'Dietary patterns in toddlers'.	2012	1 ECTS
-	Oral and poster presentation WEON 2012 'Health and disease during lifecourse'	2012	2 ECTS
International conferences (International conferences without presentations)			
-	Celiac Disease – International symposium, Amsterdam.	2009	1 ECTS
-	WCRF International Conference on Nutrition, Physical Activity & Cancer Prevention: Current Challenges, New Horizons	2010	1 ECTS
-	9th edition of the Unilever Nutrition Symposium on 26th and 27th May, Essential Fats for Future Health.	2011	0.3 ECTS
Seminars, workshops and symposia			
-	Dag voor Jonge onderzoeker 2009, NVK	2009	0.3 ECTS
-	Minicursus "Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidi eaanvragen"(Congresbureau)	2009	0.3 ECTS
-	Dag voor Jonge onderzoeker 2010, NVK	2010	0.3 ECTS

-	Nutricia symposium 'Voedingstekosten en functionele darmarmziekten en allergie'	2010	0.3 ECTS
-	Voedingscentrum Symposium 'Lastige of kritische eters'	2010	0.3 ECTS
-	Dag voor de jonge onderzoeker 2011, NVK	2011	0.3 ECTS
-	LUMC symposium Nieuwe richtlijnen voor coeliakie bij kinderen	2011	0.3 ECTS
-	EUCCONET International workshop: Nutrition resources in longitudinal studies; what can we learn form each other?	2011	1 ECTS
-	Danone Research Symposium 'Bringing Science to Early Life Nutrition'	2011	0.3 ECTS
-	ErasmusAGE Workshop on Systematic Reviews and Meta-analysis	2012	0.9 ECTS
-	Symposium 'Developmental Origins of Health and Disease (DOHaD): New results and hypotheses'	2012	0.3 ECTS
-	Seminars Department of Epidemiologie / Generation R	2009-2012	1 ECTS
2. Teaching activities			
		Year	**Workload (ECTS)**
Didactic skills Course, Onderwijscentrum, Erasmus MC		2011	1 ECTS
Supervising Master's theses			
-	MSc. student Ilse tromp 'Timing of introduction food allergens and wheezing and atopic dermatitis'.	2010	1.5 ECTS
-	MSc. student Sandra Briedé 'Determinants of solid introduction'	2011	1.5 ECTS
Supervising Bachelors theses			
-	BSc. student: Saskia & Orianne Graaff 'Validation of food frequency questionnaire against 24h recalls'.	2009	3.0 ECTS
-	BSc. student: Roxanne van Oeveren 'Dietary patterns and wheezing and atopic dermatitis in children'.	2010	1.5 ECTS
-	Medical students: Joanne Pijnacker Hordijk & Rianne Teeuw 'Use of probiotics and development of wheezing and atopic dermatitis.	2011	3.0 ECTS
-	Medical student Lisa Driessen 'Physical activity and constipation and asthma-like symptoms'.	2012	1.5 ECTS
Lectures			
-	Nutrition and infectious disease in low- and middle income countries – *public health in low and middle income countries, NIHES.*	2012	1.0 ECTS
Other			
Peer review of articles for scientific journals: *British Journal of Nutrition, Journal of Epidemiology and Community Health, Plos One,* and *Nutrients.*		2011-2012	1.0 ECTS

About the author

ABOUT THE AUTHOR

Jessica Christina Kiefte-de Jong was born on June 19th 1983 in Den Helder, the Netherlands. In 2000 she completed secondary school at the Etty Hillesum College in Den Helder. In the same year, she started her study Nutrition & Dietetics at the Amsterdam University of Applied Sciences and obtained her degree as a Dietician in 2004. She received an award from the Network of Food Experts and the Novartis Prize for Dietetics for her Bachelor thesis 'Gluten introduction and Breast-feeding: theory and practice' (Supervisors: Dr. E.G. Hopman and Dr. P.J Weijs). After she obtained her Bachelors degree, she studied Health Sciences at the VU University in Amsterdam, the Netherlands. She obtained her Master of Sciences degree in Public Health Research with focus on Nutrition and Health in August 2008, where she explored the influence of HLA-DR expression and nutritional status on clinical outcome in head and neck cancer receiving radiotherapy (Supervisors: Dr. H.M. Kruizinga and Drs. J.A.E. Langius), as well as looking at DNA methylation and anti-oxidant therapy in uremic patients (Supervisors: Prof.dr. Y.M. Smulders, Dr. P.W. Nanayakkara and Dr. M.R. Olthof). Prior to starting her PhD she worked as a dietician in the Groene Hart Hospital in Gouda and the VU Medical Center in Amsterdam, and combined her work later on with her research for her Master of Science degree. From January 2009 onwards she started working as a PhD student at the Generation R Study (Supervisor: Prof.dr. H.A. Moll). Her research was focused on early life nutrition and gastrointestinal and respiratory outcomes of which the results are presented in this thesis. In 2010 she was awarded a Fellowship from the World Cancer Research Fund for attendance of the International Course in Nutrition Epidemiology at the Imperial College in London, UK. In addition to her PhD project, she provided additional assistance for other research projects on nutrition embedded in the Rotterdam Study.

Jessica Kiefte- de Jong is married to Simon Kiefte and they live together in the city of Delft.

Dankwoord

DANKWOORD

Als iemand mij 10 jaar geleden zou vertellen dat ik ooit zou promoveren, had ik diegene fronsend aangekeken. Ook mijn biologieleraar van de middelbare school had er geloof ik niet zo veel vertrouwen in. Enfin, een balletje kan dan toch raar rollen…

Als eerste wil ik mijn promotor, Prof. Dr. H.A. Moll bedanken voor haar begeleiding in de afgelopen 3,5 jaar. Henriette; als groentje maakte ik in 2004 met jou en Sacha kennis naar aanleiding van een afstudeerstage en als iets minder dan een groentje klopte ik in 2008 bij je aan voor 'onderzoek op gebied van voeding'. Ik ben je dankbaar voor het vertrouwen dat je me hebt gegeven, de ruimte om mezelf te ontwikkelen en de vrijheid om met eigen onderzoeksideeën te komen. Daar heb ik veel van geleerd (Sorry voor de politieke relletjes die ik daardoor soms veroorzaakte…). Je bent een prettig en toegankelijk persoon om voor te werken.

Om te komen waar ik nu ben, had ik wel wat duwtjes in de goeie richting nodig. Na het opzeggen van een leuke baan, was het verder studeren op de VU toch een spannende keuze. Gelukkig hebben een aantal mensen mij geïnspireerd om verder het onderzoek in te gaan en daarvoor de nodige bagage te geven en deze wil ik dan toch graag even noemen in dit dankwoord. Prof.dr. Yvo Smulders, Dr. Prabath Nanayakkara, Dr. Margreet Olthof; bedankt voor jullie begeleiding, hulp en vertrouwen die jullie mij gaven om als student (die zonodig nog als diëtist wilde blijven werken) zo snel te mogen publiceren met de ATIC trial. Yvo; je bent de meest toegankelijke professor in de interne geneeskunde die ik ontmoet hebt. Dank voor alles (vooral ook voor de referentie die je aan Prof. Moll hebt gegeven!).

Jacqueline Langius en Hinke Kruizinga en alle andere diëtetiek collega's van het VUMC; Het was leuk om onderzoek bij jullie te doen en dit te combineren met het werk als diëtist. Jammer dat het perioperatieve voedingsproject niet doorging. Ik moet toegeven dat ik met een 'lichte pijn in het hart' het Amsterdamse voor het Rotterdamse verruilde. Wie weet komen we elkaar nog eens tegen.

En dan kom je ineens bij Generation R terecht; Allereerst wil ik alle deelnemertjes en hun ouders bedanken voor hun deelname aan het Generation R onderzoek. Bedankt voor jullie inzet en geduld. Zonder jullie geen data en geen proefschrift. Ook de onderzoeksmedewerkers die zich inzetten in het onderzoekscentrum wil ik erg bedanken. Eerst bij de focusgroep en later ook op focus op 5 (en nu focus op 9) weten jullie weer creatief om te gaan met de last minute beslissingen en soms de hectiek (naar 14 kinderen op een dag, kapotte apparatuur, herziene protocollen enz, enz). Jullie zorgen altijd dat deelnemers lachend naar huis kunnen gaan. Yvonne Knap: Yvon, door jou wordt iedereen gastvrij ontvangen bij Generation R, je hebt echt een gave om mensen zich thuis te laten voelen. Heel erg jammer dat je weg bent. Toni Noorda: bedankt voor het reviewen van Engels teksten van mijn proefschrift.

Medewerkers van het bureau: Claudia (met de snelheid van het licht data uitgeven), Patricia, Natalia, Karin, Rose, Karien en Ronald (en eerder ook Elise, Majanka, en Eran). Zonder jullie was er geen Generation R.

Vincent Jaddoe, projectleider van Generation R: Bij het typen van dit dankwoord herinner ik me dat je in de scanner van Mei 2010 aangaf ooit eens "Generation R te willen koppelen aan andere onderzoeken in ontwikkelingslanden". Ik daag je van harte uit om die uitdaging aan te gaan.

Medewerkers van de afdeling Humane Voeding aan de Wageningen Universiteit: Jeanne de Vries, Corine Peerenboom, Saskia Meijboom en Els Siebelink: Ik wist me in het begin geen raad met het verwerken van de voedingsdata van Generation R en had er een hard hoofd in dat ik nog voedingspapers tijdens mijn promotietraject zou kunnen schrijven. Gelukkig konden jullie mij verder helpen. Bedankt voor jullie inzet en samenwerking. Zonder jullie had het 'Early life nutrition'-deel van mijn proefschrift het daglicht niet gezien.

Prof.dr. Hooijkaas en Diana Dufour van het laboratorium Immunologie: bedankt voor jullie werk voor de anti-tTG bepalingen en de prettige samenwerking.

Prof.dr. Uitterlinden, Mila Jhamai, en Ramazan Buykcelik van het laboratorium Inwendige geneeskunde: Bedankt voor de last minute SNP's bepaling ten behoeve van de HLA-typeringen en dat jullie deze zo snel konden leveren.

Prof.dr. E.J.M. Feskens en Prof.dr. J.P. Mackenbach: hartelijk bedankt voor het plaatsnemen in mijn leescommissie en het beoordelen van mijn manuscript. Prof.dr. J.C. de Jongste, bedankt voor het kritisch doornemen van mijn manuscripten met betrekking tot de respiratoire uitkomsten en dat u als secretaris plaats wilde nemen in mijn leescommissie.

Sarah Timmermans: Beste Sarah, ondanks wat kleine strubbelingen is het artikel in J Nutr er toch gekomen. Met dank aan jou want jij kwam met het idee.

De overige co-auteurs van de manuscripten in dit proefschrift wil ik ook bedanken voor zijn of haar bijdrage aan de betreffende publicatie: Prof.dr. A. Hofman, Prof.dr. H. Raat, Prof.dr. E.A.P. Steegers, Prof.dr. H. Tiemeier, Drs. N. Saridjan, Dr. J.C. Escher, Dr. S.E. Bleeker, Prof.dr. O.H. Franco, Dr. C.W. Looman, Prof.dr. A.C. Hokken-Koelega, Drs. S.P. Willemsen en Dr. C.M. Renders.

Tijdens mijn promotietraject heb ik ook veel hulp gekregen van mijn studenten. Het valideren van de FFQ was niet gelukt zonder Orianne Graaff, Saskia Dorhout Mees, en Roxanne van Oeveren: bedankt voor jullie inzet met het rekruteren van deelnemers en het afnemen van de 24-h recalls (voordat je ze een keer te pakken had aan de telefoon…). Sandra Briedé, Lisa Driessen, Joanne Pijnacker Hordijk, en Rianne Teeuw: ik kwam zelf lang niet altijd aan alle interessante vraagstellingen toe maar jullie hebben er allemaal wat moois van gemaakt. Vast en zeker een basis voor interessante publicaties. Een speciaal bedankje aan Ilse Tromp: Ilse, je hebt besloten om na gezondheidswetenschappen geneeskunde te gaan studeren. Je bent een goede onderzoeker en ik twijfel er niet aan dat je ook een goede arts zal worden! Bedankt voor jouw inzet. Jouw artikelen verdienen met recht een plekje in dit proefschrift en ik hoop dat we samen nog een paar mooie papers gaan schrijven.

Lenie van Rossem: Ik heb de afgelopen jaren prettig met je samengewerkt en ik miste onze epidemiologische discussies wel toen je naar Utrecht vertrok. Analyseren op vrijdagmiddag met ons slaperige hoofd was alleen niet zo aan ons besteed (met als gevolg dat de analyses weer opnieuw konden op maandag). Het resultaat mag er toch wezen. Op naar de volgende bijkletsborrel of drijfzandvoorstelling.

Edith van den Hooven, Denise Heppe en Romy Gaillard: Bedankt voor de gezellige koffiemomentjes en dat ik mijn hart zo nu en dan bij jullie kon luchten. Ook mijn kamergenootjes, Anne Marie Visser, Ankie Lebon en Michelle Jansen wil ik bedanken. We konden heel hard werken maar er werd ook regelmatig gelachen.

Mijn paranimfen Lianne Kerkhoven en Thamar Post: Lianne, al sinds de middelbare school kennen we elkaar. Ik vind nog mappen met rare tekeningen en berichtjes. We zien elkaar niet zo veel meer als vroeger maar iedere keer als we elkaar spreken is het weer als vanouds. Je stond al naast me als getuige bij mijn trouwdag en ik ben blij dat je ook bij deze mijlpaal naast me staat. Thamar: we hebben veel leuke en minder leuke dingen met elkaar gedeeld. Onze weekendjes weg zijn altijd weer een feest (alhoewel we ook echt gek kunnen worden van elkaars geratel en lompe acties). Vroeger waren paranimfen de lijfelijke beschermers van de promovendus. Je bent echt een taaie en een vrouw met ballen dus de rol als paranimf is zeker aan je besteed.

Overige vrienden en familie: ik ga jullie niet allemaal opnoemen want ik ben bang dat ik iemand vergeet maar bedankt voor alle gezelligheid, welkome afleiding, brownies, muziek, of wijze raad. Pa en Ma de Jong: bedankt voor jullie bijdrage aan het ontwerp van mijn proefschrift. Tamarah en Wouter, Arjan en Hinko, Pa en Ma Kiefte, Tim en Ineke, Stefan en Cindy: het was soms best lastig uit te leggen waar ik in hemelsnaam zo druk mee was… maar ik ben nu eindelijk 'afgestudeerd' hoor! Tijd voor een feestje (Tamarah: volgende feestje is vast van jou!).

Lieve Simon, al de helft van ons leven samen en nog steeds zou ik niets liever wensen. Je weet als geen ander wat een complex persoon ik soms kan zijn en je geeft me de ruimte die ik nodig heb. Dank voor al je liefde en steun. Het leven is zoveel mooier als je het kunt delen. Ik houd van je!

Last but not least, dank ik God, waar ik veel niet van begrijp maar waarvan ik weet dat Hij er is en met me meegaat.