Nutrition, body composition, and cardiometabolic health in children

Trudy Voortman

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Voortman T, Braun KVE, Kiefte-de Jong JC, Jaddoe VWV, Franco OH, van den Hooven EH. Protein intake in early childhood and body composition at the age of 6 years: the Generation R Study. *Submitted for publication.*

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Chapter 3 Fatty acids

Voortman T, van den Hooven EH, Braun KVE, van den Broek M, Bramer WM, Chowdhury R, Franco OH. Effects of polyunsaturated fatty acid intake and status during pregnancy, lactation, and early childhood on cardiometabolic health: a systematic review. *Progress in Lipid Research* 2015;59:67–87.

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Chapter 4 Vitamin D status

Voortman T*, van den Hooven EH*, Heijboer AC, Hofman A, Jaddoe VWV, Franco OH. Sociodemographic and lifestyle determinants of vitamin D deficiency in a multiethnic cohort of school-age children: the Generation R Study. *Journal of Nutrition* 2015;145:781–8.

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Chapter 5 Dietary patterns

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Voortman T*, Leermakers ETM*, Jaddoe VWV, Hofman A, Franco OH, van den Hooven EH, Kiefte-de Jong JC. *A priori* and *a posteriori* dietary patterns at the age of 1 year and body composition at the age of 6 years: the Generation R Study. *Submitted for publication.*

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* *Denotes equal contribution within a manuscript*

Chapter 1

General introduction

BACKGROUND

Adequate nutrition is essential for optimal growth, development, and health of children.[1] In addition to that, early-life nutrition may also have consequences for health in later phases of life.[2-3] The origins of several non-communicable disorders, including cardiometabolic diseases, have been linked to nutritional factors in early life.[3-4] Cardiometabolic diseases, such as cardiovascular diseases and type 2 diabetes, predominantly become apparent at older ages. Hence, research into determinants of these diseases has primarily focused on adults. However, deviations from cardiometabolic health already occur in childhood.[5] An example of such a deviation is obesity. Childhood obesity has become a major public health concern worldwide and – although in some age groups rates seem to be stabilizing –, its overall prevalence continues to increase.[6] Obesity is not only a burden on the physical and emotional well-being of a child,[7] it can also adversely affect cardiometabolic health. Already during childhood, obesity plays a significant role in disturbances in blood lipids, insulin sensitivity, and blood pressure.[5] Although not all these cardiometabolic disturbances are detectable or clinically meaningful in childhood, small changes in cardiometabolic risk factors during childhood may be relevant in predicting future health.[8] Childhood adiposity and disturbances in cardiometabolic risk factors have been shown to track to later life and are associated with increased risk of cardiovascular diseases and type 2 diabetes, and with premature mortality in adulthood.[9-11] Hence, preventive measures should put their focus already on childhood in order to optimize prevention of obesity and cardiometabolic diseases throughout life. For early prevention it is crucial to identify early-life determinants of body composition and cardiometabolic health. Important modifiable determinants of these health outcomes are lifestyle and dietary factors.

NUTRITION IN EARLY CHILDHOOD

Nutrition is vital for health throughout the life course, but may be particularly important during early life. Early childhood is a critical period for nutrition, not only because dietary behaviors develop during this period that tend to remain stable throughout life,[12] but also because nutrition during early life is involved in metabolic and endocrinal changes that may have long-term consequences for health.[2-3]

Breastfeeding is a well-known example of a nutritional exposure in early life with long-term beneficial health consequences, such as low risks of obesity, hypertension, and diabetes.[13-14] After the lactation period, infants make a gradual transition from a completely breastmilk- or infant formula-based diet to a varied table foods diet. Although many studies have examined the health effects of breastfeeding versus formula feeding or the timing of the introduction of complementary foods, studies on nutritional quality shortly after the lactation and weaning period are scarce. As a consequence, not much is known about the effects of diet during this period on long-term health, whereas a better understanding of optimal nutrition in early childhood may be highly relevant for primary prevention of obesity and cardiometabolic disease.

Research described in this thesis therefore focuses on nutrition in early life, particularly in early childhood, and the associations of these early-life nutritional factors with body composition and cardiometabolic health later in childhood. We studied several nutritional factors that previous

studies suggest are important in early life, but for which research gaps remain.[2] These include protein intake, which has been suggested to be too high in the first years of life;[15] fatty acids, for which the composition in the diet may not be optimal;[16] and vitamin D status, because concern has been raised about a reemergence of deficiency.[17] Finally, we evaluated diet quality and dietary patterns of young children, because not much is known about overall diet among preschool children.

Protein

Protein is an indispensable component of infant and child nutrition, as it provides essential amino acids required for growth. However, a high protein intake in early childhood has been linked to a higher risk of obesity.[18] The 'early protein hypothesis' states that high protein intake during infancy may cause obesity in later life via hormonal responses.[18] Although several studies have shown that a high protein intake in early childhood is associated with a higher body mass index in later childhood,[18-19] it is not clear whether this reflects a higher fat mass and hence more adiposity, or also a difference in lean mass. Furthermore, because in childhood obesity is already related to adverse cardiometabolic health outcomes,[5] high protein intake in infancy may also lead to unfavorable effects on cardiometabolic outcomes. However, studies among adults report favorable effects of high protein intake on blood pressure, insulin sensitivity, and triacylglycerol concentrations,[20] and potentially harmful effects on kidney function.[21] Whether dietary protein has a – direct or indirect – effect on blood pressure, kidney function, insulin sensitivity, or blood lipids in children is unclear.

Fatty acids

Lipids, especially polyunsaturated fatty acids (PUFAs), have received considerable interest in relation to cardiometabolic health, because of their diverse roles in gene expression, inflammatory processes, and their incorporation into cell membranes.[22] Among adults, higher circulating PUFA concentrations have been associated with improved cardiometabolic health.[23-25] Furthermore, lowering dietary intake of saturated fat and replacing it with polyunsaturated fat has been shown to reduce the risk of cardiometabolic disease.[26] Exposure to different levels of fatty acids in early life may also influence health in later life. Several animal studies have for example suggested that fatty acid composition of the diet during pregnancy and lactation may influence obesity risk in the offspring.[27] During pregnancy and lactation, fatty acids are transferred from mother to fetus or infant, and may influence its growth and metabolism.[28] However, whether early-life fat intake or circulating fatty acid concentrations are associated with later body composition or cardiometabolic health in humans needs further study.[27]

Vitamin D

Vitamin D is a nutrient, but its concentration in blood primarily depends on production in the skin. Because this production of vitamin D occurs in response to exposure to sunlight,[29] changes in lifestyle, such as spending less time outdoors and using sun protection, have been suggested to result in an increase in vitamin D deficiency.[29-30] An increased prevalence of vitamin D deficiency

is of important public health interest, because vitamin D may play a role in various aspects of health.[31] Vitamin D is well known for its role in bone health, and severe vitamin D deficiency is known to cause rickets and osteomalacia.[29] In addition to skeletal outcomes, studies have suggested that vitamin D is associated with several other aspects of health, including body composition and cardiometabolic health.[29] Studies in adults have reported that lower vitamin D concentrations are associated with higher risk of obesity[32] and cardiovascular diseases.[33] Nevertheless, few studies have examined vitamin D status of young children or studied whether vitamin D deficiency is associated with obesity and cardiometabolic health in childhood.

Dietary patterns

Protein, fatty acids, and vitamin D are nutrients. However, individuals do not consume single nutrients, but consume foods that are combined into meals and dietary patterns. Well-known examples of dietary patterns are the Mediterranean diet, or a diet according to the Healthy Eating Index (HEI). Because intake of certain foods tends to cluster, nutrient intakes often correlate and nutrients may interact in influencing metabolic processes. Studying dietary patterns instead of single nutrients may better capture these interactions and the totality of diet.[34-35] In the last decades, analysis of dietary patterns has emerged as an important research field, complementary to studies focusing on single nutritional compounds.[34-35] Two main approaches have been used to identify dietary patterns in nutritional research. Firstly, dietary patterns can be defined *a priori,* using prior knowledge, for instance on the basis of existing dietary guidelines. Secondly, they can be defined *a posteriori*, using data from the study population. This latter method can be applied using only information about dietary intake of the population, or it can be applied using information about both diet and markers related to health, such as blood glucose concentrations.[36] Although many previous studies have examined different types of dietary patterns and their associations with health, these studies were primarily conducted in adult populations, and studies on dietary patterns and overall diet quality in young children are scarce.[1]

THIS THESIS

Objectives

The aim of this thesis was to study nutrition in early life, particularly in early postnatal life, and its association with body composition and cardiometabolic health. Most studies were carried out in children participating in the Generation R Study, a population-based prospective cohort from fetal life onward. In addition, we performed systematic reviews of the literature. As described above, nutritional factors of interest were protein intake, fatty acids, vitamin D concentrations, and dietary patterns.

Systematic reviews

For the systematic reviews, we conducted extensive literature searches in several databases. No limits were set on language or year of publication.[39] Working in pairs, two researchers independently reviewed the articles to determine whether the studies satisfied the predefined

selection criteria. All relevant information was extracted from the studies, and the quality of the individual studies was assessed with a predefined scoring system. We constructed this quality score on the basis of previously used scoring systems,[37-38] which we modified to be able to assess the quality of different study designs with the same score. For each review, we provided a comprehensive overview of all included studies, which we reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.[39]

The Generation R Study

The Generation R Study is a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[40] In total, 9,778 women with a delivery date between April 2002 and January 2006 were enrolled in the study, which was designed to identify early determinants of normal and abnormal growth, development, and health over the life course. During pregnancy, physical examinations were performed and information on several sociodemographic and lifestyle factors was collected using questionnaires. From birth onward, we obtained information on health and growth of the children from child health centers and with questionnaires. Child food intake around the age of 1 year was assessed with a 211-item semi-quantitative food-frequency questionnaire, which was specifically developed for this age group.[41] When the children were 6 years of age, they were invited to our dedicated research center in the Sophia Children's Hospital in Rotterdam where different health outcomes were measured by trained staff.[42] We measured the children's height and weight, their fat and fat-free mass, and their blood pressure, and blood samples were drawn in which we determined concentrations of triacylglycerol, cholesterol, and insulin. In addition to these individual cardiometabolic outcomes, we examined the overall cardiometabolic health of the children with the use of a combined cardiometabolic risk factor score, which we calculated as the sum of age- and sex-specific standard deviation scores of body fat percentage, blood pressure (systolic and diastolic), insulin, triacylglycerol concentrations and the inverse of HDL cholesterol.

Outline

Subsequent to this General Introduction, Chapter 2 of this thesis focuses on protein intake in childhood. Chapter 2.1 provides an overview of the current scientific literature on the associations between protein intake and cardiometabolic health in children. Chapters 2.2 to 2.4 describe research within the Generation R Study on protein intake in early childhood in relation to body composition (Chapter 2.2), cardiometabolic health (Chapter 2.3), and kidney health (Chapter 2.4) at school age.

Chapter 3 describes our research on fatty acids in pregnancy and in early childhood in relation to children's body composition and cardiometabolic health. In Chapter 3.1, we provide an overview of the literature on the effects of PUFAs during pregnancy, lactation, and early childhood on child obesity and cardiometabolic health. Chapter 3.2 focuses on fatty acid concentrations during mid-pregnancy in the women participating in the Generation R Study. In this chapter we describe the identification of maternal fatty acid patterns and its association with cardiometabolic health and body composition in the offspring. Chapter 3.3 describes the relation between dietary intake of

different types of fatty acids in early childhood and cardiometabolic health among children participating in the Generation R Study.

In Chapter 4¸ the focus is on vitamin D concentrations in childhood. The first study, presented in Chapter 4.1, describes the vitamin D status of the children participating in the Generation R Study when they were 6 years of age. In addition, the sociodemographic and lifestyle determinants of vitamin D deficiency in this population are reported. Subsequently, we describe the cross-sectional associations of child vitamin D status with body composition (Chapter 4.2) and with cardiometabolic health (Chapter 4.3).

In Chapter 5, we evaluate overall diet in early childhood. Chapter 5.1 describes the development of a diet quality score for preschool children and its validation and determinants in the Generation R Study. Subsequently, we describe the associations of this predefined diet quality score and of two types of data-driven dietary patterns with childhood body composition (Chapter 5.2) and with cardiometabolic health (Chapter 5.3).

The final chapter, Chapter 6, provides an overview of the main findings from all studies described in this thesis. In this chapter, we also review the major strengths and limitations of the studies. In addition, public health implications are discussed and recommendations for future research are provided.

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Chapter 2

Protein intake

Chapter 2.1

Protein intake & cardiometabolic health in children: a systematic review

Manuscript based on this chapter:
Trudy Voortman, Anna Vitezova, Wichor M. Bramer, Charlotte L. Ars, Paula K. Bautista, Adriana Buitrago-Lopez, Janine F. Felix, Elisabeth T.M. Leermakers, Ayesha Sajjad, Sanaz Sedaghat, Anne Tharner, Oscar H. Franco, Edith H. van den Hooven. Effects of protein intake on cardiometabolic health in children: a systematic review. *British Journal of Nutrition* 2015;113:383–402.

ABSTRACT

Background: High protein intake in early childhood is associated with obesity, suggesting possible adverse effects on other cardiometabolic outcomes. However, studies in adults suggest beneficial effects of protein intake on blood pressure and lipid profile. Whether dietary protein is related to cardiovascular and metabolic outcomes in children is unclear. Therefore, we aimed to systematically review evidence on the associations between protein intake and blood pressure, insulin sensitivity, and blood lipids in children.

Methods: We searched Medline, Embase, Cochrane Central, and PubMed for interventional and observational studies in healthy children up to the age of 18 years, in which associations between total, animal, and/or vegetable protein intake and one or more of the following outcomes were reported: blood pressure, measures of insulin sensitivity, cholesterol levels, or triacylglycerol levels.

Results: In the search we identified 6,636 abstracts, of which 56 studies met all selection criteria. In general, the quality of the included studies was low. Most studies were cross-sectional and many did not control for potential confounders. No overall associations were observed for protein intake in relation to insulin sensitivity or blood lipids. A few studies suggested an inverse association between dietary protein and blood pressure, but evidence was inconclusive. Only four studies examined effects of vegetable or animal protein intake, with inconsistent results.

Conclusion: The literature to date provides insufficient evidence for effects of protein intake on blood pressure, insulin sensitivity, or blood lipids in children. Future studies could be improved by adequately adjusting for key confounders, such as energy intake and obesity.

INTRODUCTION

Protein is an important component of infant and child nutrition, as it provides essential amino acids required for growth.[1-2] However, a high protein intake in early childhood has also been related to the development of obesity.[3-5] Already during childhood, obesity can lead to adverse cardiometabolic health outcomes, such as hypertension, high cholesterol levels and insulin resistance.[6-7] This suggests that high protein intake in children may lead to unfavorable effects on these outcomes. Studies in adults, however, report beneficial effects of protein intake on blood pressure, and insulin and triacylglycerol concentrations.[8-11]

Whether dietary protein has a – direct or indirect – effect on blood pressure, insulin sensitivity, or blood lipids in children is unclear. Because cardiometabolic risk factors in childhood continue into later life and have been shown to predict cardiovascular disease and type 2 diabetes in adulthood,[12-13] it is important to study determinants of cardiometabolic risk already in childhood. Our aim was therefore to conduct a systematic review on the associations between protein intake and blood pressure, insulin sensitivity, and blood lipids among children. In addition, we aimed to explore whether the reported effects differ between protein from vegetable and from animal sources.

METHODS

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.[14] Ethical approval was not required as this was a secondary data analysis.

Literature search

An extensive literature search was conducted with the help of a medical librarian in the databases Medline (via OvidSP), Embase (via Embase.com), and Cochrane Central. In addition we searched PubMed for articles that were not yet available via Medline. All databases were searched from their inception until 31 May 2013. The search strategy consisted of three elements: infants, children, or adolescents; protein intake; and cardiovascular or metabolic health outcomes. To capture studies that did not explicitly mention protein in the title or abstract, we also included general terms referring to diet and nutrient intake. All elements were searched using both controlled vocabulary terms (MeSH or Emtree) and free text words in title or abstract. Limits were applied to include only human studies and to exclude conference papers, editorials, and letters. No limits were set on language or year of publication. The full search strategy for Embase is provided in Supplement 2.1.1. In addition to the systematic search, we consulted authors and searched reference lists for the most recent 10% of articles in our review.

Selection criteria

Studies identified from the literature search were selected on the basis of the following predefined selection criteria:

Inclusion criteria

- Cross-sectional studies, case-control studies, cohort studies and intervention studies.
- Studies conducted among children ≤ 18 years old.
- Studies reporting total, animal, and/or vegetable protein intake, either in absolute amounts (e.g., g/d or kJ/d) and/or relative to total energy intake (e.g., energy percentage (E%)).
- Studies investigating associations between protein intake and one or more of the following outcomes: blood pressure (systolic or diastolic blood pressure, mean arterial pressure, hypertension); insulin sensitivity (insulin levels, glucose levels, glucose tolerance, homeostatic model assessment (HOMA), type 2 diabetes mellitus); or blood lipids (total, HDL and LDL cholesterol levels, triacylglycerol levels).

Exclusion criteria

- Studies among children with congenital diseases, phenylketonuria, type 1 diabetes, or kidney disease.
- Studies from which the exclusive effects of protein cannot be extracted (e.g., when protein supplements were combined with other nutrients without proper control).
- Letters, conference abstracts, reviews, or editorials.
- Studies not in humans.

Study selection

Working in pairs, two authors independently reviewed the titles and abstracts to determine whether the studies satisfied the selection criteria. Any disagreement with article selection was resolved through discussion or with help of a third reviewer. Full-text articles were retrieved for the selected titles and were assessed once more by two independent reviewers. For articles in languages other than English, colleagues fluent in the language assisted with translating.

Data extraction

Data were extracted with use of a structured data extraction form designed prior to data collection. Detailed study-level characteristics were collected including study design, study size, study duration, details on exposure and outcome assessment, and characteristics of the study population. We also derived information on the statistical analyses and covariate adjustments. All types of summary statistics were extracted, both for the entire study population and for subgroups; and both for crude models and for adjusted models where applicable. Authors were contacted if insufficient data were published (e.g., if effect estimates were not stated in the paper). A second reviewer checked the data extraction for a random 20% of the studies.

Quality assessment

Two reviewers independently evaluated the quality of included studies with a predefined scoring system. The quality score was constructed on the basis of previously used scoring systems and was modified to assess the quality of studies with different study designs.[15-16] A score of 0, 1 or 2 points was allocated to each of the following five items: study design; study size; exposure assessment; outcome assessment; and adjustment for potential confounders or randomization. This resulted in a total score ranging from 0 to 10 points, with a score of 10 representing the highest quality. Details on the quality score are presented in Supplement 2.1.2.

Synthesis of evidence

Because of the diversity in study designs, outcome measures and low methodological quality of the studies, a meta-analysis could not be performed. Instead, a qualitative analysis (best-evidence synthesis) was performed to synthesize the results and quality of the included studies.[17] In line with previous systematic reviews,[18-19] we defined the following four levels of evidence: 1) strong evidence is provided if at least two higher-quality studies are available and if these report consistent findings; 2) moderate evidence is provided if one higher-quality and one or more lower-quality studies are available with generally consistent findings; 3) limited evidence is provided if only one higher-quality study is available or if multiple lower-quality studies report generally consistent findings; and 4) insufficient evidence is provided if no higher-quality studies are available or if studies report inconsistent findings. Studies were considered as generally consistent if at least 75% of the studies showed statistically significant results in the same direction.[17] Studies were considered as higher-quality if they had a quality score of 6 or higher. If two or more studies on the same outcome were of higher quality, we disregarded the lower-quality studies in the evidence synthesis.[17]

RESULTS

Study selection

In the systematic search 6,636 unique references were identified. Of these references 6,305 articles were excluded on the basis of title and abstract. For the remaining 331 references, the full-text was retrieved and critically reviewed. After the selection process, 60 papers were included, reporting on unique 56 studies. Figure 2.1.1 shows the flowchart of the selection process.

Study characteristics

Table 2.1.1 shows the characteristics of the included studies and study populations. The 56 studies included a total number of 22,040 participants (ranging from 19 to 4,508 subjects per study), with a variation in mean age from 0 to 17.5 years. We decided to include three studies (reported in four papers) among subjects with an age range up to 19 or 20 years since their age ranges were very wide.[20-23] Most studies included both boys and girls, except for one study in boys only[24] and one study in girls only.[25]

Table 2.1.1 Characteristics of the 60 included papers (56 studies) on protein intake and cardiometabolic health in children

First author, year	Country	Study design (follow-up)	n	% female	Age (y) at baseline (mean (range))	Population characteristics	Quality score
Aeberli et al, 2007[44*a]	Switzerland	Cross-sectional	74	45.9%	10.1 (6-14)	Normal weight (mean BMI 15.9 kg/m^2) and overweight (mean BMI 23.4 kg/m^2)	5
Aeberli et al, 2009[45*a]	Switzerland	Cross-sectional	79	46.8%	10.2 (6-14)	Normal weight (mean BMI 15.9 kg/m^2) and overweight (mean BMI 23.6 kg/m^2)	5
Akerblom et al, 1984[46]	inland	Cross-sectional	237	45.6%	12	General population	3
Andersen et al, 1979[47]	Denmark	Cross-sectional	95	46.32	Two groups: 0.7 and 4	Healthy children participating in a screening program	4
Berenson et al, 1979[48]	USA	Cross-sectional	224	~50%	0.5	General population	3
Boulton et al, 1995[49]	New Zealand	Cross-sectional	232	47.8%	Four moments: 8, 11, 13 and 15	General population	3
Casazza et al, 2009a[50*b]	USA	Cross-sectional	202	47.0%	9.6 (7-12)	Healthy children	4
Casazza et al, 2009b[51*b]	USA	Cross-sectional	250	53.6%	9.6 (7-12)	Healthy children	6
Colin-Ramirez et al, 2009[52]	Mexico	Cross-sectional	1239	49.5%	9.4 (8-10)	General population, low SES area	4
Cowin et al, 2001[53]	UK	Longitudinal (13 mo)	389	45.0%	1.5	General population	3
Damsgaard et al, 2013[43]	8 European countries[‡]	Interventional (6 mo)	253	50.1%	13.0 (5-18)	Children with at least one overweight parent (BMI>27 kg/m^2)	9
Davis et al, 2005[32]	USA	Cross-sectional	63	49.2%	11.5 (9-13)	Overweight otherwise healthy (BMI>85th percentile, based on age and gender specific population data)	6
Davis et al, 2009[33]	USA	Longitudinal (18 mo)	85	43.5%	14.2 (11-17)	Overweight otherwise healthy (BMI>85th percentile, based on age and gender specific population data)	6
Duckworth et al, 2009[34]	UK	Interventional (1 mo)	95	64.2%	14.4 (9-18)	Overweight (mean BMI 33.9 kg/m^2)	6
Frank et al, 1977[54]	USA	Cross-sectional	68	NR	NR (10-16)	General population	1
Frank et al, 1978[55]	USA	Cross-sectional	185	NR	10.5 (9-11)	General population	1
Garemo et al, 2006[56]	Sweden	Cross-sectional	95	44.2%	4.3 (3.9-4.6)	General population	4

Table 2.1.1 (continued) Characteristics of the 60 included papers (56 studies) on protein intake and cardiometabolic health in children

First author, year	Country	Study design (follow-up)	n	% female	Age (y) at baseline (mean (range))	Population characteristics	Quality score
Garnett et al, 2013[35]	Australia	Interventional (6 mo)	111	59.5%	13.1 (10-17)	Overweight with pre-T2DM and/or features of insulin resistance, but without T2DM	8
Gately et al, 2007[36]	UK	Interventional (1 mo)	98	61.2%	14.2 (11-17)	Overweight (mean BMI 33.1 kg/m^2), without medication use	7
Glueck et al, 1982[20*c]	USA	Cross-sectional	1234	46.4%	13.5 (6-19)	General population	5
Gonzalez-Requejo et al, 1995[57]	Spain	Cross-sectional	1682	46.1%	6.3 (2-12)	General population	5
Hermelo et al, 1995[37]	Cuba	Case-control[†]	80	50.0%	11.7 (10.7-12.7)	Overweight and matched normal weight	4
Heyman et al, 2012[25]	France	Cross-sectional	19	100%	16.6	Healthy controls from a case-control study on T1DM	4
Hitchcock et al, 1977[31]	Australia	Cross-sectional	58	NR	NR (6-17)	Low (<3.9 mmol/L), average (3.9-5.2 mmol/L) or high (>6.2 mmol/L) cholesterol in both mother and child, selected from a larger population-based study (*n*=929)	1
Hong et al, 2009[58]	Korea	Cross-sectional	246	47.6%	12.6 (12-13)	General population	4
Il'chenko et al, 1989[59]	Russia	Cross-sectional	250	NR	NR (11-14)	General population	1
Jenner et al, 1988[60]	Australia	Cross-sectional	884	50.9%	9.0 (7.5-10.6)	General population	6
Keser et al, 2010[38]	Turkey	Cross-sectional	308	NR	NR (11-18)	Overweight, otherwise healthy	2
Knuiman et al, 1983[24]	Netherlands; Finland; Italy; Philippines; Ghana	Cross-sectional	589	0%	9.0 (7.6-10.2)	General population	3
Kouvalainen et al, 1982[61]	Finland	Cross-sectional	415	46.4%	Two groups: 3 and 12	General population	4

Table 2.1.1 (continued) Characteristics of the 60 included papers (56 studies) on protein intake and cardiometabolic health in children

First author, year	Country	Study design (follow-up)	n	% female	Age (y) at baseline (mean (range))	Population characteristics	Quality score
Larsen et al, 1989[62]	Denmark	Cross-sectional	42	52.4%	NR (7-11)	Low (3.1-4.1 mmol/L), average (4.3-4.8 mmol/L) and high (5.0-5.9 mmol/L) cholesterol, selected from a larger population-based study (*n*=947)	1
Lindquist et al, 2000[63]	USA	Cross-sectional	95	NR	9.6 (6.5-13)	General population	4
Lucas et al, 1994[64]	UK	Longitudinal (7.5 y)	758	50.4%	Birth	Low birthweight (<1850 g)	5
Menghetti et al, 2004[65]	Italy	Cross-sectional	293	46.1%	NR (11-14)	General population	3
Mia et al, 2000[66]	South-Africa	Cross-sectional	321	50.5%	NR (16-18)	South African Indian children	4
Morrison et al, 1980[21*c]	USA	Cross-sectional	949	48.2%	NR (6-19)	General population	4
Nicklas et al, 1993[67]	USA	Cross-sectional	1281	56.5%	10.6 (10-11)	General population	5
Obuchowicz et al, 1997[39]	Poland	Cross-sectional	191	50.8%	7.6 (5.8-9.6)	Normal weight (mean BMI 16.1 kg/m^2) and overweight (mean BMI 23.8 kg/m^2) children	4
Perry et al, 1997[68]	USA	Cross-sectional	22	54.6%	11.7 (11.0-12.6)	General population	4
Pistulkova et al, 1992[30]	Czech Republic	Case-control[f]	200	52.2%	NR (11-12)	High vs. low cholesterol (>95th and 5-10th percentile, selected from a larger cohort (*n*=2,000)	3
Potter et al, 1989[69]	U.S.	Cross-sectional	53	56.6%	12.4 (9-16)	General population	2
Räsänen et al, 1978[70]	Finland	Cross-sectional	1496	49.1%	5, 9 & 13	General population	2
Regan et al, 2006[71]	New Zealand	Longitudinal (6.6 y)	37	64.9%	Birth	Healthy, developmentally normal children, who were born preterm (≤32 weeks gestation)	5
Rinaldi et al, 2012[40]	Brazil	Cross-sectional	147	51.7%	7.9	Overweight (BMI>85th percentile, based on age and gender specific population data)	6
Sanchez-Bayle et al, 2008[72]	Spain	Cross-sectional	673	47.7%	6	General population	5
Sarría Chueca et al, 1997[22]	Spain	Cross-sectional	89	48.3%	NR (4-20)	High cholesterol levels (≥5.2 mmol/L), or a family history of coronary heart disease or hyperlipidemia	2

Table 2.1.1 (continued) Characteristics of the 60 included papers (56 studies) on protein intake and cardiometabolic health in children

First author, year	Country	Study design (follow-up)	n	% female	Age (y) at baseline (mean (range))	Population characteristics	Quality score
Schachter et al, 1979[73]	USA	Cross-sectional	150	NR	0.5	Healthy, full-term infants, born in a hospital	4
Schutte et al, 2003a[74*d]	South-Africa	Cross-sectional	631	53.1%	12.5 (10-15)	General population	6
Schutte et al, 2003b[75*d]	South-Africa	Cross-sectional	694	53.7%	12.5 (10-15)	General population	6
Sharma et al, 2009[76]	USA	Cross-sectional	80	55.0%	NR (9-11)	Overweight (BMI >85th percentile for age and sex)	6
Simons-Morton et al, 1997[77]	USA	Longitudinal (3 y)	662	46.1%	9.6 (8-11)	High LDL-C (80th to 98th percentile, based on age and gender specific population data), participating in a trial, otherwise healthy	8
Smith et al, 2003[41]	USA	Cross-sectional	102	47.1%	NR (9-18)	Normal weight and overweight	2
Starc et al, 1998[28]	USA	Cross-sectional	67	65.7%	5.8 (2-10)	Hyperlipidemia, not taking lipid-lowering medications, without a congenital disease that might affect blood lipids	4
Sugiyama et al, 2007[23]	USA	Cross-sectional	4508	49.1%	16.0 (12-19)	General population	7
Suter et al, 1993[78]	Canada	Cross-sectional	97	59.8%	13.0 (10-15)	General population	4
Ulbak et al, 2004[79]	Denmark	Cross-sectional	73	NR	2.5	General population	6
Ventura et al, 2008[42]	USA	Cross-sectional	109	43.1%	NR (10-17)	Overweight (mean BMI 31.2 kg/m^2) and family history of T2DM, but without current T2DM	4
Vobecky et al, 1979[26]	Canada	Case-control†	70	60.0%	0.5	High cholesterol (≥5.2 mmol/L) and matched controls	1
Ward et al, 1980[80]	USA	Cross-sectional	74	NR	2.6 (2.5-2.9)	General population	2
Weidman et al, 1978[27]	USA	Cross-sectional	93	NR	NR (6-16)	High (>90th percentile), median (45-55th percentile) and low (<10th percentile) cholesterol, from a larger cohort (*n*=4,021)	2

* Articles with the same character (i.e., a, b, c, or d) used data from the same study population
† All case-control studies used cross-sectional data
‡ Denmark, the Netherlands, United Kingdom, Greece, Germany, Spain, Bulgaria and the Czech Republic
Abbreviations: LDL-C, low-density lipoprotein cholesterol; NR, not reported; SES, socioeconomic status; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

Most studies were performed in Europe (*n*=20), the United States or Canada (*n*=22), and Australia or New Zealand (*n*=5). Other studies were performed in populations in South or Central America (*n*=3), South-Africa (*n*=2), Korea (*n*=1), Russia (*n*=1), Turkey (*n*=1), and one study included subjects from the Philippines, Ghana, and three European countries. Most studies examined general population-based samples of children. Some studies specifically included high-risk populations, i.e., children with high cholesterol levels[22, 26-31] or overweight.[32-42]

Only four of the 56 studies were randomized controlled trials comparing high (22.5 to 30 E%) to low (10 to 15 E%) protein diets, for one or six months.[34-36, 43] In three of the four trials, the lower and higher protein diets were isocaloric and energy-restricted, the calories from protein being replaced by carbohydrate.[34-36] In the fourth trial, protein was also replaced by carbohydrate, but without energy restriction.[43] The remaining 52 studies were observational, of which five had a longitudinal design (follow-up ranging from 1.1 to 7.5 years), and 47 were cross-sectional studies. Mean protein intake in the observational studies ranged from 7.7 E% to 19.2 E% (Supplement 2.1.3). Twenty-three studies investigated associations of protein intake with blood pressure, 15 with insulin sensitivity, and 42 with blood lipids. Details on exposure and outcomes measurements are presented in Supplement 2.1.3.

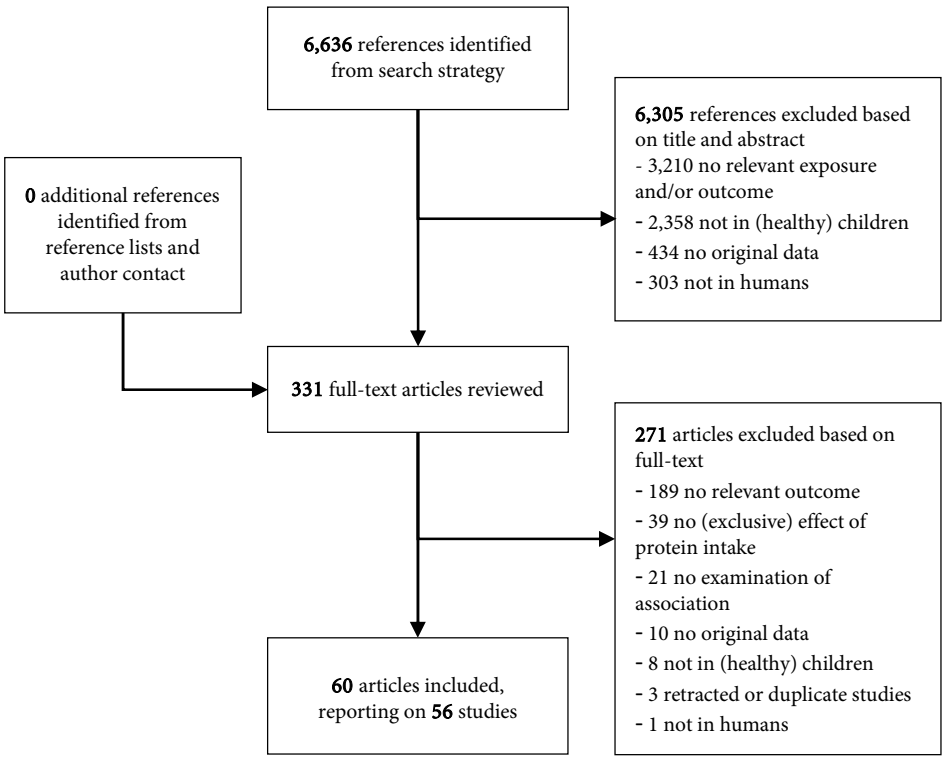

Figure 2.1.1 Flowchart of study selection

The overall quality score of the included studies ranged from 1 to 9 (Table 2.1.1), with a mean score of 4.2. Fifteen studies received a quality score of 6 or higher. Because of the large number of cross-sectional studies, most studies scored low on the item study design. Most studies did receive a high score on exposure and outcomes assessment methods. The majority of studies received a score of zero for the item on adjustments, since they did not control for important potential confounders such as age, sex, energy intake, and body weight.

Protein intake and blood pressure

Twenty-three studies reported on associations between protein intake and blood pressure (BP) in children, of which ten studies were considered higher-quality (Table 2.1.2). Four intervention studies, of which three were performed in children with overweight, did not find significant effects of a high-protein diet compared to a low-protein diet on systolic BP (SBP), diastolic BP (DBP), or mean arterial pressure.[34-36, 43] All four trials had a quality score of 6 or higher. In the trial by Damsgaard *et al.,* the authors did observe a significant beneficial effect of a high-protein diet in a subgroup of 5 to 18-year-old children who received a more intensive intervention.[43] This subgroup received free foods in addition to dietary instructions and had a higher adherence to the intervention diet. In this subgroup, children who received the high-protein diet had a 1.0 mmHg (95% CI 0.3, 1.7) lower DBP and a 6.5 mmHg (95% CI 1.5, 15.0) lower mean arterial pressure than children who received a low-protein diet.[43] Of the observational studies, six had a quality score of 6 or higher. One longitudinal and two cross-sectional studies of higher quality reported inverse associations between protein intake and BP in at least one of their subgroups.[60, 77, 79] The three remaining higher-quality studies did not find a significant association between protein intake and BP,[23, 50, 76] but two did report non-significant inverse associations.[23, 50] In contrast, one study with a quality score of 3 observed that children with high BP had a significantly higher protein intake than children with a normal BP.[65] The remaining lower-quality studies showed no significant associations.[41-42, 45, 48, 52, 54-55, 58-59, 64, 73, 75]

Protein intake and insulin sensitivity

Fifteen studies (published in 16 papers) reported on associations between protein intake and measures of insulin sensitivity in children (Table 2.1.3). The studies examined various measures of insulin sensitivity, including fasting insulin or glucose concentrations, or homeostatic model assessment of insulin resistance (HOMA-IR); or measures of insulin responses following an oral or intravenous glucose tolerance test, such as the insulin sensitivity index and acute insulin response. No studies were identified with type 2 diabetes mellitus in children as outcome.

Of the 15 studies, six had a quality score of 6 or higher. In three of these higher-quality studies a significant association was reported between protein intake and insulin sensitivity. In the previously described trial by Damsgaard *et al.*, again no effects were observed in the full study population. However, in the subgroup undergoing a more intensive intervention, insulin and HOMA-IR were significantly reduced in the high protein group compared to the low protein group.[43] In a cross-sectional study in 7 to 12-year-old children, Casazza *et al.* observed that, after extensive adjustments, higher protein intake was significantly associated with lower fasting glucose

concentrations, but not with fasting insulin concentrations or insulin sensitivity index, and that it was inversely related to the acute insulin response.[50-51] Data from another cross-sectional study showed that higher protein intake was associated with lower insulin resistance (HOMA-IR) in 9 to 11-year-old children.[76]

In summary, all three studies show a favorable effect of protein intake on one or more measures of insulin sensitivity (i.e., lower glucose concentrations and lower insulin resistance), but one of the studies also reported a harmful effect of protein (lower insulin response). The remaining three higher-quality studies did not find significant associations between dietary protein and measures of insulin sensitivity,[32-33, 35] and neither did any of the lower-quality studies.[25, 38-39, 42, 45, 56, 58-59, 63, 71]

Protein intake and blood lipids

Of the 56 included studies, 42 examined associations between protein intake and concentrations of one or more blood lipids in children (Table 2.1.4). Among these, 22 studies (published in 23 papers) investigated effects on triacylglycerol (TAG) concentrations, of which five were of higher quality. None of these higher-quality studies reported a significant effect.[35-36, 40, 43, 76] One lower-quality study reported that higher protein intake was correlated with higher TAG concentrations.[38] However, this correlation was not adjusted for potential confounders and the study had a quality score of only 2. The remaining 16 lower-quality studies did not find significant associations.[20-21, 37, 42, 48, 54-56, 58-59, 63, 66, 68-69, 72, 78]

The relation between protein intake and HDL cholesterol levels was reported in 24 studies (published in 25 papers) (Table 2.1.4). Statistically significant associations were observed in three studies: two positive[24, 28] and one negative.[38] However, all three reported only simple correlations without adjustment for potential confounders and had quality scores ranging from 2 to 4. In the remaining studies, including four higher-quality studies, no significant associations were observed.[20-22, 25, 28, 35, 37, 40, 42-43, 46, 49, 51, 53, 57-59, 61, 67, 69, 76, 78]

Associations between protein intake and total and/or LDL cholesterol were investigated in 38 studies (Table 2.1.4). Five studies reported statistically significant associations between protein intake and cholesterol concentrations, however, they all had a quality score of 5 or lower and there was no consistency in the direction of the effect.[26, 30, 48-49, 66] The remaining studies, including five higher-quality studies, did not find significant associations.[20-22, 24-25, 27, 31, 35, 37-38, 44, 46-47, 53-59, 61-62, 66, 69, 72, 76, 78, 80]

Vegetable and animal protein

Only four of all 56 studies included investigated associations between vegetable or animal protein and cardiometabolic health outcomes in children. One higher-quality cross-sectional study reported no associations of animal or vegetable protein with BP.[75] Three lower-quality studies reported inconsistent results. One found no effect of animal or vegetable protein on blood lipids in overweight children.[38] The second reported that animal protein was positively correlated to serum LDL cholesterol (r=0.20, p=0.0002), whereas vegetable protein was not.[66] In contrast, the last study reported an inverse correlation between animal to vegetable protein intake ratios and concentrations of LDL cholesterol (r=-0.28, p=0.05).[69]

Table 2.1.2 Reported associations between protein intake and blood pressure in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Higher-quality studies								
Damsgaard et al, 2013[43]	9	Linear mixed models	Effect of protein (control vs. high vs. low) on BP (mmHg) after 1 mo and 6 mo of intervention	Age, gender, family, country, participant, baseline MAP, time since randomization	SBP	Whole group	NR	NS
						Intensive group*	NR	NS
					DBP	Whole group	NR	NS
						Intensive group*	-1.0	<0.01
					MAP	Whole group	NR	0.66
						Intensive group*	-6.5	0.01
Duckworth et al, 2009[34]	6	ANOVA	Comparison of BP between groups that received high vs. low protein intervention diets	Randomized	SBP	-	NR	NS
					DBP		NR	NS
Garnett et al, 2013[35]	8	Linear mixed models	Effect of protein (high vs. low) on BP (mmHg) after 3 mo and 6 mo of intervention	Randomized	SBP	-	NR	NS
					DBP		NR	NS
Gately et al, 2007[36]	7	ANOVA	Comparison of BP between groups that received high vs. low protein intervention diets	Randomized	SBP	-	NR	NS
					DBP		NR	NS
Jenner et al, 1988[60]	6	Linear regression	Difference in BP (mmHg) per energy-adjusted (residuals method) gram higher protein intake	Age, height, body weight, SES	SBP	M	-0.01	0.85
					DBP	M	-0.10	0.06
					SBP	F	-0.14	0.05
					DBP	F	-0.03	0.64
Schutte et al, 2003a and 2003b[74-75]	6	Linear regression	Difference in BP (mmHg) per gram higher in protein intake	Sex, pubertal stage, BMI, body fat, intake of macronutrients, intake of several other nutrients	SBP	-	NR	NS
					DBP		NR	NS
					MAP		NR	NS
					Pulse pressure		NR	NS
Sharma et al, 2009[76]	6	Linear regression	Standardized regression coefficient (protein intake in g/d)	Sex, pubertal stage, waist circumference, intake of carbohydrate and fat	SBP	-	0.00	NS
					DBP		0.02	NS
Simons-Morton et al, 1997	8	Linear regression	Difference in BP (mmHg) per gram higher in protein intake	Sex, height, body weight, and energy intake	SBP	Baseline, cross-sectional	-0.01	NS
					DBP		-0.01	NS

Table 2.1.2 (continued) Reported associations between protein intake and blood pressure in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Simons-Morton et al, 1997 (cont'd)	8	Linear regression	Difference in BP (mmHg) per gram higher in protein intake	Sex, height, body weight, and energy intake	SBP DBP	3 y follow-up, model 1†	-0.01 -0.01	<0.01 <0.01
				Sex, height, body weight, energy intake, intake of macronutrients and several micronutrients	SBP DBP	3 y follow-up, model 2†	-0.02 -0.03	NS NS
Sugiyama et al, 2007[23]	7	Linear regression	Difference in BP (mmHg) per 10 gram increase in protein intake (energy-adjusted)	Sex, age, height, body weight, BMI, energy intake, SES, physical activity, intake of macronutrients and several micronutrients	SBP DBP	-	-0.02 NR	0.07 NS
Ulbak et al, 2004[79]	6	Linear regression	Difference in BP (mmHg) per E% increase in protein intake	Sex, age, height, body weight, and outside temperature	SBP DBP	-	-0.56 -1.86	0.035 0.028
Lower-quality studies								
Aeberli et al, 2009[45]	5	Linear regression	Standardized regression coefficient (protein intake in E%)	Sex, age, BMI	SBP	-	-0.13	0.23
Berenson et al, 1979[48]	3	Pearson correlation	Correlation between protein intake (g/1000 kcal) and BP	-	BP BP	0.5 y 1 y	NR NR	NS NS
Casazza et al, 2009a[50]	4	Linear regression	Standardized regression coefficient (protein intake in E%)	Sex, age, total body fat, SES	SBP	-	-0.15	NS
Colin-Ramirez et al, 2009[52]	4	ANOVA	Comparison of protein intake (E%) in children with normal BP vs. diastolic hypertension vs. systolic hypertension	-	Hypertension Diastolic hypertension Systolic hypertension	-	NR NR NR	NS NS NS
Frank et al, 1977[54]	1	Pearson correlation	Correlation between protein intake (g/1000 kcal) and BP	-	BP	-	NR	NS

Table 2.1.2 (continued) Reported associations between protein intake and blood pressure in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Frank et al, 1978[55]	1	Pearson correlation	Correlation between protein intake (g/1000 kcal) and SBP	-	SBP	-	NR	NS
Hong et al, 2009[58]	4	Correlation	Partial correlation between protein intake (g/d) and BP	Sex, age, Tanner stage	SBP DBP	-	0.08 -0.02	NS NS
Il'chenko et al, 1989[59]	1	t-test‡	Comparison of protein intake (g) in children with elevated vs. normal arterial pressure	-	Arterial pressure	-	NR	NS
Lucas et al, 1994[64]	5	Linear regression	Difference in BP (mmHg) per gram increase in protein intake	Height, BMI, BP measuring device, mid-arm circumference	SBP DBP	-	NR NR	NS NS
Menghetti et al, 2004[65]	3	t-test	Comparison of protein intake (E%) between children with hypertension vs. normal BP	-	Hypertension	-	positive	<0.05
Schachter et al, 1979[73]	4	Correlation	Correlation between protein intake (g) and BP	-	BP	-	NR	NS
Smith et al, 2003[41]	2	Spearman correlation	Partial correlation between protein intake (g) and BP	Age	SBP DBP	-	0.05 0.10	NS NS
Ventura et al, 2008[42]	4	Pearson correlation	Correlation between protein intake (E%) and BP	-	SBP DBP	-	NR NR	<0.05 <0.05

* The intensive intervention group received most of their foods for free and had a higher adherence to the intervention diets.
† See column with covariate adjustments: model 2 additionally adjusted for intake of other nutrients.
‡ Statistical analysis used was not clearly reported.
Abbreviations: BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; E%, energy percent; MAP, mean arterial pressure; NR, not reported; NS, not significant; QS, quality score; SES, socioeconomic status; SBP, systolic blood pressure.

Table 2.1.3 Reported associations between protein intake and measures of insulin sensitivity in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Higher-quality studies								
Damsgaard et al, 2013[43]	9	Linear mixed models	Effect of protein (control vs. high vs. low) on insulin levels (pmol/L) and HOMA-IR (points) after 1 mo and 6 mo of intervention	Age, gender, family, country, participant, baseline insulin or HOMA-IR, time since randomization	Fasting insulin	Whole group	NR	0.63
						Intensive group*	-6.0	0.01
					HOMA-IR	Whole group	NR	0.66
						Intensive group*	-0.8	0.02
Casazza et al, 2009a and 2009b[50-51]	4/6	Linear regression	Standardized regression coefficient (protein intake in E%)	Sex, age, total body fat, SES	Fasting glucose	-	-0.43	<0.05
				Sex, Tanner stage, fast mass, lean mass, SES, ethnicity, (and Si for AIR)	Fasting insulin	-	0.03	0.61
					Si		0.01	0.83
					AIR		-0.13	0.02
Davis et al, 2005[32]	6	Linear regression	Standardized regression coefficient (protein intake in g/d)	Sex, age, Tanner stage, lean tissue mass, fat mass, (and Si for AIR)	Si	-	NR	NS
					AIR		NR	NS
					Disposition index (β cell function)		NR	NS
Davis et al, 2009[33]	6	Correlation	Partial correlation 2y change in protein intake and 2y change in Si, AIR, or disposition index	Sex, Tanner stage, time, baseline protein intake, baseline insulin measures, body composition	Si	-	NR	>0.20
					AIR		NR	>0.20
					Disposition index (β cell function)		NR	>0.20
Garnett et al, 2013[35]	8	Linear mixed models	Effect of protein (high vs. low) on Si after 3 mo and 6 mo of intervention	-	Si	-	NR	NS
Sharma et al, 2009[76]	6	Linear regression	Standardized regression coefficient (protein intake in g/d)	Sex, pubertal stage, waist circumference, intake of carbohydrate and fat	HOMA-IR	-	-0.341	<0.05

Table 2.1.3 (continued) Reported associations between protein intake and measures of insulin sensitivity in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Lower-quality studies								
Aeberli et al, 2009[45]	5	Linear regression	Standardized regression coefficient (protein intake in E%)	BMI-SDS	Log fasting insulin	Normal weight	-0.03	0.87
						Overweight	0.05	0.79
					QUICKI	Normal weight	NR	NS
						Overweight	NR	NS
Garemo et al, 2006[56]	4	Pearson correlation	Correlation between protein intake (E%) and glucose, insulin, or HOMA	-	Fasting glucose, insulin, HOMA-IR, HOMA-β	-	NR	NS
Heyman et al, 2012[25]	4	Pearson/Spear-man correlation	Correlation between protein intake (E%) and glucose and insulin	-	Fasting glucose, insulin	-	NR	NS
Hong et al, 2009[58]	4	Correlation	Partial correlation between protein intake (g/d) and glucose	Sex, age, Tanner stage	Fasting glucose	-	-0.08	NS
Keser et al, 2010[38]	2	Correlation	Correlation between protein intake (g) and measures of insulin sensitivity	-	HOMA-IR, QUICKI, insulin, glucose	-	NR	NS
Lindquist et al, 2000[63]	4	Linear regression	Log increase in Si (10^{-4} L×min^{-1}×$pmol^{-1}$) per g increase in protein intake	Fat mass, ethnicity, SES, intake of carbohydrate and fat	Si	-	0.00	0.45
					AIR		0.00	0.76
Obuchowicz et al, 1997[39]	4	Correlation	Correlation between protein intake (E%) and insulin levels	-	Fasting insulin	Obese	-0.03	0.76
					Fasting insulin	Normal weight	-0.00	0.99
Regan et al, 2006[71]	5	Linear regression	Difference in Si (10^{-4} L×min^{-1} mU/L) per g increase in protein intake	Sex, age, weight SDS, height SDS, energy intake, birthweight, intake carbohydrate and fat	Si	-	NR	NS
Ventura et al, 2008[42]	4	Pearson correlation	Correlation between protein intake (E%) and post-challenge glucose levels	-	2h post-challenge glucose levels	-	NR	NS

*The intensive intervention group received most of their foods for free and had a higher adherence to the intervention diets.
Abbreviations: AIR, acute insulin response; BMI, body mass index; E%, energy percent; HOMA-β, homeostatic model assessment-β-cell function; HOMA-IR, homeostatic model assessment for insulin resistance; NR, not reported; NS, not significant; QS, quality score; QUICKI, quantitative insulin sensitivity check index; SES, socioeconomic status; Si, insulin sensitivity index.

Table 2.1.4 Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Higher-quality studies								
Damsgaard et al, 2013[43]	9	Linear mixed models	Effect of protein (control vs. high vs. low) on blood lipids (mmol/L) after 1 mo and 6 mo of intervention	Age, gender, family, country, participant, baseline TAG, time since randomization	TAG TAG TC TC LDL-C LDL-C HDL-C HDL-C	Whole group Intensive group* Whole group Intensive group* Whole group Intensive group* Whole group Intensive group*	NR NR NR NR NR NR NR NR	0.59 NS 0.58 NS 0.62 NS 0.84 NS
Garnett et al, 2013[35]	8	Linear mixed models	Effect of protein (high vs. low) on blood lipids (mmol/L) after 3 mo and 6 mo of intervention	-	TAG LDL-C HDL-C	-	NR NR NR	NS NS NS
Gately et al, 2007[36]	7	ANOVA	Comparison of lipid levels in children that received high vs. low protein intervention diets	Randomized	TAG TC LDL-C HDL-C	-	NR NR NR NR	NS NS NS NS
Rinaldi et al, 2012[40]	6	Linear regression	Difference in TAG concentration (mmol/L) per E% higher protein intake	Sex, age, BMI, total energy intake, intake of other nutrients	TAG TC LDL-C HDL-C	-	-0.01 0.08 0.20 0.12	0.95 0.56 0.26 0.43
Sharma et al, 2009[76]	6	Linear regression	Standardized regression coefficient (protein intake in g/d)	Sex, pubertal stage, waist circumference, intake of carbohydrate and fat	TAG TC LDL-C HDL-C	-	-0.09 0.29 0.30 0.04	NS NS NS NS

Table 2.1.4 (continued) Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Lower-quality studies								
Aeberli et al, 2007[44]	5	Linear regression	Standardized regression coefficient (protein intake in E%)	Waist-hip ratio	LDL-C particle size	-	-0.18	0.10
Andersen et al,	4	Spearman rank correlation	Correlation between protein intake (g/d) and cholesterol levels	-	TC	Infants	-0.29	NS
Akerblom et al, 1984[46]	3	t-test	Comparison of protein intake (g/1000 kcal) in lowest vs. highest quartile of cholesterol levels	-	TC HDL-C	-	NR NR	NS NS
Berenson et al, 1979[48]	3	Pearson correlation	Correlation between protein intake (g/1000 kcal) and cholesterol levels	-	TAG TAG TC TC	0.5 y 1 y 0.5 y 1 y	NR NR NR 0.30	NS NS NS <0.01
Boulton et al, 1995[49]	3	Correlation	Correlation between protein intake (g/d) and cholesterol levels	-	TC TC TC TC LDL-C HDL-C	8 y 11 y 13 y 15 y - -	-0.08 -0.13 -0.19 -0.16 NR NR	NS NS <0.01 <0.05 NR NS
Cowin et al, 2001[53]	3	Spearman correlation	Correlation between protein intake (energy-adjusted, residuals method) and cholesterol levels	-	TC TC LDL-C LDL-C HDL-C HDL-C	M F M F M F	0.00 -0.07 -0.04 -0.17 -0.07 0.06	0.98 0.38 0.68 0.08 0.36 0.49
Frank et al, 1977[54]	1	Pearson correlation	Correlation between protein intake (g/1000 kcal) and lipid levels	-	TAG TC	-	NR NR	NS NS
Frank et al, 1978[55]	1	Pearson correlation	Correlation between protein intake (g/1000 kcal) and lipid levels	-	TAG TC	-	NR NR	NS NS

Table 2.1.4 (continued) Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Glueck et al, 1982[20] †	5	Pearson correlation	Correlation between protein intake (g/d) and lipid concentrations	-	TAG	M, 6-12 y	-0.04	NS
					TAG	F, 6-12 y	-0.03	NS
					TAG	M, 13-19 y	0.02	NS
					TAG	F, 13-19 y	-0.06	NS
					TC	M, 6-12 y	0.04	NS
					TC	F, 6-12 y	-0.08	NS
					TC	M, 13-19 y	0.01	NS
					TC	F, 13-19 y	0.02	NS
					LDL-C	M, 6-12 y	0.00	NS
					LDL-C	F, 6-12 y	-0.04	NS
					LDL-C	M, 13-19 y	-0.03	NS
					LDL-C	F, 13-19 y	-0.04	NS
					HDL-C	M, 6-12 y	0.07	NS
					HDL-C	F, 6-12 y	-0.02	NS
					HDL-C	M, 13-19 y	0.02	NS
					HDL-C	F, 13-19 y	0.11	NS
Gonzalez-Requejo et al, 1995[57]	5	t-test	Comparison of cholesterol levels in highest vs. lowest tertile of protein intake (E%)	-	TC	-	NR	NS
					LDL-C		NR	NS
					HDL-C		NR	NS
Hermelo et al, 1995[37]	4	Pearson correlation	Correlation between protein intake (E%) and cholesterol levels	-	TC	M, obese	0.07	NS
					TC	M, not obese	0.06	NS
					TC	F, obese	0.11	NS
					TC	F, not obese	0.11	NS
					HDL-C	M, obese	-0.09	NS
					HDL-C	M, not obese	-0.1	NS
					HDL-C	F, obese	0.16	NS
					HDL-C	F, not obese	0.14	NS
Heyman et al, 2012[25]	4	Correlation	Correlation between protein intake (E%) and lipid levels	-	TAG	-	NR	NS
					TC		NR	NS
					LDL-C		NR	NS
					HDL-C		NR	NS

Table 2.1.4 (continued) Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Hitchcock et al, 1977[31]	1	ANOVA‡	Comparison of protein intake (E%) in tertiles of cholesterol levels	-	TC	-	NR	NS
Hong et al, 2009[58]	4	Correlation	Partial correlation between protein intake (g/d) and lipid levels	Sex, age, Tanner stage	TAG	-	0.04	NS
					TC		-0.05	NS
					HDL-C		-0.04	NS
Il'chenko et al, 1989[59]	1	t-test‡	Comparison of protein intake in children with high vs. normal TAG levels	-	TAG	-	NR	NS
					TC		NR	NS
					HDL-C		NR	NS
Keser et al, 2010[38]	2	Correlation	Correlation between protein intake and blood lipids	-	TAG	-	0.24	<0.05
					TC		NR	NS
					LDL-C		NR	NS
					HDL-C		-0.18	<0.05
Knuiman et al, 1983[24]	3	Pearson correlation	Correlation between protein intake (E%) and cholesterol levels	-	TC	Finland	0.00	NS
					TC	Netherlands	0.01	NS
					TC	Italy	0.08	NS
					TC	Philippines	0.14	NS
					TC	Ghana	-0.08	NS
					HDL-C	Finland	-0.01	NS
					HDL-C	Netherlands	-0.02	NS
					HDL-C	Italy	0.04	NS
					HDL-C	Philippines	0.32	<0.001
					HDL-C	Ghana	-0.01	NS
Kouvalainen et al, 1982[61]	4	t-test	Comparison of protein intake (g/1000 kcal) in highest vs. lowest quartile of cholesterol levels		TC	3 y	NR	NS
					TC	12 y	NR	NS
					HDL-C	3 y	NR	NS
					HDL-C	12 y	NR	NS
Larsen et al, 1989[62]	1	Mann-Whitney test	Mann-Whitney on tertiles of cholesterol	-	TC	-	NR	NS

Table 2.1.4 (continued) Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Lindquist et al, 2000[63]	4	Linear regression	Log difference in lipid levels (mmol/L) per g increase in protein intake	Fat mass, ethnicity, SES, intake of carbohydrate and fat	TAG TC	-	-0.000 0.001	0.79 0.06
Mia et al, 2000[66]	4	Pearson correlation	Correlation between protein intake (g/d) and lipid levels	-	TAG LDL-C LDL-C	- Total protein Animal protein	NR NR 0.20	NS NS 0.0002
Morrison et al, 1980[21] †	4	Correlation	Partial correlation between protein intake (g/d) and lipid levels	Sex, age, weight, height, ethnicity	TAG TC LDL-C HDL-C	-	-0.05 0.02 0.03 0.00	NS NS NS NS
Nicklas et al, 1993[67]	5	Correlation	Correlation between protein intake (g/d) and blood lipids	-	TC LDL-C HDL-C	-	-0.02 -0.01 -0.037	NS NS NS
Perry et al, 1997[68]	4	Linear regression	Difference in TAG levels (mg/dL) per E% increase in protein intake	Energy intake, intake of carbohydrates, fat, cholesterol, fiber, sodium, and vitamins A and C	TAG	-	NR	NS
Pistulkova et al, 1992[30]	3	t-test	Comparison of protein intake (E%) in children with high vs. low cholesterol levels	-	TC TC	M F	NR positive	NS <0.05
Potter et al, 1989[69]	2	Correlation	Partial correlation between the ratio of animal to vegetable protein intake and lipid levels	Sex	TAG TC LDL-C HDL-C	-	-0.26 -0.22 -0.28 0.08	0.06 0.114 0.047 0.566
Räsänen et al, 1978[70]	2	t-test	Comparison of protein intake (g/1000 kcal) in children with high vs. median vs. low cholesterol levels	-	TC	-	NR	NS

Table 2.1.4 (continued) Reported associations between protein intake and blood lipids in children

First author, year	QS	Statistical analysis	Measure of association	Covariate adjustments	Outcome	Subgroups	Effect estimate	*p*-value
Sanchez-Bayle et al, 2008[72]	5	ANOVA	Comparison of TAG levels in tertiles of protein intake (E%)	-	TAG	-	NR	NS
					TC		NR	NS
					LDL-C		NR	NS
Sarría Chueca et al, 1997[22]	2	Linear regression	Difference in cholesterol levels (mg/dL) per E% increase in protein intake	Body size and dietary variables (unspecified)	TC	-	NR	NS
					LDL-C		NR	NS
					HDL-C		NR	NS
Starc et al, 1998[28]	4	Pearson correlation	Correlation between protein intake (energy-adjusted) and HDL-C levels	-	HDL-C	-	0.28	<0.05
Suter et al, 1993[78]	4	Spearman correlation	Correlation between protein intake (E%) and lipid levels	-	TAG	-	NR	NS
					TC		NR	NS
					LDL-C		NR	NS
					HDL-C		NR	NS
Ventura et al, 2008[42]	4	Pearson correlation	Correlation between protein intake (E%) and TAG levels	-	TAG	-	NR	NS
					HDL-C		NR	<0.05
Vobecky et al, 1979[26]	1	t-test‡	Comparison of protein intake in infants with high vs. normal cholesterol levels	-§	TC	M	NR	NS
					TC	F	positive	<0.01
					TC	total	positive	<0.01
Ward et al, 1980[80]	2	Stepwise linear regression	Difference in cholesterol levels (mg/dL) per E% increase in protein intake	Dietary and biochemical variables (unspecified)	TC	-	NR	NS
Weidman et al, 1978[27]	2	Linear regression	Difference in cholesterol levels (mg/dL) per E% increase in protein intake	Weight, height, energy intake, intake of fat, carbohydrate, fiber, and cholesterol	TC	-	NR	NS

* The intensive intervention group received most of their foods for free and had a higher adherence to the intervention diets.
† Partly overlapping populations
‡ Statistical analysis used not clearly reported.
§ Matched for sex and age.
Abbreviations: BMI, body mass index; E%, energy percent; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NR, not reported; NS, not significant; QS, quality score; SES, socioeconomic status; TC, total cholesterol; TAG, triacylglycerol; VLDL-C, very low-density lipoprotein cholesterol; WC, waist circumference.

DISCUSSION

To our knowledge, this systematic review is the first to summarize the published literature on the effects of protein intake on blood pressure, insulin sensitivity, and blood lipids in children. In this review, 56 studies (published in 60 papers) on the association between protein intake and one or more of these cardiometabolic outcomes in children were identified. Overall, the literature shows insufficient evidence for an effect of animal, vegetable, or total protein intake on blood pressure, insulin sensitivity, or blood lipids, because of a lack of high-quality studies and inconsistency in the results.

Summary of main findings and potential mechanisms

Of the ten higher-quality studies that studied the relation between protein intake and blood pressure in children, four reported inverse associations in one or more of their subgroups,[43, 60, 77, 79] whereas the other studies found no significant effects. Although these results suggest a possible inverse association, there is insufficient evidence to draw meaningful conclusions. Also, the observed effect estimates for reductions in blood pressure are small and not clinically relevant on an individual level. Nevertheless, they may be relevant on a population level. More studies are needed to verify whether protein intake is associated with BP already during childhood, and whether this effect tracks into adulthood. A possible inverse association between protein intake and BP is in line with meta-analyses of studies among adults.[8-10] Mechanisms underlying a beneficial effect of protein intake on blood pressure have not yet been clarified.[81] Proposed pathways include the increased synthesis of cellular ion channels in response to protein intake; an increase in renal plasma flow, a higher glomerular filtration rate; or the vasodilating effects of certain amino acids.[82] For the association between protein intake and measures of insulin sensitivity in children, three of the six higher-quality studies report significant results, but in different directions. Therefore we conclude that there is insufficient evidence for an association between protein intake and insulin sensitivity in children. Two systematic reviews of intervention studies in adults showed inverse associations between protein intake and fasting insulin concentrations, but no significant effect on fasting glucose.[9, 83] Effects of protein intake on insulin sensitivity are suggested to act through the stimulation of insulin secretion.[84]

Many studies assessed the associations between protein intake and blood lipids in children, but only five had a quality score of 6 or higher and none of these higher-quality studies reported a significant effect. Meta-analyses of trials in adults also reported no significant effects of protein intake on LDL or HDL cholesterol concentrations.[9, 83] For TAG levels, one meta-analysis reported that subjects following a higher protein diet had lower TAG concentrations than subjects consuming a diet lower in protein,[9] whereas another meta-analysis, which included only trials with a duration of more than a year, observed no effect.[83]

In children, endpoint measures of cardiometabolic health can usually not be observed, since they occur later in life. Several studies in adults investigated the relation of protein intake with cardiovascular disease and type 2 diabetes. Findings from three cohorts were that total and animal, but not vegetable protein intake were associated with an increased risk for type 2 diabetes.[85-87] Three large cohort studies in women reported that total protein intake was inversely associated with the

incidence of coronary heart disease,[88] stroke,[89] and ischemic heart disease.[90] In contrast, data from two other large cohorts showed that diets high in protein were associated with a higher risk of cardiovascular disease.[91-92] Finally, in a Dutch prospective cohort, a U-shaped association was observed between protein intake and cardiovascular events after 6.4 years of follow-up, with higher incidence rates in subjects with low or high protein intake compared to median protein intake levels.[93]

Different types of protein

Studies on the effects of vegetable or animal protein on blood pressure, insulin sensitivity, and blood lipids in children are scarce and the results are inconsistent. Studies in adults also reported inconsistent effects of either animal or vegetable protein on cardiometabolic risk factors. Meta-analyses of trials and prospective studies reported no differences between effects of vegetable and animal protein on BP.[8, 10] In contrast, prospective cohort studies reported that animal, but not vegetable, protein intake decreases risk of stroke[89, 94] but increases diabetes risk.[85, 87] More studies, both in adult and child populations, are needed to elucidate the differential effects of animal and vegetable protein on cardiometabolic health.

Some of the inconsistencies in the results of the studies in our review may be explained by the type of dietary protein intake. If vegetable and animal protein differently affect cardiometabolic risk factors and the ratio of vegetable versus animal protein intake varies between populations, this might explain some of the discrepancies in the results for total protein intake. Furthermore, if vegetable and animal protein intake affect outcomes in opposite directions, their effects might cancel each other out when studying total protein intake.

Not only the ratio between vegetable and animal protein, but also the main food sources and therefore the amino acid composition of protein might vary between populations. For example, a study in children reported a positive association of dairy protein with BMI and body fat, but no associations of meat or cereal protein intake with these outcomes.[95] Besides the source and type of protein, also the total amount of protein consumed in the population might have affected the results. Mean protein intake in the studies included in our review ranged from 8 to 19 E% (Supplement 2.1.3). A potential relation between total protein intake and cardiometabolic risk factors might not be linear.[93] Effects could therefore be different for various levels of protein intake and might not be identified when statistical approaches are used that assume a linear relation.

Quality of the included studies

We applied a scoring system with a theoretical range from 0 to 10 to assess the quality of the included studies. Only 15 studies were regarded as having a relative high quality (score ≥ 6). Many studies received a low score for the items study design and adjustment for potential confounders. Most of the studies included in this review (47 of the 56) were cross-sectional, only five were longitudinal, and four were intervention studies. In observational studies, even after adjustment for multiple potential confounders, residual confounding may exist. Intakes of several other nutrients for example could be correlated not only to protein intake, but also to several other (unmeasured) determinants of cardiometabolic health such as exercise, BMI, and dietary patterns. In many studies

included in this review, results were not at all or not sufficiently adjusted for important confounding variables, which limits the validity of their results.

An important potential mediator in the relation between protein intake and cardiometabolic health is body weight or body fat. Protein intake has been positively linked to childhood obesity,[3-5] whereas in adults it has been inversely associated with obesity.[9] Since obesity is strongly related to cardiometabolic health, it is interesting to investigate the association of protein intake with cardiometabolic outcomes both with and without adjustment for measures of body composition. However, in only 9 of the 52 observational studies results were adjusted for a measure of body weight or composition,[28, 32, 41, 44, 51, 63, 64, 75-76] and we did not observe clear differences in results that were versus those that were not adjusted for measures of obesity. Ten of the included studies were performed in overweight children only,[32-36, 38, 40-42] and three studies included both overweight and normal-weight children.[37, 39, 45] The latter three studies reported no clear differences in associations between protein intake and insulin sensitivity or blood lipid concentrations among the overweight versus the normal-weight group.

Only four intervention studies met the selection criteria for this review, of which two were short-term (29 and 31 days),[34, 36] limiting the ability to observe an effect. The other two trials had a duration of six months, but consisted of dietary advice only.[35, 43] In one of these trials the actual protein intake (mean ± SEM) did not even differ significantly between the two groups among participants who received dietary instructions only (18.6 ± 1.3 E% vs. 17.6 ± 1.3 E%, p=0.31).[43] However, the latter trial also included a subgroup that received free food products and had a higher adherence to the intervention diet (23.7 ± 1.4 vs. 16.9 ± 1.3 E%, p=0.001). In this more intensive treatment group, beneficial effects of protein intake on BP and insulin sensitivity were observed, whereas in the total group no differences were observed.[43]

Strengths and limitations of this review

The main strength of this review is that it gives a comprehensive overview of the currently available evidence for effects of dietary protein on blood pressure, insulin sensitivity, and blood lipids in children. A very extensive literature search in multiple databases was used to identify articles. We aimed to reduce the problem of publication bias by also searching for publications that did not explicitly mention protein intake in their title or abstract, and by contacting authors to identify unpublished studies. Studies were independently screened and data extracted by two reviewers through a predefined and meticulous procedure.

We assessed the quality of the included studies with a scoring system in order to more objectively distinguish between higher and lower quality studies. Unfortunately, many of the included studies were of relatively low quality. This made it difficult to draw conclusions regarding the absence or presence of the association evaluated. A meta-analysis was not possible, due to the large heterogeneity in study design, outcomes, and age range of the children. Moreover, we were limited by a lack of reported effect estimates. Therefore we conducted only a qualitative synthesis of evidence, in which we took into account the quality score of the included studies and the consistency of the reported results.

Conclusions

The 56 studies included in this systematic review provide insufficient evidence for effects of protein intake on blood pressure, insulin sensitivity, or blood lipids in children. Although a substantial number of studies addressed these associations, data from high-quality studies investigating the independent effects of protein intake are scarce. Results from the few high-quality studies were not consistent. Further research of high methodological quality is needed to understand the effects of protein intake on cardiometabolic health in children. Specifically, in order to investigate the independent effects of protein intake, future studies should take into consideration important potential confounding factors, such as total energy intake and other dietary factors, and measures of body weight. A better evaluation of the effect of protein on cardiometabolic outcomes in children is important, since cardiometabolic risk factors in childhood have been shown to predict cardiovascular disease and type 2 diabetes in adulthood. Insight into early life determinants of cardiometabolic risk may therefore contribute to the prevention of cardiovascular disease and type 2 diabetes in adulthood.

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SUPPLEMENT CHAPTER 2.1

Supplement 2.1.1 Literature search

Example for Embase (via Embase.com)

Population	((infan* OR newborn* OR (new NEXT/1 born*) OR baby OR babies OR neonat* OR perinat* OR postnat* OR child OR 'child s' OR childhood* OR children* OR kid OR kids OR toddler* OR teen* OR boy* OR girl* OR minors* OR underag* OR (under NEXT/2 ag*) OR juvenil* OR youth* OR kindergar* OR puber* OR pubescen* OR prepubescen* OR prepuberty* OR pediatric* OR peadiatric* OR school* OR preschool* OR highschool* OR suckling):de,ab,ti OR (adoles*:de,ab,ti NOT adult/exp) OR child/exp OR newborn/exp)
Exposure	AND ('protein intake'/de OR (protein NEXT/1 supplement*) OR ((diet* OR intake* OR consumption* OR consumed* OR feeding OR food* OR nutrition* OR 'energy percentage' OR 'percentage of energy' OR 'caloric percentage' OR 'percentage of calories') NEAR/6 (protein* OR macronutrient* OR nutrient*)):ab,ti OR (diet* NEXT/1 intake*):ab,ti OR (diet* NEAR/3 composition*):ab,ti)
Outcomes	AND (('cardiovascular disease'/exp NOT ('cardiovascular disease'/exp/dm_cn OR 'congenital disorder'/exp)) OR 'non insulin dependent diabetes mellitus'/de OR hypertension/exp OR obesity/exp OR 'body mass'/de OR 'Metabolic Syndrome X'/de OR (((cardiovascular OR cardiac OR heart OR vascular OR cardiometabolic) NOT congenital) OR (diabetes NEAR/6 ('type 2' OR 'type ii' OR 'non insulin' OR noninsulin)) OR ((glucose OR insulin) NEAR/3 (level* OR concentration OR plasma OR blood OR serum OR metabolism OR tolerance OR intolerance OR sensitivit* OR insensitivity* OR resistance OR homeosta*)) OR 'blood pressure' OR hypertensi* OR ((cholesterol OR LDL* OR HDL* OR triglyceride* OR triacylglycer* OR lipoprotein* OR lipid*) NEAR/3 (plasma OR blood OR serum OR level* OR profile*)) OR hyperlip* OR dyslip* OR obesity OR obese OR 'over weight' OR overweight OR adiposity OR 'metabolic syndrome' OR 'body mass index' OR BMI OR Quetelet OR (body NEXT/1 (composition* OR fat* OR weight*)) OR (weight NEXT/1 gain*) OR 'abdominal fat' OR (fat NEAR/3 (mass OR percentage*)) OR skinfold* OR (waist NEAR/3 (hip OR circumference*))):ab,ti)
Limits	NOT ([animals]/lim NOT [humans]/lim) NOT ([Review]/lim OR [Conference Abstract]/lim OR [Conference Paper]/lim OR [Short Survey]/lim OR [Editorial]/lim OR [Letter]/lim OR [Note]/lim OR [Conference Review]/lim OR [Erratum]/lim)

Supplement 2.1.2 Quality score

1. Study design

0 for cross-sectional studies
1 for longitudinal studies (prospective or retrospective) or non-randomized intervention studies
2 for randomized intervention studies

2. Population

Observational studies
0 if *n* < 200
1 if *n* 200 to <1000
2 if *n* ≥ 1000

Intervention studies
0 if *n* < 50
1 is *n* 50 to <100
2 if *n* ≥ 100

3. Exposure

Observational studies
0 if the study used no appropriate standard dietary assessment method (see below) or if not reported.
1 if the study used a one-day food record, one 24-h recall, or a short FFQ that did not cover the full diet.
2 if the study used multiple-day food records, multiple 24-h recalls, or a full-diet FFQ.

Intervention studies
0 if the intervention diet was not described or not adequately blinded.
1 if the intervention diet was adequately single-blinded.
2 if the intervention was adequately double-blinded.

4. Outcome

0 if the study used no appropriate outcome measurement method (see below) or if not reported.
1 if the study used moderate quality outcome measurement methods:
- Blood pressure: only one measurement, in resting position, by a trained observer;
- Insulin sensitivity: only either glucose or insulin measures, blood sampling after 12h or overnight fast;
- Blood lipids: non-fasting blood sampling.

2 if the study used adequate outcome measurement methods*:
- Blood pressure: at least two measurements, in resting position, by a trained observer;
- Insulin sensitivity: both glucose and insulin or a composite measure, with blood sampling after 12h or overnight fast, or an appropriate glucose or insulin tolerance test;
- Blood lipids: blood sampling after 12h or overnight fast.

5. Adjustments

0 if a study was not randomized (for intervention studies) or if findings were not controlled** for at least the four key covariates stated below.
1 if findings were controlled for:
- age or Tanner stage,
- sex,
- energy intake (including E%), and
- at least one measure of body weight (e.g., BMI, body weight, or body fat).

2 if a study was adequately randomized (for intervention studies) or if findings were additionally controlled for at least two of the following covariates:
- intake of other macronutrients,
- intake of micronutrients,
- physical activity,
- growth,
- birth characteristics (e.g., birthweight, gestational age),
- maternal characteristics (e.g., maternal BMI),
- socioeconomic status, or
- ethnicity.

* Based on guidelines from the American Heart Association (blood pressure and blood lipids) and the American Diabetes Association (insulin sensitivity)
** 'controlled for' here refers to: adjusted for in the statistical analyses (e.g., with multivariable regression); stratified for in the analyses (e.g. boys and girls separately); or narrow selection criteria of study participants on this covariate (e.g., including only 7-year-olds would count as sufficiently controlling for age and including girls only does not require controlling for sex).

Supplement 2.1.3 Exposures and outcomes measured in the included studies

First author, year	Dietary assessment	Mean protein intake (g/d)	Mean protein intake (E%)	Outcomes
Aeberli et al, 2007	2x 24hR + 1d weighed FR	62.9	13.8%	LDL particle size
Aeberli et al, 2009	2x 24hR + 1d weighed FR	62.6	13.9%	SBP, fasting insulin concentration, QUICKI
Akerblom et al, 1984	48hR	82.0	14.0%	Total and HDL cholesterol, TAG
Andersen et al, 1979	1wk FR + 1 24hR	43.0 (0.7 y) 65.0 (4 y)	18.7% (0.7 y) 17.3% (4 y)	Total cholesterol
Berenson et al, 1979	4d weighed FR	30.0	12.6%	Total, HDL and LDL cholesterol
Boulton et al, 1995	1x 24hR	NR	NR	Total cholesterol, TAG, BP
Casazza et al, 2009a	2x 24hR	71.9	15.3%	SBP, glucose, TAG, HDL cholesterol
Casazza et al, 2009b	2x 24hR	NR	~15% (estimated from figure)	Fasting insulin, insulin sensitivity index, acute insulin response
Colin-Ramirez et al, 2009	1x 24hR	67.3	13.7%	Hypertension, diastolic hypertension, and systolic hypertension[†]
Cowin et al, 2001	3x 1d FR	42.7 (M)/ 40.1 (F)[*]	15.0% (M)/ 15.1% (F)[*]	Total, HDL and LDL cholesterol
Damsgaard et al, 2013	Trial diet (target 10-15 vs. 23-28 E% protein), adherence measured with 3d weighed FR	NR	18.6 vs. 17.6% (whole group) 23.7 vs. 16.9% (intensive group)	MAP, fasting glucose and insulin levels, HOMA-IR, total, LDL and HDL cholesterol, TAG
Davis et al, 2005	3d FR	78.3	16.5%	Insulin sensitivity, acute insulin response, disposition index
Davis et al, 2009	2x 24hR	67.7	15.3%	Insulin sensitivity, acute insulin response, disposition index
Duckworth et al, 2009	Trial diet (15 vs. 25 E% protein)	NR	15.0% (control) 25.0% (intervention)	SBP and DBP
Frank et al, 1977	1x 24hR	15 to 210	13%	BP, total cholesterol, TAG
Frank et al, 1978	1x 24hR	69.0	12.9%	SBP, total cholesterol, TAG
Garemo et al, 2006	7d FR (split in 2)	52.2	15.0%	Fasting glucose, insulin, HOMA-IR, HOMA-β, total cholesterol, TAG
Garnett et al, 2013	Trial diets (15 vs. 25-30 E%)	NR	15% (control) 25-30% (intervention)	Insulin sensitivity index, TAG, LDL and HDL cholesterol, SBP and DBP
Gately et al, 2007	Trial diets (15 vs. 22.5 E%)	NR	15.0% (control) 22.5% (intervention)	Total, LDL and HDL cholesterol, TAG, SBP and DBP
Glueck et al, 1982	1x 24hR	75.7	14.2%	Total, HDL and LDL cholesterol, TAG
Gonzalez-Requejo et al, 1995	1d FR	NR	14.0%	Total, HDL and LDL cholesterol
Hermelo et al, 1995	3d FR	NR	NR	Total, HDL, LDL, and VLDL cholesterol, TAG
Heyman et al, 2012	4d FR	72.4	15.4%	Total, HDL and LDL cholesterol, TAG, fasting glucose and insulin
Hitchcock et al, 1977	1x 24hR	NR	12.4%	SBP, DBP, total cholesterol
Hong et al, 2009	3d FR	76.0	15.9%	Total and HDL cholesterol, TAG, fasting glucose
Il'chenko et al, 1989	1d FR	61.5	11.7%	Total and HDL cholesterol, TAG, arterial pressure
Jenner et al, 1988	FFQ	62.7	13.93%	SBP and DBP
Keser et al, 2010	1x 24hR	NR	15-24%	Total, HDL and LDL cholesterol, TAG, glucose, insulin
Knuiman et al, 1993	48hR	76.6 (Finland) 70.9 (Netherlands) 74.4 (Italy)	13.8% (Finland) 15.5% (Netherlands) 13.4% (Italy)	Total and HDL cholesterol

Supplement 2.1.3 (continued) Exposures and outcomes measured in the included studies

First author, year	Dietary assessment	Mean protein intake (g/d)	Mean protein intake (E%)	Outcomes
Knuiman et al, 1993 (cont'd)	48hR	56.0 (Philippines) 39.2 (Ghana)	11.7% (Philippines) 9.2% (Ghana)	Total and HDL cholesterol
Kouvalainen et al, 1982	7d FR	55.0 (3 y) 82.7 (12 y)	14.7% (3 y) 14.2% (12 y)	Total and HDL cholesterol
Larsen et al, 1989	1x 24hR	70.5	13.8%	Total cholesterol
Lindquist et al, 2000	3x 24hR	63.7	14.0%	Total cholesterol, TAG, insulin sensitivity, acute insulin response
Lucas et al, 1994	Lab analysis of breast milk and formula samples	2.3	9.4%	SBP and DBP
Menghetti et al, 2004	1x 24hR	NR	15-16%	Hypertension[$]
Mia et al, 2000	FFQ	91.7	13.0%	Total and LDL cholesterol, TAG
Morrison et al, 1980	1x 24hR	78.2	14.0%	Total, HDL and LDL cholesterol, TAG
Nicklas et al, 1993	1x 24hR	72.5	13.4%	Total, HDL and LDL cholesterol
Obuchowicz et al, 1997	3d diet history	65.4 (normal weight) 104.7 (overweight)	12.7% (normal weight) 13.6% (overweight)	Fasting insulin concentrations
Perry et al, 1997	3x 1d FR	63.0	15.0%	TAG
Pistulkova et al, 1992	3d FR	86.8	12.9%	Total cholesterol
Potter et al, 1989	3x 1d FR	NR	NR	Total, HDL and LDL cholesterol, TAG
Räsänen et al, 1978	1x 24hR	NR	NR	Total cholesterol
Regan et al, 2006	Hospital records	2.5	7.7%	Insulin sensitivity
Rinaldi et al, 2012	3x 24hR	NR	16.0%	Total, LDL and HDL cholesterol, TAG
Sanchez-Bayle et al, 2008	1x 24hR & FFQ	NR	14.8%	Total, LDL and HDL cholesterol, TAG
Sarría Chueca et al, 1997	1x 24hR	102.0	19.2%	Total, LDL and HDL cholesterol
Schachter et al, 1979	2d FR	NR	NR	BP
Schutte et al, 2003a	1x 24hR	60.5	NR	SBP, DBP, and MAP
Schutte et al, 2003b	1x 24hR	60.5	NR	Pulse pressure
Sharma et al, 2009	3d FR	65.5	14.8%	Total, HDL and LDL cholesterol, TAG, HOMA-IR
Simons-Morton et al, 1997	3x 24hR	63.0	14.8%	SBP and DBP
Smith et al, 2003	1x 24hR	NR	NR	SBP and DBP
Starc et al, 1998	3d FR	57.5	16.5%	HDL cholesterol
Sugiyama et al, 2007	1x 24hR	79.2	13.5%	SBP and DBP
Suter et al, 1993	3d FR	75.3	15.8%	Total, HDL and LDL cholesterol, TAG
Ulbak et al, 2004	7d FR	42.0	11.9%	SBP and DBP
Ventura et al, 2008	2x 24hR	71.8	16.1%	HDL cholesterol, TAG, glucose tolerance, SBP and DBP
Vobecky et al, 1979	1x 24hR	4.3	NR	Total cholesterol
Ward et al, 1980	3d FR	47.8	14.1%	Total cholesterol
Weidman et al, 1978	7d FR	77.6	14.0%	Total cholesterol

†Defined on the basis of population-based percentiles for sex, age, and height: Hypertension SBP and DBP ≥90th percentile; diastolic hypertension DBP ≥95thpercentile and SBP<90thpercentile; systolic hypertension SBP ≥95thperentile and DBP<90thpercentile.
*Retrieved from another paper from the same study (Cowin et al, 2001)
$Based on reference values for the Italian population
Abbreviations: 24hR, 24-hour recall; BP, blood pressure; DBP, diastolic blood pressure; E%, energy percentage; FFQ, food-frequency questionnaire; FR, food record; HOMA-β, homeostatic model assessment-β-cell function; HOMA-IR, homeostatic model assessment-insulin resistance; MAP, mean arterial pressure; NR, not reported; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TAG, triacylglycerol.

Chapter 2.2

Protein intake in early childhood & body composition

Manuscript based on this chapter:

Trudy Voortman, Kim V.E. Braun, Jessica C. Kiefte-de Jong, Vincent W.V. Jaddoe, Oscar H. Franco, Edith H. van den Hooven. Protein intake in early childhood and body composition at the age of 6 years: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Previous studies suggest that high protein intake in infancy leads to a higher body mass index in later childhood, but most studies did not distinguish between body fat and lean mass. We examined the associations of total, animal and vegetable protein intake in early childhood with detailed measures of body composition at the age of 6 years.

Methods: This study was performed in 2,911 children participating in a population-based cohort. Protein intake at the age of 1 year was assessed with a validated food-frequency questionnaire and was adjusted for total energy intake. At the children's age of 6 years, we measured their anthropometrics and body fat (with dual-energy X-ray absorptiometry). We calculated age- and sex- specific SD-scores for body mass index (BMI), fat mass index (FMI), and fat-free mass index (FFMI).

Results: After adjustment for confounders, a 10 g/d higher total protein intake at the age of 1 year was associated with a 0.05 SD (95% CI 0.00, 0.09) higher BMI at the age of 6 years. This association was fully driven by a higher FMI (0.06 SD (95% CI 0.01, 0.11)) and not FFMI (-0.01 SD (95% CI -0.06, 0.05)). The associations of protein intake with BMI and FMI at the age of 6 years remained significant after adjustment for BMI at age 1 year. Additional analyses showed that the associations of protein intake with BMI and FMI were stronger in girls than in boys (p for interaction=0.03), stronger among children who had catch-up growth in the first year of life (p for interaction<0.01), and stronger for animal protein than for vegetable protein intake.

Conclusions: Our results suggest that high protein intake in early childhood is associated with higher body fat mass, but not fat-free mass. Future studies are needed to investigate whether these changes persist into adulthood and to examine the optimal range of protein intake for infants and young children.

INTRODUCTION

Protein is an important component of early life nutrition, as it provides essential amino acids required for growth. However, a high protein intake in early childhood has been linked to a higher risk of obesity.[1-3] In contrast, studies in adults suggest beneficial effects of a higher total protein intake on weight maintenance and body composition.[4] Potential mechanisms underlying these effects include increased satiety and energy expenditure.[5-6] In infancy, different mechanisms may play a role. The 'early protein hypothesis' states that high protein in early childhood may is linked to obesity in later life through hormonal responses,[1] including enhanced secretion of insulin-like growth factor 1 (IGF-1),[7] which may increase growth and adipogenesis.[8] This effect might have a specific risk window around the age of 1 year, when most children undergo a transition from complementary feeding to table foods, with a corresponding rapid increase in protein intake.[9] Furthermore, certain groups of children may be extra sensitive to potential adverse effect of high protein intake in early life, for example those who are genetically predisposed to obesity or children who experienced catch-up growth in early childhood.[2]

Although a number of studies showed that a high protein intake in early childhood is associated with a higher body mass index (BMI) in later childhood,[1-2, 10] most studies did not distinguish between body fat and lean mass. Because variation in fat-free mass is an important source of variability in BMI in infants and children, BMI is considered to be of limited use to measure childhood adiposity.[11] Therefore, we examined the associations of protein intake at the age of 1 year with BMI, fat mass index, and fat-free mass index at the age of 6 years. Furthermore, we evaluated these associations in a subgroup of the children for protein intake at the age of 2 years and we examined whether the associations were different by sex, ethnicity, genetic risk score for BMI, birthweight, catch-up growth, and between animal or vegetable protein.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[12] A total of 7,893 children were available for follow-up studies in early childhood. A questionnaire on infant diet was introduced from 2003 onward, and was sent to 5,088 mothers who provided consent for follow-up and had sufficient mastery of the Dutch language The questionnaire was returned for 3,650 (72%) children, of whom 3,629 provided valid dietary data.[13-14] Of this group, 2,911 children visited the research center at the age of 6 years and had body composition measured (Figure 2.2.1).

Dietary assessment

Food intake was assessed at a median age of 12.9 months (95% range 12.2 to 19.0) with a semi-quantitative food-frequency questionnaire (FFQ) consisting of 211 food items.[13] On the basis of standardized portion sizes and the Dutch Food Composition Table 2006,[15] food frequencies were converted into energy and nutrient intakes. Protein intake was converted from grams per day to energy percentage (E%) assuming that 1 g of protein provides 4 kcal. Evaluation of the FFQ against

three 24-h recalls showed an intraclass correlation coefficient of 0.7 for protein intake (more details in Chapter 5.1).[13-14] Mothers of a subgroup of 899 Dutch children,[12] received an additional FFQ at their child's median age of 24.9 months (95% range 24.3 to 27.6).[14] This questionnaire was completed for 844 children (94%), of whom 698 children had body composition measures at the age of 6 years available. In total, 649 children had dietary data at both 1 and 2 years and body composition at age 6 years available.

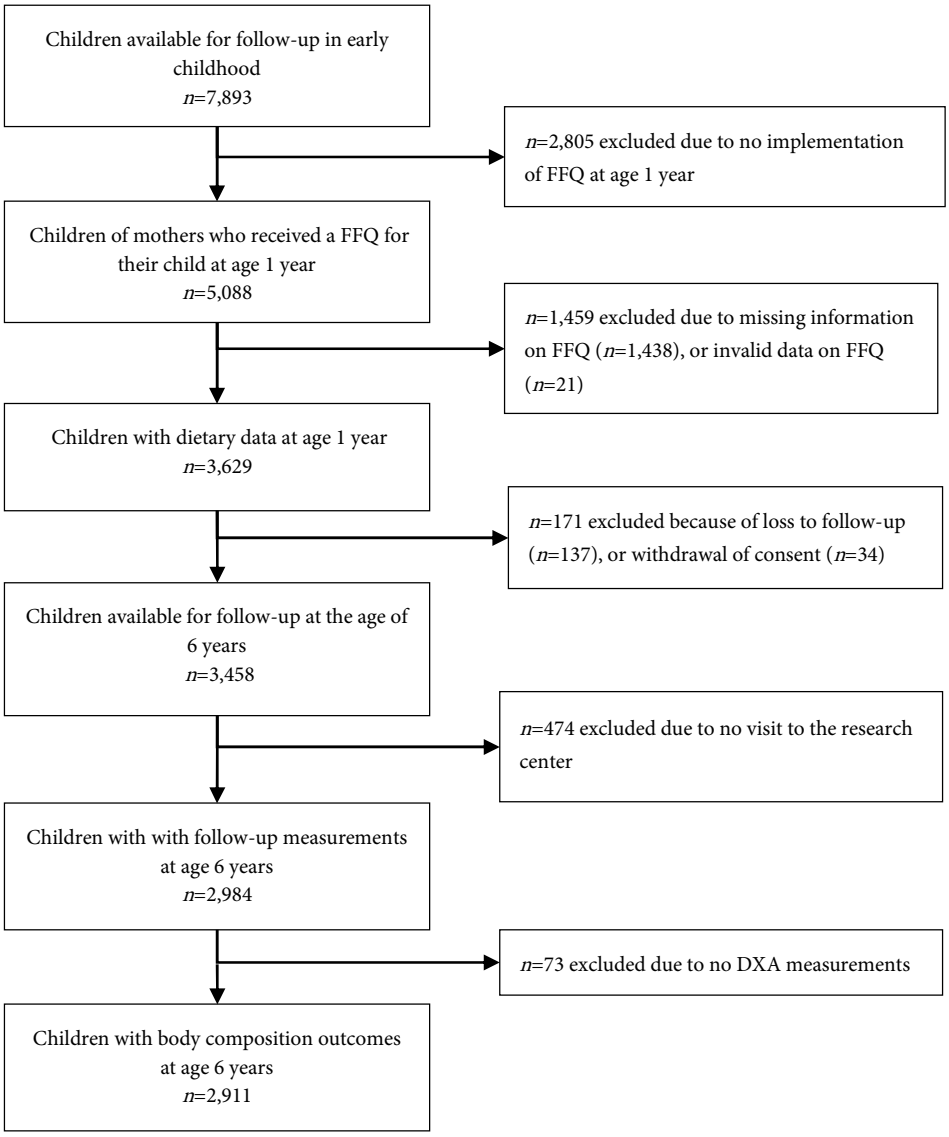

Figure 2.2.1 Flowchart of study participants included for the main analysis

Body composition assessment

Children's anthropometrics and body composition were measured at a median age of 5.9 years (95% range 5.6 to 6.6) by well-trained staff in our research center.[12] Height was determined with a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.) and weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). Total and regional body fat mass, lean mass, and bone mass were measured with a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA), using enCORE software version 13.6. We calculated body mass index (BMI) [weight (kg) /height (m)^2], fat mass index (FMI) [fat mass (kg) /height (m)^2], and fat-free mass index (FFMI) [fat-free mass (kg) /height (m)^2].[16] As secondary outcomes we calculated body fat percentage (BF%) [fat mass (kg) /weight (kg)], and android/gynoid fat ratio (A/G ratio) [android fat mass (kg) /gynoid fat mass (kg)]. For all outcomes we calculated age- and sex-specific SD-scores (SDS) on the basis of the total Generation R Study population with body composition data at 6 years of age (n=6,491).[12]

Covariates

Information on maternal age, maternal educational level, folic acid supplement use, and net household income was obtained with questionnaires at enrollment in the study.[12] Education and income were categorized into high or low according to Dutch standard classifications.[17] Maternal smoking and alcohol consumption during pregnancy were assessed with the use of questionnaires in each trimester and both variables were categorized into never; until pregnancy was known; or continued during pregnancy. Maternal anthropometrics were measured at enrollment at the research center and BMI was calculated.[12]

Information on child's sex, birthweight, and gestational age was available from medical records and hospital registries, and sex- and gestational age-specific SD-scores for birthweight were calculated.[18] Child's ethnicity (Dutch or non-Dutch) was defined as Dutch if both parents were born in the Netherlands.[19] In a subgroup of 1,909 children, cord blood samples were collected at birth and were genotyped using Illumina Infinium II HumanHap610 Quad Arrays following manufacturer's protocols. As described in detail previously,[20] we calculated a weighted genetic risk score for BMI using 29 independent variants that have been shown to be robustly associated with BMI.[20-21] Information on breastfeeding was obtained from delivery reports and postnatal questionnaires and was categorized as never; partial in the first 4 months; or exclusive in the first 4 months.[13] Timing of introduction of solids in the first year of life was obtained from the FFQ administered at the age of 1 year.[22] Information on doctor-diagnosed food allergies was obtained with questionnaires at the child's ages of 6 and 12 months.[12] Child's height and weight were measured at several time points between birth and the age of 4 years at the Community Child Health Centers and age- and sex-specific height, weight, and BMI SD-scores were calculated.[23] Catch-up growth was defined as a weight change in SD-scores greater than 0.67 between the age of 1 month and the age of 11 months.[24] Child fat and carbohydrate intake were measured using the FFQs and adjusted for total energy intake with the residual method.[25] A diet score for preschool children (details in Chapter 5.1) was used to assess overall diet quality with use of data obtained with the FFQs.[14]

For 1,966 children, non-fasting blood samples were obtained at the research center at the children's age of 6 years and serum concentrations of insulin were measured with enzymatic methods (Cobas 8000, Roche, Almere, the Netherlands).[26] Screen time (time spent watching television or using a computer) and participation in sports (yes/no) at the age of 6 years were assessed with a questionnaire and were used as proxies for physical activity during early childhood.

Statistical analyses

Because we were interested in the effect of protein independent of its energy content, we adjusted protein intake for total energy intake with the nutrient residual method.[25] To enhance interpretability, predicted protein intake for the mean energy intake (1310 kcal/d) was added to the residuals as a constant.

Using natural cubic splines, we found no indication for non-linear associations between protein intake at the age of 1 year and body composition outcomes at age 6 years. Therefore we used linear regression models to assess the associations of energy-adjusted total, animal, and vegetable protein intake with body composition outcomes (BMI, FMI, and FFMI). Crude models were adjusted for child's sex, total energy intake, and age at outcome measurement, and analyses with vegetable protein intake were additionally adjusted for animal protein intake and vice versa (model 1). Multivariable models were further adjusted for maternal age, educational level, BMI, and smoking during pregnancy; household income; and child's ethnicity, birthweight Z-score, breastfeeding, total fat intake, diet quality score, screen time, and participation in sports (model 2). The covariates in model 2 were selected on the basis of theory and previous literature and were included in case of a significant change (≥10%) in effect estimates when included in model 1. The following covariates were considered, but not included because they did not fulfill the ≥10% change criterion: maternal parity, alcohol consumption during pregnancy, folic acid supplement use during pregnancy, child's timing of introduction of solids, and child's food allergies. The final model was additionally adjusted for child BMI-SDS at the age of 1 year to assess whether protein intake at the age of 1 year predicted body composition at age 6 years independent of baseline BMI (model 3). To assess whether the associations were different by sex, ethnicity, birthweight, genetic risk score for BMI, catch-up growth, or age at dietary assessment, we evaluated the statistical interaction by adding the product term of the potential effect modifier and protein intake to model 3.

We performed several sensitivity analyses. Firstly, because the FFQ was developed and validated for Dutch children,[13] we performed a sensitivity analysis in Dutch children only. Secondly, we examined the associations of protein intake at the age of 2 years with body composition at 6 years of age in the subgroup of children with dietary data at age 2 years (n=698), using the same multivariable linear regression models as for protein intake at the age of 1 year (model 1 and 2). Thirdly, to examine whether changes in BMI were explained by differences in height and/or weight, we analyzed the associations between protein intake and repeatedly measured height and weight between the ages of 1 and 6 years linear mixed models. Fourthly, to test whether it made a difference whether protein was consumed at the expense of fat or carbohydrate intake, we performed macronutrient substitution analyses in which we adjusted our models for carbohydrate intake, or for saturated, monounsaturated, and polyunsaturated fat intake, instead of total fat intake.

Finally, to examine whether associations could be explained by differences in insulin concentrations (as described in Chapter 2.3),[27] we additionally adjusted the multivariable models for blood insulin concentrations at 6 years of age.

To reduce potential bias associated with missing data, missing values of covariates were multiple imputed (n=10 imputations),[28] using the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. We report the pooled effect estimates after the multiple imputation procedure. All statistical analyses were performed with the use of SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Subject characteristics

Characteristics of the children and their mothers are presented in Table 2.2.1. Because we observed significant interactions between protein intake and sex on body composition, we present characteristics of the whole group as well as for boys and girls separately. Mean (± SD) total protein intake at the age of 1 year was 41.2 g (± 12.9), corresponding to 12.9% of total energy intake. This is higher than recommended for this age group,[29] but similar to intakes observed in other Dutch and other Western pediatric populations.[30-31] Mean animal protein intake was 8.1 E% (± 2.4) and mean vegetable protein intake 4.7 E% (± 1.4). Boys had a higher total energy and absolute protein intake than girls, but E% from protein was the same for boys and girls (12.9 E%). At the age of 6 years, boys were taller and heavier than girls. Mean BMI was similar, but girls had a higher mean FMI and a lower mean FFMI than boys (Table 2.2.1).

Associations between protein intake and body composition

The associations between protein intake at the age of 1 year and body composition at the age of 6 years are presented in Table 2.2.2. In confounder-adjusted models (model 2), a 10 g/d higher total protein intake at the age of 1 year was associated with a 0.05 SD (95% CI 0.00, 0.09) higher BMI and a 0.06 SD (95% CI 0.01, 0.11) higher FMI at age 6 years, but not with FFMI (-0.01 SD (95% CI -0.06, 0.05)). The positive association with FMI slightly attenuated but remained statistically significant after additional adjustment for baseline BMI (model 3). Associations between total protein intake and BF% were similar to those observed for FMI, but protein intake was not associated with A/G fat ratio (Supplement 2.2.1). Associations between protein intake and FMI were stronger for animal protein than for vegetable protein intake (Table 2.2.2). We performed *post hoc* analyses in which we further separated animal protein into protein from dairy versus non-dairy sources. Associations with FMI were similar for protein from dairy (0.04 SD (95% CI 0.00, 0.08) versus protein from meat, fish, and eggs (0.05 SD (95% CI 0.00, 0.10).

Table 2.2.1 Subject characteristics

	All (*n*=2,911)	Boys (*n*=1,422)	Girls (*n*=1,489)	*p*-value*
Parental characteristics				
Maternal age (y)	31.9 (21.7-39.7)	31.8 (21.7-39.9)	31.8 (21.7-39.7)	*0.77*
Maternal BMI at enrollment (kg/m^2)	23.7 (18.9-35.2)	23.4 (18.7-35.2)	23.6 (19.0-36.0)	*0.34*
Higher maternal education (%)	58.4	60.0	57.3	*0.30*
High household income (%)	66.9	66.4	67.7	*0.40*
Smoking during pregnancy (%)				*0.06*
Never	77.9	78.5	78.0	
Until pregnancy was known	10.1	8.7	11.2	
Continued	12.0	12.8	10.8	
Child characteristics				
Girls (%)	51.2	-	-	-
Dutch ethnicity (%)	68.9	69.4	68.4	*0.54*
Birthweight (g)	3452 (568)	3521 (577)	3387 (553)	*<0.01*
Breastfeeding in the first 4 months (%)				*0.92*
Exclusive	30.4	30.7	30.1	
Partial	60.1	59.9	60.2	
Never	9.5	9.4	9.7	
Child characteristics at age 1 year				
Body mass index (kg/m^2)	17.4 (1.6)	17.6 (1.4)	17.2 (1.3)	*<0.01*
Catch-up growth first year (%)	21.4	22.9	20.0	*<0.01*
Age at dietary measurement (mo)	12.9 (12.2-19.0)	12.9 (12.2-19.1)	12.9 (12.2-18.8)	*0.87*
Total energy intake (kcal/d)	1265 (676-2207)	1315 (691-2205)	1222 (651-2232)	*<0.01*
Diet quality score**	4.2 (1.3)	4.2 (1.3)	4.1 (1.3)	*0.04*
Total protein intake (g/d)	41.8 (12.7)	42.4 (13.3)	40.0 (12.4)	*<0.01*
Animal protein intake (g/d)	26.3 (10.2)	26.3 (10.6)	25.1 (10.0)	*<0.01*
of which dairy protein (g/d)†	18.5 (8.3)	18.7 (8.1)	17.6 (8.4)	*<0.01*
Vegetable protein intake (g/d)	15.0 (5.6)	15.5 (5.5)	14.4 (5.7)	*<0.01*
Total protein intake (E%)	12.9 (2.4)	12.9 (2.4)	12.9 (2.4)	*0.94*
Total fat intake (E%)	28.6 (5.6)	28.6 (5.6)	28.6 (5.6)	*0.87*
Total carbohydrate intake (E%)	58.4 (6.0)	58.4 (5.9)	58.4 (6.0)	*0.95*
Child characteristics at 6-year visit				
Age (y)	5.9 (5.7-6.6)	5.9 (5.7-6.6)	5.9 (5.7-6.6)	*0.60*
Screen time (h/d)	1.2 (0.3-4.4)	1.3 (0.2-4.5)	1.2 (0.2-4.3)	*<0.01*
Participation in sports (%)	44.3	43.0	45.6	*0.15*
Height (cm)	118.2 (5.2)	118.5 (5.1)	117.9 (5.2)	*<0.01*
Weight (kg)	22.4 (3.4)	22.5 (3.4)	21.7 (3.4)	*0.15*
Body mass index (kg/m^2)	16.0 (1.6)	16.0 (1.6)	16.0 (1.7)	*0.75*
Fat mass index (kg/m^2)	3.8 (1.2)	3.5 (1.1)	4.2 (1.2)	*<0.01*
Fat-free mass index (kg/m^2)	11.9 (0.9)	12.2 (0.8)	11.6 (0.8)	*<0.01*
Overweight or obese (%)#	13.5	10.9	16.1	*<0.01*

Values are percentages, means (SD), or medians (95% range).

* p-values for differences in means between boys and girls, assessed with independent t-tests for continuous variables with a normal distribution, Mann Whitney U tests for continuous variables with a skewed distribution, and chi-square tests for categorical variables.

** Diet quality score for preschool children with a theoretical range of 0 to 10 (Chapter 5.1).[14]

† Protein from dairy products including dairy-based infant formulas (mean 6.9 g/d) and breast milk (mean 0.6 g/d).

According to international age- and sex-specific cut-offs for BMI.[32]

Table 2.2.2 Associations of protein intake at the age of 1 year with body composition at the age of 6 years

	BMI (SDS)	FMI (SDS)	FFMI (SDS)
Total protein intake (10 g/d)			
Model 1 (crude)	**0.06 (0.01, 0.10)**	**0.06 (0.02, 0.10)**	0.02 (-0.03, 0.06)
Model 2 (confounders)	**0.05 (0.01, 0.09)**	**0.06 (0.02, 0.09)**	0.02 (-0.03, 0.06)
Model 3 (baseline BMI)	0.03 (-0.01, 0.06)	**0.05 (0.00, 0.09)**	-0.01 (-0.05, 0.03)
Animal protein intake (10 g/d)			
Model 1 (crude)	**0.05 (0.01, 0.09)**	**0.06 (0.01, 0.10)**	0.01 (-0.03, 0.06)
Model 2 (confounders)	**0.06 (0.01, 0.10)**	**0.05 (0.01, 0.09)**	0.02 (-0.03, 0.07)
Model 3 (baseline BMI)	0.02 (-0.02, 0.06)	**0.05 (0.00, 0.10)**	-0.02 (-0.06, 0.03)
Vegetable protein intake (10 g/d)			
Model 1 (crude)	0.04 (-0.04, 0.11)	0.02 (-0.06, 0.09)	0.04 (-0.04, 0.12)
Model 2 (confounders)	0.01 (-0.07, 0.08)	-0.01 (-0.08, 0.07)	0.00 (-0.09, 0.08)
Model 3 (baseline BMI)	0.02 (-0.05, 0.09)	0.00 (-0.07, 0.07)	0.01 (-0.07, 0.09)

Values are based on multivariable linear regression models and reflect differences (95% CI) in body composition outcomes (age- and sex-specific SD-scores) per 10 g/d higher energy-adjusted protein intake. **Bold** values indicate statistically significant effect estimates.
Model 1 is adjusted for child sex, total energy intake at 1 year, and age at body composition measurement; models with animal protein intake are additionally adjusted for vegetable protein intake and vice versa.
Model 2 is additionally adjusted for maternal age, pre-pregnancy BMI, educational level, and smoking during pregnancy; household income; and child's ethnicity, birthweight Z-score, breastfeeding in the first 4 months of life, age at dietary assessment, total fat intake at 1 year, diet quality score at 1 year, screen time at 6 years, and participation in sports at 6 years.
Model 3 is additionally adjusted for BMI at the age of 1 year
Abbreviations: BMI, body mass index; FMI, fat mass index; FFMI, fat-free mass index, SDS, standard deviation score.

Stratified analyses

No significant interactions were observed of protein intake at the age of 1 year with ethnicity, age at dietary measurement, birthweight, or the genetic risk score for BMI on any of the outcomes. We observed statistically significant interactions between protein intake at age 1 year and child's sex for FMI (p=0.03), but not for BMI (p=0.15) or FFMI (p=0.24) at age 6 years. After stratifying our analyses for sex, we observed that the association between protein intake and FMI was similar in direction for boys and girls, but that effect estimates were slightly stronger among girls than among boys (Table 2.2.3).

We also observed a significant interaction of protein intake with catch-up growth in the first year of life on BMI and FMI at the age of 6 years (both p<0.01) Results of stratified analyses showed that associations between protein intake and measures of body composition were stronger among children who had experienced catch-up growth in the first year of life (0.19 SD (95% CI 0.08, 0.30) higher FMI per 10 g/d protein), than among those without catch-up growth in the first year of life (0.02 SD (95% CI -0.02, 0.06)) (Table 2.2.4), whereas protein intake was similar in both groups (12.9 E% versus 13.1 E%). Among the children with catch-up growth, a higher protein intake was also associated with a higher BF% and a higher A/G ratio (Supplement 2.2.1).

Table 2.2.3 Associations of protein intake at the age of 1 year with body composition at the age of 6 years, stratified by child sex

Total protein intake (10 g/d)	BMI (SDS)	FMI (SDS)	FFMI (SDS)
Girls (*n*=1,489)			
Model 1 (crude)	**0.07 (0.01, 0.13)**	**0.09 (0.03, 0.15)**	0.00 (-0.07, 0.07)
Model 2 (confounders)	**0.06 (0.00, 0.12)**	**0.08 (0.02, 0.13)**	-0.01 (-0.08, 0.05)
Model 3 (baseline BMI)	0.05 (-0.01, 0.11)	**0.07 (0.02, 0.12)**	-0.02 (-0.08, 0.04)
Boys (*n*=1,422)			
Model 1 (crude)	0.05 (-0.01, 0.11)	0.04 (-0.02, 0.10)	0.03 (-0.03, 0.20)
Model 2 (confounders)	0.04 (-0.02, 0.11)	0.05 (-0.02, 0.12)	0.02 (-0.06, 0.10)
Model 3 (baseline BMI)	0.01 (-0.06, 0.07)	0.03 (-0.04, 0.09)	-0.02 (-0.09, 0.06)

See footnote Table 2.2.4

Table 2.2.4 Associations of protein intake at the age of 1 year with body composition at the age of 6 years, in children with or without catch-up growth in the first year of life

Total protein intake (10 g/d)	BMI (SDS)	FMI (SDS)	FFMI (SDS)
No catch-up growth (*n*=2,287)			
Model 1 (crude)	0.02 (-0.02, 0.07)	0.02 (-0.02, 0.07)	0.00 (-0.06, 0.05)
Model 2 (confounders)	0.02 (-0.02, 0.06)	0.02 (-0.02, 0.06)	-0.01 (-0.06, 0.05)
Model 3 (baseline BMI)	0.00 (-0.04, 0.04)	0.01 (-0.03, 0.05)	-0.02 (-0.07, 0.02)
Catch-up growth (*n*=624)			
Model 1 (crude)	**0.18 (0.06, 0.29)**	**0.18 (0.07, 0.30)**	0.08 (-0.03, 0.20)
Model 2 (confounders)	**0.18 (0.08, 0.29)**	**0.19 (0.08, 0.30)**	0.09 (-0.02, 0.20)
Model 3 (baseline BMI)	**0.14 (0.04, 0.23)**	**0.15 (0.05, 0.26)**	0.05 (-0.06, 0.15)

Values are based on multivariable linear regression models and reflect differences and 95% confidence intervals in body composition outcomes (age- and sex-specific SD-scores) per 10 g/d higher energy-adjusted protein intake. **Bold** values indicate statistically significant effect estimates.
Model 1 is adjusted for child sex, total energy intake at 1 year and age at body composition measurement; models with animal protein intake are additionally adjusted for vegetable protein intake and vice versa.
Model 2 is additionally adjusted for maternal age, pre-pregnancy BMI, educational level, and smoking during pregnancy; household income; and child's ethnicity, birthweight Z-score, breastfeeding in the first 4 months of life, age at dietary assessment, total fat intake at 1 year, diet quality score at 1 year, screen time at 6 years, and participation in sports at 6 years.
Model 3 is additionally adjusted for BMI at the age of 1 year.
Abbreviations: BMI, body mass index; FMI, fat mass index; FFMI, fat-free mass index, SDS, standard deviation score.

Sensitivity analyses

At the age of 2 years, protein intake was slightly higher (13.9 ± 1.9 E%) than at the age of 1 year (12.9 ± 2.4 E%).[33] Among the subgroup with dietary data at both the age of 1 and 2 years (*n*=649), associations with body composition were similar for protein intake at both ages (Supplement 2.2.2). Analyses using repeated growth measures showed that higher intake of total and animal protein, but not vegetable protein, was associated with both a higher height and weight of the children (Supplement 2.2.3). Macronutrient substitution models in which we replaced total fat intake by either carbohydrate intake or by saturated, monounsaturated and polyunsaturated fat intake revealed similar effect estimates for protein intake (data not shown). Analyses restricted to children with a Dutch background revealed similar effect estimates as for the whole group (Supplement 2.2.4). Among the children with information on insulin concentrations at the age of 6 years (*n*=1,966), additional adjustment for insulin levels did not change the effect estimates for protein intake on BMI and FMI (data not shown).

DISCUSSION

This large prospective population-based study suggests that protein intake at the age of 1 year is associated with higher fat mass index, but not with fat-free mass index at school age. These associations were stronger for protein from animal than from vegetable sources. Furthermore, associations were slightly larger in girls than in boys, and were stronger among children who had experienced catch-up growth in the first year of life.

Comparison with previous studies

A number of observational studies showed that a higher protein intake in the complementary feeding period is associated with a higher BMI in later childhood.[1, 35-37] The causal effect of a higher protein intake in infancy on a higher BMI in later childhood was also confirmed in a large randomized trial in which children received high-protein or lower-protein infant formulas in their first year of life.[2] We also observed that a higher protein intake at the age of 1 year is associated with a higher BMI at the age of 6 years, and we additionally show that it is specifically associated with a higher fat mass, but not with fat-free mass index.

To our knowledge, only four previous studies examined protein intake in early life in relation to later measures of body fat.[1, 3, 38-39] Mean protein intake in these populations ranged from 13 to 15 E%, which is close to the 12.9 E% in our study population. In the first study, Rolland-Cachera *et al.* reported that, among 112 French children, protein intake at the age of 2 years was associated with a higher BMI and higher subscapular skinfold thickness at the age of 8 years.[1] In the second study, performed by Hoppe *et al.*, protein intake at the age of 9 months was not associated with child BF% (assessed with DXA) at the age of 10 years in 142 Danish children.[38] The absence of an association in this study might be explained by the earlier age at which protein intake was measured, as results of the third study, performed by Günther *et al.,* suggests that the effect of protein intake on obesity risk may have a specific risk window, i.e., around the age of 12 months.[9] In this study in 203 German children, higher protein intake at the age of 12 months, but not at 6 or 18-24 months, was associated with a higher BF% (calculated from skinfold thicknesses) at the age of 7 years.[3] However, this relation was no longer present when the children reached young adulthood (18-25 years).[40] In the fourth study, neither protein intake at 6 months nor at 12 months was associated with fat mass assessed with DXA at the age of 4 years in 556 British children.[39] In a smaller subgroup of our study population, we did not observe differences in associations for protein intake at the ages of 1 year or 2 years and no interaction between age of dietary assessment and protein intake on body composition. Thus, our results do not support a specific risk window for protein intake at the age of 1 year, however, we only measured diet twice and the group of children with dietary data at the age of 2 years was small.

We observed no associations between protein intake at the age of 1 year and later FMI. This is in contrast to findings among adults or older children, in whom several studies reported associations between high protein intake and higher FFMI or sparing of FFMI loss during weight loss.[41-43] To our knowledge, only one previous study has examined the association of protein intake at preschool age with measures of later fat-free mass. In this follow-up of 159 children participating in the previously described study by Günther *et al.,* Assmann *et al.* reported that protein intake in

early childhood was no longer associated with measures of body fat and was not associated with FFMI in young adulthood (18-25 years).[40] However, in line with studies in adults, a higher protein intake between the ages of 9 and 15 years was associated with a higher FFMI in young adulthood.[40] Results of our study further support the existing literature that suggests that higher protein intake in early childhood may have very different effects on body composition than protein intake in later life.[1]

Potential mechanisms

In adults and older children, a higher protein intake seems to promote weight loss while sparing fat-free mass.[43] A potential mechanism underlying this effect is a higher satiety from high-protein diets, via increased release of satiety hormones in the small intestine and consequently a lower energy intake.[5-6] However, a higher protein intake in adults has also been associated with weight loss independent of energy intake, possibly because of the high thermic effect of protein resulting in a slightly increased energy expenditure.[5]

In contrast, a high protein intake in early childhood may lead to a higher fat mass without affecting fat-free mass. In early childhood, different mechanisms than in adults may play a role. According to the 'early protein hypothesis', high protein intake in infancy may play a role in metabolic programming of later risk of obesity and associated disorders. This hypothesis postulates that high protein in infancy triggers hormonal responses that may cause rapid weight gain in early life and persistent changes in later obesity risk.[1, 44-45] Protein intake stimulates the secretion of insulin and insulin-like growth factor 1 (IGF-1),[7, 27] which may in turn increase growth and adipogenesis[8, 46] and have a long-term effect on body fat.[47] Unfortunately, in our study we did not measure IGF-1 or other growth hormone levels at the age of 1 year. However, when we adjusted our analyses for insulin concentration at the moment of outcome measurement, the association between protein intake and FMI attenuated marginally, suggesting that insulin only partially mediates this association.

In our study population, the associations between protein intake and FMI were stronger for animal than for vegetable protein intake. This supports the hypothesis that protein intake may lead to adiposity via endocrine responses, because a previous study reported that intake of animal, but not vegetable protein in 2.5-year-old children was associated with higher IGF-1 concentrations.[8] To our knowledge, only one previous study, the previously referred to study by Günther *et al.,* reported associations for intake of protein from different sources in early childhood in relation body composition. In line with our results, they found that animal but not vegetable protein intake was associated with child body fat.[9] More specifically, they observed that dairy protein, but not meat or cereal protein intake was associated with BMI and body fat at the age of 7 years.[9] Contrary to these results, additional analyses in our study showed no differences in associations for protein intake from dairy versus non-dairy animal sources.

Previous studies suggested that the IGF-1 axis response to high protein intake may be stronger in girls than in boys.[48] This may explain why we observed slightly stronger associations between protein intake and FMI in girls than in boys. In line with this, we have previously shown that protein intake in early childhood was associated with a higher insulin concentration in later childhood in

girls, but not in boys.[27] The observed sex differences could be explained by a difference in timing of adiposity rebound (AR) or in peak BMI at AR. The AR indicates a rise in BMI curve that occurs between the age of 5 and 7 years,[49] which corresponds with the age of outcome measurements in our study (6 years). An early AR and a higher BMI at AR are associated with a higher risk of obesity.[49] One previous study reported that a higher protein intake in early childhood is associated with an earlier AR,[1] although in the study by Günther *et al.* no consistent association was observed between protein intake between the ages of 1 and 2 years and timing of AR.[50] Günther *et al.,* however, did observe that a higher protein intake in early childhood was associated with a higher BMI at AR in girls, but not in boys.[50]

Our results suggest that growth patterns in infancy may also influence the relation between protein intake and later body composition, as we observed an interaction between protein intake at the age of 1 year and catch-up growth in the first year of life – but not with birthweight – on later BMI and FMI. Catch-up growth in early childhood is an important risk factor for obesity in later life,[51-53] and our results suggest that especially children who experienced catch-up growth in the first year of life may have a higher risk of obesity with higher protein consumption. These children may already have a 'thrifty' energy metabolism leading to increased fat accumulation.[54] which may be further exacerbated by high protein intake. It has been previously suggested that certain subgroups may be extra sensitive to the adverse effect of high protein intake in early life.[2] Our results suggest that children who grew rapidly in the first year of life may be one of these susceptible groups. Previous studies also suggested that the effect of protein intake on obesity may be stronger among children who are at genetic risk to become obese,[55] but we did not find evidence for this interaction effect in our population. Future studies should further examine which children are at increased risk for adverse effects of high protein intake.

Methodological considerations

Important strengths of this study are its prospective population-based design and the large number of children being studied. Also, we were able to perform a meticulous stepwise adjustment for a large number of potential child and parental confounders. However, because of the observational design of our study, residual confounding of other lifestyle-related variables, such as physical activity or other dietary factors, might still occur. Not all mothers and children who participated in the study were included in the current analysis, mainly because of missing dietary data. On average, mothers who filled out the FFQ had a higher educational level, a higher household income, and a healthier lifestyle than mothers who did not.[14] This selection toward a more healthy and affluent population could have led to bias if the associations between protein intake and body composition are different in the children who did not participate. We do not expect this, but it cannot be ruled out.

A limitation of our study is that the FFQ was constructed for Dutch children, whereas children with several ethnic backgrounds were included in our cohort. However, we observed similar effect estimates when we restricted our analyses to children with a Dutch ethnic background compared to those observed in the whole group. Another limitation of our dietary assessment method is that food intake reported using an FFQ is subject to substantial measurement error.[56] By adjusting for

total energy intake, we aimed to reduce this measurement error.[56-57] However, when keeping energy intake constant, a higher protein intake involves a lower intake of fat and/or carbohydrate. Adjusting our models for either total fat, fat subtypes, or carbohydrate intake in macronutrient substitution models resulted in similar effect estimates for protein intake, suggesting that it does not matter whether fat or carbohydrate is exchanged for protein in children's diets with respect to later body composition. Strengths of our dietary assessment are that an FFQ measures habitual diet rather than dietary intake at just one or a few days, and that we calculated not only total protein intake, but also protein intake from different sources. A limitation is that we did not have dietary data in the children at the age of 6 years. Future studies should examine the role of current diet and whether an interaction exists between protein intake in early life and diet in later life with respect to obesity.

An important strength of our study is that we performed detailed measurements of body composition. We not only measured height and weight to calculate BMI, but we also measured body fat and fat-free mass with DXA. Because a given BMI can encompass a wide range of fat mass in children, BMI is considered to be of limited use to measure adiposity in childhood.[11]

Implications

Previously, a large randomized controlled trial observed that a higher protein intake in early childhood leads to a higher BMI in later childhood.[2] In our study, we observed that a higher BMI in relation to protein intake was specifically explained by a higher fat mass, and not higher fat-free mass. Therefore, results of our study and previous studies combined,[1-3] suggest that high protein intake in early childhood increases the risk of adiposity. A reduction in protein intake in infancy and early childhood may therefore be advised to prevent obesity, for example by lowering the amounts of protein in infant formula and toddler foods. However, a too low protein intake may restrict growth. Therefore future studies should explore what the optimal range of protein intake is for infants and young children for optimal growth and other aspects of later health.[34] Furthermore, future studies should explore whether intake of different types of protein or certain amino acids are specifically associated with increased adiposity, for example by studying the endocrinal response to different protein subtypes. Results from these studies can be used to further optimize dietary recommendations regarding protein intake in early childhood.

Conclusions

In this prospective cohort, high protein intake in early childhood is associated with higher fat mass, but not fat-free mass index in later childhood. This association was stronger for animal protein than for vegetable protein, and was stronger among children who had experienced catch-up growth in the first year of life. Further research should explore the underlying mechanisms and study the optimal amount of protein intake needed in early childhood for optimal growth and body composition.

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SUPPLEMENT CHAPTER 2.2

Supplement 2.2.1 Associations of protein intake at the age of 1 year with body fat percentage and android/gynoid ratio at the age of 6 years (*n*=2,911)

Total protein intake (10 g/d)	BF% (SDS)	A/G ratio (SDS)
All children (*n*=2,911)		
Model 1 (crude)	**0.06 (0.02, 0.11)**	0.03 (-0.01, 0.07)
Model 2 (confounders)	**0.06 (0.02, 0.10)**	0.04 (-0.01, 0.09)
Model 3 (baseline BMI)	**0.05 (0.00, 0.10)**	0.04 (-0.02, 0.09)
Girls (*n*=1,489)		
Model 1 (crude)	**0.10 (0.03, 0.16)**	0.05 (-0.01, 0.11)
Model 2 (confounders)	**0.09 (0.02, 0.16)**	0.06 (-0.02, 0.14)
Model 3 (baseline BMI)	**0.08 (0.01, 0.15)**	0.06 (-0.02, 0.14)
Boys (*n*=1,422)		
Model 1 (crude)	0.04 (-0.03, 0.10)	0.01 (-0.05, 0.08)
Model 2 (confounders)	0.05 (-0.02, 0.12)	0.02 (-0.06, 0.09)
Model 3 (baseline BMI)	0.03 (-0.04, 0.09)	0.01 (-0.06, 0.09)
No catch-up growth (*n*=2,287)		
Model 1 (crude)	0.03 (-0.02, 0.08)	0.00 (-0.05, 0.04)
Model 2 (confounders)	0.02 (-0.02, 0.07)	-0.01 (-0.06, 0.04)
Model 3 (baseline BMI)	0.01 (-0.03, 0.06)	-0.01 (-0.06, 0.03)
Catch-up growth (*n*=624)		
Model 1 (crude)	**0.17 (0.06, 0.29)**	**0.16 (0.04, 0.27)**
Model 2 (confounders)	**0.17 (0.06, 0.28)**	**0.16 (0.04, 0.27)**
Model 3 (baseline BMI)	**0.15 (0.04, 0.26)**	**0.14 (0.02, 0.26)**

See footnote Supplement 2.2.2

Supplement 2.2.2 Associations of protein intake at the age of 1 and 2 years with childhood body composition at the age of 6 years (*n*=649)

Total protein intake (10 g/d)	BMI (SDS)	FMI (SDS)	FFMI (SDS)
Protein intake at age 1 year (*n*=649)			
Model 1 (crude)	0.05 (-0.03, 0.12)	-0.01 (-0.08, 0.07)	0.08 (-0.02, 0.18)
Model 2 (confounders)	0.03 (-0.06, 0.13)	-0.02 (-0.11, 0.08)	0.07 (-0.04, 0.19)
Model 3 (baseline BMI)	0.00 (-0.09, 0.08)	-0.04 (-0.13, 0.04)	0.03 (-0.07, 0.14)
Protein intake at age 2 years (*n*=649)			
Model 1 (crude)	0.03 (-0.06, 0.11)	-0.03 (-0.11, 0.05)	0.09 (-0.02, 0.20)
Model 2 (confounders)	0.06 (-0.05, 0.16)	-0.01 (-0.11, 0.09)	0.10 (-0.02, 0.23)
Model 3 (baseline BMI)	-0.02 (-0.11, 0.07)	-0.07 (-0.17, 0.02)	0.04 (-0.08, 0.16)

Values are based on multivariable linear regression models and reflect differences and 95% confidence intervals in body composition outcomes (age- and sex-specific SD-scores) per 10 g/d higher energy-adjusted protein intake. **Bold** values indicate statistically significant effect estimates.
Model 1 is adjusted for total energy intake at dietary assessment and age at body composition measurement; models with animal protein intake are additionally adjusted for vegetable protein intake and vice versa.
Model 2 is additionally adjusted for maternal age, pre-pregnancy BMI, educational level, and smoking during pregnancy; household income; and child's ethnicity, birthweight Z-score, breastfeeding in the first 4 months of life, age at dietary assessment, total fat intake at 1 year, diet quality score at 1 year, screen time at 6 years, and participation in sports at 6 years.
Model 3 is additionally adjusted for BMI at age of dietary assessment.
Abbreviations: A/G ratio, android/gynoid fat ratio; BF%, body fat percentage; SDS, standard deviation score.

Supplement 2.2.3 Associations of protein intake at the age of 1 year with repeatedly measured height, weight, and BMI between the age of 1 and 6 years

	Height (SDS)	Weight (SDS)	BMI (SDS)
Total protein intake (10 g/d)	**0.03 (0.01; 0.06)**	**0.06 (0.04; 0.09)**	**0.05 (0.03; 0.08)**
Animal protein intake (10 g/d)	**0.04 (0.01; 0.07)**	**0.07 (0.04; 0.10)**	**0.06 (0.03; 0.08)**
Vegetable protein intake (10 g/d)	0.01 (-0.02; 0.04)	0.01 (-0.02; 0.04)	0.02 (-0.02; 0.04)

Values are regression coefficients and 95% confidence intervals based on linear mixed models and reflect differences in growth measures (age- and sex-specific SD-scores) per 10 g/d increase in energy-adjusted protein intake. **Bold** values indicate statistically significant effect estimates.
Models are adjusted for child sex, ethnicity, birthweight Z-score, breastfeeding in the first 4 months, age at dietary measurement, total energy intake at 1 y, diet quality score at 1 year, playing sports; and for household income, maternal BMI, maternal educational level, folic acid supplement use during pregnancy, and smoking during pregnancy, Models including animal protein intake were adjusted for vegetable protein intake and vice versa.

Supplement 2.2.4 Associations of protein intake at the age of 1 year with childhood body composition at the age of 6 years, in Dutch children only (*n*=2,006)

Total protein intake (10 g/d)	BMI (SDS)	FMI (SDS)	FFMI (SDS)
All children (*n*=2,006)			
Model 1 (crude)	**0.08 (0.04, 0.13)**	**0.06 (0.02, 0.10)**	**0.08 (0.02, 0.13)**
Model 2 (confounders)	**0.08 (0.03, 0.14)**	**0.07 (0.02, 0.12)**	0.06 (-0.01, 0.13)
Model 3 (baseline BMI)	0.04 (-0.01, 0.09)	0.04 (-0.01, 0.09)	0.02 (-0.04, 0.08)
Girls (*n*=1,018)			
Model 1 (crude)	**0.10 (0.03, 0.16)**	**0.10 (0.03, 0.16)**	0.04 (-0.04, 0.12)
Model 2 (confounders)	**0.12 (0.04, 0.20)**	**0.12 (0.05, 0.20)**	0.04 (-0.06, 0.13)
Model 3 (baseline BMI)	**0.10 (0.03, 0.17)**	**0.11 (0.04, 0.18)**	0.02 (-0.07, 0.10)
Boys (*n*=988)			
Model 1 (crude)	**0.07 (0.01, 0.14)**	0.03 (-0.03, 0.08)	**0.11 (0.04, 0.19)**
Model 2 (confounders)	0.05 (-0.03, 0.12)	0.02 (-0.05, 0.09)	0.08 (-0.01, 0.18)
Model 3 (baseline BMI)	-0.01 (-0.08, 0.06)	-0.01 (-0.08, 0.05)	0.03 (-0.06, 0.15)
No catch-up growth (*n*=2,287)			
Model 1 (crude)	0.02 (-0.02, 0.07)	0.02 (-0.02, 0.07)	0.00 (-0.05, 0.05)
Model 2 (confounders)	0.02 (-0.04, 0.07)	0.03 (-0.02, 0.08)	-0.03 (-0.09, 0.04)
Model 3 (baseline BMI)	0.00 (-0.05, 0.05)	0.02 (-0.03, 0.07)	-0.05 (-0.11, 0.01)
Catch-up growth (*n*=624)			
Model 1 (crude)	**0.18 (0.06, 0.29)**	**0.18 (0.06, 0.30)**	0.08 (-0.03, 0.20)
Model 2 (confounders)	**0.18 (0.05, 0.31)**	**0.19 (0.06, 0.33)**	0.09 (-0.05, 0.22)
Model 3 (baseline BMI)	**0.15 (0.03, 0.27)**	**0.17 (0.04, 0.30)**	0.05 (-0.07, 0.17)

Values are based on multivariable linear regression models and reflect differences and 95% confidence intervals in body composition outcomes (age- and sex-specific SD-scores) per 10 g/d higher energy-adjusted protein intake. **Bold** values indicate statistically significant effect estimates.
Model 1 is adjusted for child sex, total energy intake at 1 year and age at body composition measurement; models with animal protein intake are additionally adjusted for vegetable protein intake and vice versa.
Model 2 is additionally adjusted for maternal age, pre-pregnancy BMI, educational level, and smoking during pregnancy; household income; and child's ethnicity, birthweight Z-score, breastfeeding in the first 4 months of life, age at dietary assessment, total fat intake at 1 year, diet quality score at 1 year, screen time at 6 year, and participation in sports at 6 years.
Model 3 is additionally adjusted for BMI at the age of 1 year.
Abbreviations: BMI, body mass index; FMI, fat mass index; FFMI, fat-free mass index; SDS, standard deviation score.

Chapter 2.3

Protein intake in early childhood & cardiometabolic health

Manuscript based on this chapter:

Trudy Voortman, Edith H. van den Hooven, Myrte J. Tielemans, Jessica C. Kiefte-de Jong, Albert Hofman, Vincent W.V. Jaddoe Oscar H. Franco. Protein intake in early childhood and cardiometabolic health at school age: the Generation R Study. *European Journal of Nutrition* 2015; doi:10.1007/s00394-015-1026–7.

ABSTRACT

Background*:* High protein intake in infancy has been linked to obesity. Whether dietary protein is also related to other cardiometabolic outcomes in children is unclear. We aimed to examine the associations of protein intake in early childhood with cardiovascular and metabolic outcomes at school age.

Methods: This study was performed in 2,965 children participating in a population-based prospective cohort. Protein intake at the age of 1 year was assessed with a food-frequency questionnaire and was adjusted for energy intake. At the children's age of 6 years we measured their body fat percentage (BF%), blood pressure (BP), and insulin, HDL cholesterol, and triacylglycerol serum concentrations. These measures were incorporated into a cardiometabolic risk factor score, using age- and sex-specific SD-scores.

Results*:* In covariate-adjusted models, higher protein intake was associated with a higher BF%, lower diastolic BP, and lower triacylglycerol. We observed a significant interaction of protein intake with child sex on metabolic outcomes. Stratified analyses showed that protein intake was positively associated with BF% (0.07 SD (95% CI 0.02, 0.13) per 10 g/d) and insulin levels in girls, but not in boys. In boys, but not in girls, higher protein intake was associated with a lower triacylglycerol concentration (-0.12 SD (95% CI -0.20, -0.04) per 10 g/d) and a lower cardiometabolic risk factor score. Protein intake was not consistently associated with systolic BP or HDL cholesterol.

Conclusion*:* Protein intake in early childhood was associated with a higher BF% and higher insulin levels at the age of 6 years in girls, and with lower triacylglycerol levels in boys. Further studies are needed to explore these sex differences and to investigate whether the observed changes persist into adulthood.

INTRODUCTION

Already during childhood, adiposity, high blood pressure, dyslipidemia, and insulin resistance are highly prevalent.[1] These cardiometabolic risk factors in childhood have been shown to track to later life, and are suggested to predict adult cardiovascular disease and type 2 diabetes,[2-3] highlighting the need to study determinants of cardiometabolic health already in early childhood.[4]

Studies in adults suggest favorable effects of high dietary protein intake on cardiometabolic risk factors, including a lower blood pressure, lower triacylglycerol concentrations and a reduction in body weight.[5-7] Mechanisms underlying these effects remain to be elucidated, but may include increased satiety, a higher energy expenditure, and metabolic effects of specific amino acids.[8] In contrast, a high protein intake in early childhood has been associated with a higher risk of obesity,[9-12] suggesting that high protein intake in early life may lead to unfavorable effects on cardiometabolic health. A high protein intake in infancy may enhance the secretion of insulin-like growth factor 1 (IGF-1) and insulin,[13-14] which could in turn increase adipogenesis.[15] The period around the age of 1 year has been suggested to be a critical phase with respect to protein intake and later obesity risk, possibly because this period is often characterized by a transition from complementary feeding to family diet and a corresponding rapid increase in protein intake.[16] Thus far, studies investigating the effects of protein intake on insulin concentrations, blood lipids, and blood pressure in children are scarce and report inconsistent results (Chapter 2.1).[17]

We examined the associations of protein intake at the age of 1 year with body fat percentage, insulin levels, blood lipids, and a combined cardiometabolic risk factor score at the age of 6 years in 2,965 children participating in a population-based prospective cohort study. In addition, we aimed to evaluate whether the associations differed for different sources of protein; and whether associations differed by child sex, ethnicity, birthweight, or weight status at age 6 years.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[18] Written informed consent was provided by caregivers for all children the study was approved by the local medical ethics committee. In total, 7,893 children were available for follow-up studies in early childhood.[18] A questionnaire on child diet was implemented at a later stage of the study and was sent to 5,088 mothers who provided consent for follow-up and had sufficient mastery of the Dutch language. In total, 3,650 (72%) of these mothers returned the questionnaire, of whom 3,629 provided valid dietary data.[19] Of this group, 2,984 children visited the research center at the age of 6 years (Chapter 2.2, Figure 2.2.1), of whom 2,965 had one or more cardiometabolic measurements available. Not all children had information available on each outcome, mainly because some parents or children did not give consent for blood collection.[20] The number of children included in this study therefore differs per outcome, ranging from 1,894 for the cardiometabolic score to 2,911 for body fat measurements (Table 2.3.2).

Dietary assessment

Food intake was assessed at a median age of 12.9 months (95% range 12.2 to 19.0) using a semi-quantitative food-frequency questionnaire (FFQ), covering the previous month, that was filled out by the caregivers.[19] This FFQ consisted of 211 food items that, according to a Dutch National Food Consumption Survey in 2002, are frequently consumed by children aged 9 to 18 months.[21] On the basis of standardized portion sizes and the Dutch Food Composition Table 2006,[22] food frequencies were converted into average daily nutrient intakes. Evaluation of the FFQ against three 24-h recalls in a representative sample of 32 Dutch children with a median age of 14 months (95% range 6 to 20) living in Rotterdam, showed an intraclass correlation coefficient of 0.7 for total protein intake (Chapter 5.1).[19, 23] Mothers of a subgroup of 899 Dutch children[18] received an additional FFQ around their child's age of 2 years (median 24.9 months, 95% range 24.3 to 27.6), which was similar to the FFQ applied around the age of 1 year (Chapter 5.1).[23] Of the children with dietary data at the age of 2 years, 714 had cardiometabolic measurements available at the age of 6 years.

Cardiometabolic outcome assessment

Children's cardiometabolic health outcomes were measured at their median age of 5.9 years (95% range 5.6 to 6.6) in our research center by well-trained staff.[20] Weight was determined with a mechanical personal scale (SECA, Almere, the Netherlands) and height was measured with a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Body fat mass was measured with a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA) and with use of enCORE software version 13.6. Body fat percentage (BF%) was calculated by expressing total fat mass as percentage of total body weight.

Non-fasting blood samples were obtained, transported, and stored as described in detail previously.[20] Serum concentrations of insulin, C-peptide, triacylglycerol, and total, HDL, and LDL cholesterol were measured at Erasmus Medical Center with enzymatic methods (Cobas 8000, Roche, Almere, the Netherlands).[20] Quality control samples demonstrated intra-assay coefficients of variation ranging from 0.77 to 1.17%, and inter-assay coefficients ranging from 0.87 to 1.69%.

While the children were lying, systolic (SBP) and diastolic blood pressure (DBP) were measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmomanometer Datascope Accutorr Plus™ (Paramus, NJ, USA). We used mean SBP and mean DBP of the last three measurements. For all cardiometabolic outcomes we calculated age- and sex-specific SD-scores (SDS), on the basis of the total Generation R Study population with data on cardiometabolic health at the ae of 6 years (*n* ranging from 4,414 to 6,491).[18]

In addition to the individual cardiometabolic outcomes, we calculated a continuous score for overall cardiometabolic risk. In line with previous studies that defined scores for a metabolic syndrome-like phenotype in children,[24] we created a continuous score including five components: BF%, blood pressure (SPB and DBP), HDL cholesterol (HDL-C), triacylglycerol (TAG), and insulin concentrations. The cardiometabolic risk factor score was calculated as the sum of age- and sex-specific SD-scores of these five variables, as proposed previously for pediatric populations.[24] The SD-scores for HDL-C were multiplied by -1 since higher HDL-C levels reflect better cardiometabolic health. The SD-scores for SBP and DBP were multiplied by 0.5 so that each

contributed half to the blood pressure component. The final cardiometabolic risk factor score was thus calculated as: BF%SDS + 0.5 × SBP SDS + 0.5 × DBP SDS + TAG SDS + (-1 × HDL-C SDS) + insulin SDS, with a higher score reflecting a higher cardiometabolic risk.

Covariates

Information on maternal age and educational level at enrollment was obtained with a questionnaire.[26] Maternal height and weight were measured at our research center at enrollment in the study and body mass index (BMI, kg/m^2) was calculated.[18] Maternal smoking during pregnancy was assessed using questionnaires in each trimester and was categorized into never; quit in the first trimester; or continued. Information on child's sex, birthweight and gestational age was available from medical records and hospital registries, and gestational age- and sex- specific birthweight Z-scores were calculated.[25] Child's ethnicity was defined as Dutch when both parents were born in the Netherlands and as non-Dutch when one or both parents were born in another country.[27] Information on breastfeeding (never, partial, or exclusive in the first 4 months) was obtained from delivery reports and postnatal questionnaires.[19] Child's height and weight around the age of 1 year were measured at the Community Child Health Centers and age- and sex-specific BMI SD-scores were calculated using Dutch reference curves.[28] Child fat and carbohydrate intake was derived from the FFQs and energy-adjusted using the residual method.[29] Child BMI (kg/m^2) at the age of 6 years was calculated from measured height and weight, and expressed in age- and sex- specific SD-scores.[28] Child weight status (underweight, normal weight, or overweight) was defined with use of international age- and sex-specific BMI cut-offs.[30] Screen time and participation in sports (yes/no) at the age of 6 years were assessed with a questionnaire as proxies for physical activity level.

Statistical analyses

Because we were interested in the effect of protein independent of its energy content, we adjusted protein intake for total energy intake using the nutrient residual method.[29] To enhance interpretability, predicted protein intake for mean energy intake (1312 kcal/d) was added as constant. Protein intake was analyzed both as continuous variable and categorized into tertiles. Insulin concentrations were square-root transformed to obtain a normal distribution and subsequently, age- and sex-specific SD-scores were created for all outcomes.

We used linear regression models to assess the associations of total, animal, and vegetable protein intake at the age of 1 year with cardiometabolic outcomes at the age of 6 years. Crude models for total protein intake were unadjusted. Animal and vegetable protein intakes were adjusted for each other. Potential confounders were selected based on theory or previous literature. The following covariates were included in all multivariable models: child's age, sex, ethnicity, birthweight Z-score, height-for-age, and total energy intake. Other potential confounders were entered individually in the crude models and were included in the final models in case of a significant change (≥10%) in the effect estimate of protein intake on at least one of the cardiometabolic outcomes.[31] Following this procedure, the final models were adjusted for maternal age, educational level, BMI, and smoking during pregnancy; and child's ethnicity, birthweight Z-score, breastfeeding in the first four months of life, age at dietary measurement, BMI at the age of

1 year, total energy intake, energy-adjusted fat intake, height SDS at age 6 years, screen time and participation in sports. The following covariates were considered but not included because they did not fulfill the 10%-change criterion: household income, maternal parity, maternal alcohol consumption during pregnancy, child's gestational age at birth, timing of introduction of solid foods, and food allergies.

To assess whether the associations between protein intake and cardiometabolic outcomes might differ by child sex, ethnicity, birthweight Z-score, or weight status at the age of 6 years, we evaluated the statistical interaction by adding the product term of the potential effect modifier and total protein intake to the multivariable models.

We performed several sensitivity analyses. We additionally adjusted the multivariable models for child BMI-SDS at the age of 1 year to assess whether protein intake at 1 year of age predicted childhood cardiometabolic health independent of baseline BMI. To examine whether associations with blood lipids, BP, or insulin levels could be explained by differences in body fat, we additionally adjusted these models for child BF% at 6 years of age in separate models. We performed macronutrient substitution analyses in which we adjusted our models for carbohydrate intake or for saturated, monounsaturated, and polyunsaturated fat intake, instead of total fat intake, to check whether it made a difference whether protein was consumed at the expense of different other macronutrients. To explore whether associations with the cardiometabolic score were driven by one component only, we performed sensitivity analyses in which we excluded one component of the score at a time. Because the FFQ was developed for Dutch children, we repeated the analyses in children with a Dutch ethnic background only. To explore potential selection bias due to missing blood sampling, we examined descriptive statistics and associations of protein intake with body fat and blood pressure measurements in children who had blood samples available (n=2,010). In the subgroup of children with dietary data at age 2 years, we assessed associations of protein intake at this age with cardiometabolic outcomes at 6 years of age using the same multivariable linear regression models as for protein intake at the age of 1 year.

Missing values of covariates were multiple imputed (n=10 imputations) with use of the Fully Conditional Specification method (predictive mean matching).[32] We report the pooled regression coefficients after the multiple imputation procedure. Statistical analyses were performed using SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Subject characteristics

Characteristics of the children and their mothers are presented in Table 2.3.1. Mean (±SD) total protein intake at the age of 1 year was 41.2 g (±12.9), corresponding to 12.9% (±2.4) of total energy intake (E%). This is higher than recommended for this age group, but similar to intakes observed in the general Dutch and other Western pediatric populations.[33-34] Boys had a higher total energy and absolute protein intake than girls, but E% from protein was not different. Energy-adjusted protein intake was also similar among children with and without blood samples (Supplement 2.3.1).

Table 2.3.1 Characteristics of the participating children and their mothers

	All (*n*=2,965)	Boys (*n*=1,445)	Girls (*n*=1,520)
Maternal characteristics			
Maternal age (y)	31.9 (21.8-39.8)	31.9 (21.7-40.0)	31.9 (21.9-39.6)
Maternal BMI at enrollment (kg/m^2)	23.7 (19.0-35.2)	23.4 (18.7-35.2)	23.6 (19.0-36.0)
Educational level (%)			
Primary	5.2	5.2	5.1
Secondary	36.3	34.9	37.6
Higher	58.5	59.9	57.3
Smoking during pregnancy (%)			
Never	78.1	78.3	78.6
Until pregnancy was known	10.3	8.9	10.9
Continued	11.6	12.8	10.5
Child characteristics			
Girls (%)	51.3	-	-
Dutch ethnicity (%)	68.8	69.5	68.4
Gestational age at birth (wk)	39.9 (1.8)	39.9 (1.8)	40.1 (1.8)
Birthweight (g)	3452 (569)	3524 (576)	3383 (555)
Breastfeeding in the first 4 months (%)			
Exclusive	31.4	31.8	31.0
Partial	60.7	60.6	60.9
Never	7.9	7.6	8.1
Child characteristics at dietary measurement			
Age at FFQ (mo)	12.9 (12.2-19.0)	12.9 (12.2-19.1)	12.9 (12.2-18.8)
Total energy intake (kcal/d)	1265 (678-2206)	1316 (691-2210)	1221 (652-2230)
Protein intake (g/d)			
Total protein	41.8 (12.6)	42.9 (13.0)	40.6 (12.2)
Animal protein	25.6 (10.2)	26.9 (10.5)	25.7 (9.8)
Vegetable protein	14.2 (5.6)	15.5 (5.5)	14.4 (5.7)
Child characteristics at 6-year visit			
Age (y)	5.9 (5.7-6.6)	5.9 (5.6-6.6)	5.9 (5.6-6.5)
Screen time (h/d)	1.3 (0.3-4.3)	1.3 (0.3-4.8)	1.2 (0.2-4.6)
Participation in sports (%)	44.2	43.0	45.5
Height (cm)	118.2 (5.2)	118.5 (5.1)	117.9 (5.2)
Weight (kg)	22.4 (3.4)	22.5 (3.4)	21.7 (3.4)
BMI (kg/m^2)	16.0 (1.6)	16.0 (1.6)	16.0 (1.7)
Body fat percentage (%)	23.5 (16.2-36.4)	21.1 (15.7-33.5)	25.6 (18.8-37.5)
Systolic blood pressure (mmHg)	102 (8)	101 (8)	102 (8)
Diastolic blood pressure (mmHg)	60 (7)	60 (7)	61 (6)
HDL cholesterol (mmol/L)	1.35 (0.31)	1.36 (0.31)	1.33 (0.30)
Triacylglycerol (mmol/L)	0.97 (0.40-2.36)	0.96 (0.38-2.34)	0.98 (0.44-2.47)
Insulin (pmol/L)	115 (18-398)	119 (17-382)	115 (19-432)

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.
Abbreviations: BMI, body mass index; FFQ, food-frequency questionnaire.

Associations between protein intake and cardiometabolic outcomes

After adjustment for covariates, children with a protein intake in the highest tertile had a 0.08 SD (95% CI 0.01, 0.16) higher BF%, a 0.14 SD (95% CI -0.24, -0.03) lower TAG concentration, and a 0.09 SD (95% CI -0.18, 0.00) lower DBP than children in the lowest tertile of protein intake (Table 2.3.2). Protein intake was not significantly associated with SBP, insulin, HDL-C, or the cardiometabolic score.

Because we observed significant or borderline significant interactions between total protein intake and sex on BF% (p=0.05), insulin (p=0.01), C-peptide (p=0.03), HDL-C (p=0.08), TAG (p<0.01), and the cardiometabolic score (p=0.07), we also performed all analyses in boy and girls separately (Table 2.3.2). These stratified analyses revealed that higher protein intake was associated with a higher BF% and higher insulin concentrations in girls, but not in boys. In contrast, higher protein intake was associated with a lower TAG concentration in boys, but not in girls. The association between protein intake and DBP was slightly stronger in boys than in girls, but in both groups effect estimates were similar to those observed in the whole population. Finally, protein intake was not associated with the cardiometabolic score in girls, whereas in boys, higher intake was associated with a lower cardiometabolic risk factor score (-0.56 SD (95% CI -0.92, -0.20) per 10g/d of total protein intake).

Additional adjustment for BMI at 1 year of age (data not shown) or BF% at 6 years of age (Supplement 2.3.2) did not change the results. Associations of protein intake with C-peptide concentrations were similar to those observed with insulin concentrations, and no associations were observed with total or LDL cholesterol (Supplement 2.3.3). Results of crude models were similar to those from the adjusted models, with similar differences between boys and girls (Supplement 2.3.4).

Additional analyses

The association between protein intake and BF% in girls was mainly driven by animal protein intake, whereas effect estimates for other outcomes were similar for animal and vegetable protein intake (Supplement 2.3.5). Associations between protein intake and cardiometabolic outcomes did not significantly differ by the children's ethnicity, weight status at the age of 6 years, or birthweight Z-score. Adjusting the models for total carbohydrate or different fatty acids instead of total fat intake rendered similar results for protein intake (data not shown). Sensitivity analyses in Dutch children only (n=1,965) showed similar effect estimates as compared to the whole group, except for DBP for which the effect estimates were slightly larger in Dutch children (Online Supplement). Sensitivity analyses restricted to children with blood sample available rendered similar effect estimates for body fat and blood pressure (data not shown). Analyses in which we excluded one component from the cardiometabolic score at a time revealed similar associations. In the subgroup of children with dietary data at age 2 years (n=714), protein intake at the age of 2 years was no longer associated with BF% or insulin levels at the age of 6 years in girls, whereas in boys it was associated with a lower BF% (Online Supplement). Associations between protein intake at age 2 years and other cardiometabolic outcomes were similar to those observed for protein intake at age 1 year, but with wider confidence intervals.

Table 2.3.2. Covariate-adjusted associations of total protein intake at the age of 1 year with cardiometabolic outcomes at the age of 6 years

	BF% (SDS)	Insulin (SDS)	SBP (SDS)	DBP (SDS)	HDL-C (SDS)	Triacylglycerol (SDS)	Cardiometabolic risk factor score
Whole group	*n*=2,911	*n*=1,996	*n*=2,841	*n*=2,841	*n*=2,006	*n*=2,001	*n*=1,894
Protein intake† per 10 g/d	***0.04*** ***(0.00, 0.08)***	*0.02* *(-0.03, 0.08)*	*0.00* *(-0.05, 0.05)*	*-0.04* *(-0.09, 0.01)*	*0.03* *(-0.03, 0.09)*	***-0.07*** ***(-0.13, -0.01)***	*-0.09* *(-0.24, 0.05)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.03 (-0.04, 0.11)	0.04 (-0.07, 0.15)	-0.08 (-0.17, 0.01)	-0.02 (-0.12, 0.06)	0.05 (-0.05, 0.16)	-0.08 (-0.19, 0.03)	-0.12 (-0.39, 0.15)
Tertile 3	**0.08** **(0.01, 0.16)**	0.01 (-0.10, 0.12)	-0.05 (-0.13, 0.04)	**-0.09** **(-0.18, -0.00)**	0.05 (-0.06, 0.16)	**-0.14** **(-0.24, -0.03)**	-0.17 (-0.44, 0.10)
p for trend*	**0.03**	0.22	0.32	**0.04**	0.35	**0.01**	0.21
Girls	*n*=1,487	*n*=980	*n*=1,426	*n*=1,457	*n*=1,457	*n*=984	*n*=982
Protein intake† per 10 g/d	***0.07*** ***(0.02, 0.13)***	***0.10*** ***(0.01, 0.19)***	*0.00* *(-0.07, 0.07)*	*-0.03* *(-0.10, 0.04)*	*0.00* *(-0.09, 0.09)*	*-0.01* *(-0.10, 0.08)*	*0.05* *(-0.17, 0.27)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.10 (-0.01, 0.20)	0.05 (-0.06, 0.25)	-0.05 (-0.17, 0.07)	0.03 (-0.09, 0.15)	0.02 (-0.13, 0.17)	0.00 (-0.15, 0.15)	0.10 (-0.27 0.48
Tertile 3	**0.11** **(0.01, 0.21)**	**0.15** **(0.00, 0.31)**	-0.09 (-0.22, 0.03)	-0.10 (-0.22, 0.03)	0.02 (-0.14, 0.17)	-0.02 (-0.17, 0.14)	0.04 (-0.35, 0.43)
p for trend*	**0.03**	**<0.05**	0.14	0.20	0.83	0.85	0.84
Boys	*n*=1,422	*n*=1,016	*n*=1,381	*n*=1,384	*n*=1,384	*n*=1,017	*n*=1,013
Protein intake† per 10 g/d	*0.01* *(-0.05, 0.07)*	*-0.05* *(-0.13, 0.03)*	*-0.01* *(-0.08, 0.06)*	*-0.04* *(-0.11, 0.03)*	*0.05* *(-0.03, 0.13)*	***-0.12*** ***(-0.20, -0.04)***	***-0.22*** ***(-0.42, 0.02)***
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	-0.05 (-0.16, 0.06)	0.03 (-0.13, 0.17)	0.11 (-0.23, 0.01)	-0.08 (-0.21, 0.05)	0.06 (-0.10, 0.21)	**-0.16** **(-0.31, -0.01)**	-0.36 (-0.74, 0.03)
Tertile 3	0.04 (-0.07, 0.14)	-0.11 (-0.26, 0.03)	-0.03 (-0.15, 0.10)	**-0.12** **(-0.23, 0.00)**	0.11 (-0.04, 0.27)	**-0.25** **(-0.40, -0.10)**	**-0.40** **(-0.77, -0.02)**
p for trend*	0.50	0.11	0.68	0.06	0.14	**<0.01**	**0.02**

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex-adjusted SDS) and in cardiometabolic risk factor score per 10 g/d higher protein intake, and for tertiles of protein intake, as compared to the lowest tertile.

P-values for interaction between total protein intake and child sex were: 0.05 for BF%; 0.01 for insulin; 0.79 for SBP; 0.58 for DBP; 0.08 for HDL-C; <0.01 for triacylglycerol; and 0.07 for the cardiometabolic score.

Models are adjusted for maternal age, BMI, education, and smoking during pregnancy; and child's ethnicity, birthweight Z-score, breastfeeding in the first four months of life, age at dietary measurement, total energy intake, energy-adjusted fat intake, height SDS at 6 years, and participation in sports and screen time at 6 years. Significant effect estimates are indicated in **bold**.

† Protein intakes are energy-adjusted with the residual method. Tertiles are computed based on the total population for analysis (*n*=2,965), the distribution was similar in boys and girls. Mean protein intake in the tertiles was 34.5 g/d, 41.7 g/d, and 50.2 g/d.

* Tests for trend were conducted using the tertiles of protein intake as a continuous ordinal variable.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; SDS, standard deviation score.

DISCUSSION

This large prospective population-based study suggests that protein intake at the age of 1 year is associated with cardiometabolic health at school age, but that these associations differ by sex. A higher protein intake was associated with a higher BF% and higher insulin concentrations in girls; and with lower TAG concentrations, lower DBP, and a lower cardiometabolic risk factor score in boys. Protein intake was not consistently associated with SBP or HDL cholesterol concentrations at the age of 6 years. The associations with BP, insulin, and TAG were independent of BF%. Although the effect estimates were small and may not have direct consequences on an individual level, they remained statistically significant after adjustment for several confounders and may be relevant on a population level in predicting later cardiometabolic disease risk.[2-3]

Interpretation and comparison with previous studies

The association between protein intake in early life and later obesity is in line with results from a large European trial in 1,090 infants. In this study, children who received high-protein formula in infancy had a higher BMI at the age of 6 years than children who received lower-protein formula.[12] However, only a few previous studies examined associations between protein intake in the first years of life and later measures of body fat. Two observational studies reported no association between early life protein intake with BF% at the ages of 4 or 10 years,[35-36] whereas two other studies reported positive associations with skinfold thicknesses at 7 or 8 years.[9-10]

Nevertheless, none of these studies reported differences between boys and girls. The observed differences between boys and girls in our study might be explained by a difference in timing of adiposity rebound (AR) or in peak BMI at AR. The AR corresponds to a rise in BMI curve followed by a rise in fat mass index, that occurs between the age of 5 and 7 years[37-38] and tends to occur earlier in girls than in boys.[39] A previous study reported that higher protein intake in early childhood is associated with an earlier AR,[10] whereas in other studies no consistent relation was observed with timing of AR.[40-41] One of these latter studies however did observe that a higher protein intake in early childhood was associated with a higher BMI at AR in girls, but not in boys.[41]

Another potential mechanism for the observed differences between boys and girls is a difference in endocrine response to high protein intake.[42] High protein intake has been associated with increased IGF-1 secretion, which may mediate the relation between protein intake and obesity.[42] In the previously stated European trial with high- and low-protein infant formulas, although no sex differences were observed in the association between the intervention and growth,[12] girls had a stronger IGF-1 response to the higher protein formula than boys.[42] In line with these findings, we observed clear sex differences in the association between protein intake and insulin levels, which also acts as a growth hormone.[42] Protein intake was strongly associated with a higher insulin concentration in girls, but not at all in boys. When we adjusted the BF% models in girls for insulin concentrations, the association between protein intake and BF% was attenuated, which suggests that insulin might mediate the association with BF%. Studies in adults show that dietary proteins stimulate the secretion of insulin,[43] and high protein intake has been related to an increased risk of type 2 diabetes.[44] However, previous studies among children did not report effects of high protein intake on measures of insulin sensitivity (reviewed in Chapter 2.1).[17, 45-46]

We observed that higher protein intake was associated with a lower DBP, but not consistently with SBP. A previous study reported inverse associations between protein intake and both SBP and DBP in 2.5-year-old children,[47] whereas two other studies observed no associations.[48-49] In line with our findings, a large observational study in 1605 adolescents (12.5 to 17.5 years), showed an inverse association between protein intake and DBP, but not SBP in boys.[50] Several meta-analyses of studies in adults also report inverse associations between protein intake and blood pressure.[5-7] The mechanisms underlying a beneficial effect of protein intake on blood pressure have not yet been clarified, and may differ for intake of different amino acids.[50] Proposed pathways of a blood pressure-lowering effect of protein include increased synthesis of ion channels; increased renal plasma flow, increased glomerular filtration rate; or vasodilating effects of certain amino acids.[5, 7] However, as described in Chapter 2.4, in our population protein intake in early childhood is not associated with glomerular filtration rate.[51]

The present study shows that protein intake was not associated with concentrations of total, HDL or LDL cholesterol, but we did observe lower TAG concentrations in relation to higher protein intake in boys. These latter findings correspond to results from a previous study in adolescents, in which higher absolute protein intake was associated with lower TAG and cholesterol concentrations.[46] However, in this study the association was no longer significant after adjustment for total energy intake. Other previous studies among children showed no association between protein intake and cholesterol or TAG levels (reviewed in Chapter 2.1[17]). However, in line with our results, a meta-analysis of trials in adults showed that subjects consuming a higher protein diet had a lower TAG concentration than subjects with a lower protein diet.[6] Potential mechanisms of a direct effect of protein intake on lipid profile are unknown. A high-carbohydrate diet, which could correspond to a low-protein diet, has been shown to reduce triglyceride clearance and thereby increase serum concentrations of TAG.[52] In our analyses, however, effect estimates for protein intake were similar after adjusting for either carbohydrate or fat intake.

In our study we observed differences in animal versus vegetable protein intake on the association with body fat, but not on the other cardiometabolic outcomes. In one previous study it was also reported that animal, and more specifically, dairy protein, but not meat or cereal protein intake at the age of 1 year was associated with child body fat at the age of 7 years.[16] Animal and vegetable protein might have different effects on adiposity via differences in IGF-1: another study reported that intake of animal, but not vegetable protein in 2.5-year-old children was associated with higher IGF-1 concentrations.[15] Studies in adults report inconsistent results for differences in effects of animal and vegetable protein on cardiometabolic diseases.[44, 53] Further studies, both in adult and child populations, are needed to elucidate the effects of animal and vegetable protein on cardiometabolic health.

Methodological considerations

Important strengths of this study are its prospective population-based design and the large number of subjects being studied. Of all mothers who received the FFQ, 72% returned the questionnaire. These mothers were generally higher educated and had a more healthy lifestyle than mothers who did not return the FFQ.[23] Of all children of whom information was available on food intake, more

than 80% participated in the follow-up measurements at the age of 6 years. Blood samples were available in 67% of these children, who had on average higher educated mothers than children without blood samples, but were not different with regard to protein intake, body fat or blood pressure (Supplement 2.3.1). Furthermore, associations between protein intake and body fat or blood pressure were not different among the children with blood samples available than among the whole group.

The FFQ was sent to Dutch-speaking mothers only, but with different ethnic backgrounds. A limitation of our FFQ is that it was constructed for Dutch children, whereas our study population is multiethnic. However, sensitivity analyses restricted to children with a Dutch ethnic background revealed similar results, suggesting that in our analyses no large bias due to ethnicity was present. Strengths of our dietary assessment are that an FFQ measures habitual diet rather than dietary intake at just one or a few days, and that we calculated not only total protein intake, but also broken down in protein from animal and vegetable sources. A limitation is that we did not have dietary data around the age 6 years and therefore could not study whether the associations of early diet were independent of current diet. Because we adjusted our models for total energy and fat intake, the effect estimates can be interpreted as the effect of exchanging carbohydrate for a similar number of calories from protein. Adjusting the models for other macronutrients rendered similar results, which suggests that for cardiometabolic outcomes it does not matter whether fat or carbohydrate is exchanged for protein in the diets of young children.

A strength of our study is that we had information on many potential parental and child confounders for which we adjusted in our analyses. However, because of the observational design of our study, residual confounding of other lifestyle-related variables, such as physical activity, may still be present. Finally, we performed detailed measurements of childhood adiposity and cardiometabolic health. Because we evaluated multiple outcomes, this might have increased the risk of chance findings (type I errors) due to multiple testing. Nevertheless, because the cardiometabolic outcomes considered are correlated, we did not adjust for multiple comparisons. In addition, we combined the individual risk factors in a continuous cardiometabolic score. Advantages over a dichotomous metabolic syndrome definition are that a continuous score is less prone to error and more sensitive to detect differences because more information is used.[24]

Conclusions

In this prospective cohort of young children with high protein intake, we observed differences in associations of protein intake in early childhood with cardiometabolic outcomes at school age between boys and girls. A higher protein intake at the age of 1 year was associated with higher insulin levels and a higher body fat percentage at 6 years of age in girls and with lower triacylglycerol levels and a lower cardiometabolic risk factor score in boys. In both sexes, protein intake tended to be associated with a lower diastolic blood pressure. Further studies are needed to explore whether and how protein intake differently affects cardiometabolic health in boys and girls, and to investigate whether the observed changes in cardiometabolic outcomes in childhood persist into adulthood.

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SUPPLEMENT CHAPTER 2.3

Additional Supplemental Material for this chapter can be found online: http://link.springer.com/article/10.1007%2Fs00394-015-1026-7

Supplement 2.3.1. Characteristics of subjects with and without child blood samples

	With blood samples (*n*=2,006)	Without blood samples (*n*=959)
Maternal characteristics		
Maternal age (y)	31.9 (22.1-39.9)	31.7 (21.0-39.6)
Maternal BMI at enrollment (kg/m^2)	23.4 (18.9-35.2)	23.6 (18.8-36.0)
Educational level (%)		
Primary	5.6	6.2
Secondary	35.0	36.7
Higher	59.4	57.1
Smoking during pregnancy (%)		
Never	78.5	78.3
Until pregnancy was known	10.0	9.7
Continued	11.5	12.0
Child characteristics		
Girls (%)	48.8	55.2
Dutch ethnicity (%)	69.2	68.1
Gestational age at birth (wk)	39.9 (1.7)	40.0 (1.8)
Birthweight (g)	3482 (550)	3453 (552)
Breastfeeding in the first 4 months (%)		
Exclusive	29.2	27.7
Partial	61.7	61.6
Never	9.1	10.7
Child characteristics at dietary measurement		
Age at FFQ (mo)	12.9 (12.2-19.0)	12.9 (12.2-18.8)
Total energy intake (kcal/d)	1283 (664-2164)	1245 (691-2279)
Protein intake (g/d)		
Total protein	41.4 (12.7)	40.8 (13.1)
Animal protein	25.9 (10.1)	25.3 (10.6)
Vegetable protein	15.0 (5.6)	14.9 (5.8)
Child characteristics at 6-year visit		
Age (y)	5.9 (5.7-6.6)	5.9 (5.6-6.5)
Screen time (h/d)	1.3 (0.2-4.4)	1.3 (0.2-4.2)
Participation in sports (%)	43.9	43.0
Height (cm)	118.4 (5.1)	117.8 (5.3)
Weight (kg)	22.5 (3.3)	22.3 (3.5)
BMI (kg/m^2)	16.0 (1.6)	16.0 (1.7)
Body fat percentage (%)	23.3 (16.2-36.1)	24.1 (16.3-37.5)
Systolic blood pressure (mmHg)	102 (8)	103 (8)
Diastolic blood pressure (mmHg)	60 (6)	61 (7)

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

Abbreviations: BMI, body mass index; FFQ, food-frequency questionnaire.

Supplement 2.3.2 Covariate-adjusted associations of total protein intake at the age of 1 year with cardiometabolic outcomes at age 6 years, additionally adjusted for body fat percentage

	Insulin (SDS)	SBP (SDS)	DBP (SDS)	HDL-C (SDS)	TAG (SDS)	Cardiometabolic risk factor score
Whole group	*n*=1,996	*n*=2,841	*n*=2,841	*n*=2,006	*n*=2,001	*n*=1,894
Per 10 g/d	*0.02 (-0.04, 0.08)*	*-0.01 (-0.06, 0.04)*	*-0.04 (-0.09, 0.00)*	*0.03 (-0.03, 0.08)*	***-0.07 (-0.13, -0.01)***	*-0.12 (-0.25, 0.01)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.04 (-0.07, 0.15)	-0.08 (-0.17, 0.01)	-0.03 (-0.11, 0.06)	0.05 (-0.05, 0.16)	0.08 (-0.19, 0.03)	-0.13 (-0.37, 0.11)
Tertile 3	0.01 (-0.10, 0.11)	-0.06 (-0.15, 0.03)	**-0.10 (-0.18, -0.01)**	0.05 (-0.05, 0.16)	**-0.14 (-0.25, -0.03)**	**-0.25 (-0.50, -0.01)**
p for trend*	0.91	0.22	**0.03**	0.33	**0.01**	**0.04**
Girls	*n*=980	*n*=1,426	*n*=1,457	*n*=1,457	*n*=984	*n*=982
Per 10 g/d	***0.10 (0.01, 0.19)***	*-0.01 (-0.08, 0.07)*	*-0.04 (-0.11, 0.03)*	*0.01 (-0.07, 0.09)*	*-0.01 (-0.10, 0.08)*	0.02 (-0.17, 0.23)
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.09 (-0.06, 0.24)	-0.05 (-0.17, 0.07)	0.02 (-0.10, 0.14)	0.02 (-0.13, 0.17)	0.02 (-0.14, 0.17)	0.03 (-0.33 0.39)
Tertile 3	0.13 (-0.02, 0.29)	-0.08 (-0.21, 0.04)	-0.08 (-0.20, 0.05)	0.02 (-0.14, 0.17)	0.02 (-0.14, 0.17)	0.03 (-0.34, 0.40)
p for trend*	0.10	0.19	0.23	0.99	0.88	0.99
Boys	*n*=1,422	*n*=1,016	*n*=1,381	*n*=1,384	*n*=1,384	*n*=1,017
Per 10 g/d	*-0.05 (-0.13, 0.03)*	*-0.01 (-0.08, 0.06)*	*-0.05 (-0.12, 0.01)*	*0.06 (-0.01, 0.14)*	***-0.12 (-0.21, -0.04)***	***-0.24 (-0.42, -0.05)***
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.05 (-0.10, 0.21)	-0.12 (-0.24, 0.01)	-0.06 (-0.19, 0.07)	0.06 (-0.10, 0.21)	-0.08 (-0.23, 0.08)	-0.22 (-0.58, 0.14)
Tertile 3	-0.12 (-0.27, 0.04)	-0.03 (-0.16, 0.09)	**-0.13 (-0.25, 0.00)**	0.11 (-0.04, 0.27)	**-0.26 (-0.41, -0.11)**	**-0.57 (-0.92, -0.21)**
p for trend*	0.13	0.60	**0.05**	0.16	**<0.01**	**<0.01**

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age-and sex-specific SD-scores) and in cardiometabolic score per 10 g/d higher protein intake, and for tertiles of protein intake, as compared to the lowest tertile. Statistically significant effect estimates are indicated in **bold**.

Protein intakes are energy-adjusted with the residual method. Tertiles are computed based on the total population for analysis (*n*=2,965).

Models are adjusted for maternal age, BMI, education, and smoking during pregnancy; and child's ethnicity, birthweight Z-score, breastfeeding in the first four months of life, age at dietary measurement, energy intake, fat intake, height-for-age at 6 years, participation in sports at 6 years, and screen time at 6 years.

* Tests for trend were conducted using the tertiles of protein intake as a continuous ordinal variable.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; SDS, standard deviation score; TAG, triacylglycerol.

Supplement 2.3.3 Covariate-adjusted associations of total protein intake at the age of 1 year with C-peptide, total cholesterol and LDL cholesterol concentrations at age 6 years

	C-peptide (SDS)	**Total cholesterol (SDS)**	**LDL cholesterol (SDS)**
Whole group	*n*=1,996	*n*=2,006	*n*=2,006
Per 10 g/d	*0.04 (-0.02, 0.9)*	*-0.01 (-0.07, 0.05)*	*-0.01 (-0.07, 0.05)*
Tertile 1	Reference	Reference	Reference
Tertile 2	0.00 (-0.11, 0.11)	0.07 (-0.04, 0.17)	0.04 (-0.07, 0.15)
Tertile 3	0.00 (-0.11, 0.11)	0.05 (-0.06, 0.16)	0.05 (-0.05, 0.16)
p for trend	0.97	0.37	0.33
Girls	*n*=980	*n*=1,457	*n*=1,457
Per 10 g/d	***0.10 (0.02, 0.18)***	*0.01 (-0.07, 0.08)*	*-0.01 (-0.09, 0.07)*
Tertile 1	Reference	Reference	Reference
Tertile 2	0.06 (-0.09, 0.22)	0.09 (-0.06, 0.24)	0.07 (-0.08, 0.22)
Tertile 3	0.10 (-0.05, 0.26)	0.03 (-0.12, 0.18)	0.00 (-0.15, 0.15)
p for trend	0.08	0.68	0.98
Boys	*n*=1,016	*n*=1,384	*n*=1,384
Per 10 g/d	*-0.02 (-0.09, 0.06)*	*-0.01 (-0.08, 0.07)*	*-0.03 (-0.10, 0.05)*
Tertile 1	Reference	Reference	Reference
Tertile 2	-0.07 (-0.22, 0.08)	-0.06 (-0.21, 0.09)	-0.09 (-0.24, 0.07)
Tertile 3	-0.12 (-0.27, 0.03)	0.02 (-0.14, 0.17)	0.01 (-0.15, 0.16)
p for trend	0.20	0.83	0.92

Values are based on multivariable linear regression models and reflect differences (95% CI) in C-peptide and cholesterol concentrations (age- and sex-specific SD-scores) per 10 g/d higher protein intake, and for tertiles of protein intake, as compared to the lowest tertile. Statistically significant effect estimates are indicated in **bold**.

Protein intakes are energy-adjusted with the residual method. Tertiles are computed based on the total population for analysis (*n*=2,965).

Models are adjusted for maternal age, BMI, education, and smoking during pregnancy; and child's ethnicity, birthweight Z-score, breastfeeding in the first four months of life, age at dietary measurement, energy intake, fat intake, height-for-age at 6 years, weight-for-age at 6 years, participation in sports at 6 years, and screen time at 6 years.

Abbreviations: SDS, standard deviation score; LDL, low-density cholesterol.

Supplement 2.3.4 Crude associations of total protein intake at the age of 1 year with cardiometabolic outcomes at 6 years of age

	BF% (SDS)	Insulin (SDS)	SBP (SDS)	DBP (SDS)	HDL-C (SDS)	TAG (SDS)	Cardiometabolic risk factor score
Whole group	*n*=2,909	*n*=1,996	*n*=2,841	*n*=2,841	*n*=2,006	*n*=2,001	*n*=1,894
Per 10 g/d	***0.06 (0.02, 0.11)***	*0.03 (-0.02, 0.08)*	*0.01 (-0.04, 0.06)*	*-0.03 (-0.08, 0.02)*	*0.02 (-0.04, 0.08)*	***-0.07 (-0.13, -0.01)***	-0.05 (-0.20, 0.10)
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.03 (-0.05, 0.11)	0.05 (-0.06, 0.16)	-0.07 (-0.16, 0.02)	-0.03 (-0.11, 0.06)	0.05 (-0.06, 0.16)	-0.08 (-0.18, 0.03)	-0.12 (-0.40, 0.15)
Tertile 3	**0.11 (0.03, 0.19)**	0.03 (-0.08, 0.13)	-0.02 (-0.11, 0.07)	**-0.08 (-0.16, 0.00)**	0.04 (-0.07, 0.14)	**-0.14 (-0.25, -0.04)**	-0.09 (-0.37, 0.18)
p for trend	**<0.01**	0.69	0.70	0.11	0.49	**<0.01**	0.51
Girls	*n*=1,487	*n*=980	*n*=1,426	*n*=1,457	*n*=1,457	*n*=984	*n*=982
Per 10 g/d	***0.09 (0.03, 0.15)***	***0.11 (0.02, 0.20)***	*0.02 (-0.05, 0.09)*	*-0.03 (-0.10, 0.04)*	*-0.01 (-0.10,0.08)*	*-0.02 (-0.11, 0.07)*	*0.09 (-0.14, 0.32)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.08 (-0.03, 0.19)	0.10 (-0.05, 0.26)	-0.05 (-0.17, 0.07)	0.00 (-0.12, 0.11)	0.02 (-0.14, 0.16)	0.02 (-0.13, 0.18)	0.07 (-0.32, 0.46)
Tertile 3	**0.12 (0.01, 0.23)**	**0.16 (0.00, 0.31)**	-0.06 (-0.19, 0.07)	-0.08 (-0.20, 0.04)	-0.01 (-0.16, 0.15)	0.00 (-0.16, 0.15)	0.13 (-0.27, 0.54)
p for trend	**0.03**	**0.04**	0.35	0.22	0.94	0.99	0.52
Boys	*n*=1,422	*n*=1,016	*n*=1,381	*n*=1,384	*n*=1,384	*n*=1,017	*n*=1,013
Per 10 g/d	*0.04 (-0.03, 0.10)*	*-0.03 (-0.11, 0.05)*	*0.01 (-0.06, 0.08)*	*-0.03 (-0.10, 0.04)*	*0.05 (-0.03, 0.13)*	***-0.12 (-0.20, -0.04)***	*-0.15 (-0.35, 0.05)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	-0.03 (-0.15, 0.09)	0.08 (-0.07, 0.23)	-0.09 (-0.21, 0.04)	-0.05 (-0.18, 0.08)	0.04 (-0.11, 0.20)	-0.08 (-0.23, 0.07)	-0.15 (-0.54, 0.24)
Tertile 3	0.04 (-0.06, 0.17)	-0.09 (-0.24, 0.06)	0.03 (-0.10, 0.15)	-0.09 (-0.21, 0.04)	0.08 (-0.07, 0.23)	**-0.24 (-0.39, -0.09)**	-0.35 (-0.73, 0.02)
p for trend	0.38	0.21	0.68	0.19	0.29	**<0.01**	0.07

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex-adjusted SD-scores) and in cardiometabolic score per 10 g/d higher protein intake, and for tertiles of protein intake, as compared to the lowest tertile. Statistically significant effect estimates are indicated in **bold**.

Protein intakes are energy-adjusted with the residual method. Tertiles are computed based on the total population for analysis (*n*=2,965).

Models are adjusted for energy and fat intake.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; SDS, standard deviation score.

Supplement 2.3.5 Covariate-adjusted associations of animal and vegetable protein intake at the age of 1 year with cardiometabolic outcomes at age 6 years

	BF% (SDS)	Insulin (SDS)	SBP (SDS)	DBP (SDS)	HDL-C (SDS)	TAG (SDS)	Cardiometabolic risk factor score
Girls	*n*=1,487	*n*=980	*n*=1,426	*n*=1,457	*n*=1,457	*n*=984	*n*=982
Animal protein intake (g)							
Per 10 g/d	***0.07 (0.01, 0.13)***	***0.09 (0.00, 0.17)***	*-0.01 (-0.08, 0.05)*	*-0.02 (-0.08, 0.05)*	*-0.04 (-0.12, 0.04)*	*0.02 (-0.06, 0.11)*	*0.09 (-0.11, 0.30)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.02 (-0.08, 0.12)	-0.04 (-0.19, 0.11)	-0.08 (-0.20, 0.05)	-0.02 (-0.14, 0.10)	0.08 (-0.07, 0.23)	0.03 (-0.12, 0.18)	-0.21 (-0.60, 0.18)
Tertile 3	**0.10 (0.00, 0.21)**	**0.14 (0.00, 0.29)**	-0.01 (-0.14, 0.12)	-0.05 (-0.17, 0.08)	0.01 (-0.15, 0.16)	0.06 (-0.11, 0.22)	0.13 (-0.27, 0.54)
p for trend*	0.06	0.09	0.82	0.48	0.94	0.52	0.52
Vegetable protein intake (g)							
Per 10 g/d	*0.03 (-0.07, 0.13)*	***0.14 (0.00, 0.28)***	*-0.01 (-0.13, 0.11)*	*-0.04 (-0.15, 0.08)*	*0.12 (-0.03, 0.27)*	*-0.09 (-0.24, 0.06)*	*-0.20 (-0.58, 0.17)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	-0.02 (-0.13, 0.08)	0.14 (-0.01, 0.29)	-0.07 (-0.19, 0.05)	0.04 (-0.08, 0.16)	0.06 (-0.09, 0.21)	-0.08 (-0.24, 0.07)	-0.04 (-0.43, 0.35)
Tertile 3	-0.02 (-0.13, 0.09)	**0.16 (0.00, 0.32)**	-0.02 (-0.15, 0.11)	-0.05 (-0.18, 0.08)	0.08 (-0.08, 0.24)	-0.05 (-0.21, 0.11)	-0.12 (-0.53, 0.29)
p for trend*	0.72	**0.05**	0.73	0.50	0.35	0.53	0.58
Boys	*n*=1,422	*n*=1,016	*n*=1,381	*n*=1,384	*n*=1,384	*n*=1,017	*n*=1,013
Animal protein intake (g)							
Per 10 g/d	*-0.01 (-0.06, 0.05)*	*-0.03 (-0.10, 0.05)*	*-0.02 (-0.08, 0.05)*	*-0.05 (-0.11, 0.01)*	*0.07 (-0.01, 0.14)*	***-0.11 (-0.18, -0.03)***	***-0.23 (-0.42, -0.04)***
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	-0.06 (-0.17, 0.05)	0.03 (-0.13, 0.18)	-0.02 (-0.14, 0.10)	-0.05 (-0.17, 0.08)	-0.01 (-0.17, 0.14)	-0.10 (-0.26, 0.05)	-0.23 (-0.62, 0.54)
Tertile 3	0.00 (-0.11, 0.11)	-0.04 (-0.19, 0.11)	0.00 (-0.12, 0.12)	-0.07 (-0.20, 0.06)	0.10 (-0.05, 0.26)	**-0.20 (-0.35, -0.05)**	-0.34 (-0.72, 0.04)
p for trend*	0.99	0.57	0.94	0.33	0.19	**<0.01**	0.08
Vegetable protein intake (g)							
Per 10 g/d	*-0.01 (-0.12, 0.10)*	*-0.05 (-0.21, 0.11)*	*0.02 (-0.10, 0.15)*	*-0.06 (-0.19, 0.07)*	*0.09 (-0.07, 0.25)*	*-0.07 (-0.22, 0.09)*	*-0.15 (-0.54, 0.24)*
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Tertile 2	-0.01 (-0.13, 0.10)	-0.05 (-0.21, 0.11)	0.10 (-0.02, 0.23)	**0.15 (0.01, 0.28)**	-0.05 (-0.21, 0.11)	-0.08 (-0.24, 0.08)	0.09 (-0.30, 0.49)
Tertile 3	-0.01 (-0.13, 0.10)	-0.03 (-0.19, 0.13)	0.08 (-0.05, 0.20)	-0.01 (-0.14, 0.12)	0.02 (-0.15, 0.18)	-0.05 (-0.21, 0.11)	-0.02 (-0.41, 0.37)
p for trend*	0.88	0.73	0.26	0.74	0.78	0.57	0.89

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex-specific SD-scores) and in cardiometabolic score per 10 g/d higher protein intake, and for tertiles of protein intake, as compared to the lowest tertile. Statistically significant effect estimates are indicated in **bold**.

Protein intakes are energy-adjusted using the residual method. Tertiles are computed based on the total population for analysis (*n*=2,965).

Models are adjusted for maternal age, BMI, education, and smoking during pregnancy; and child's ethnicity, birthweight Z-score, breastfeeding in the first four months of life, age at dietary measurement, energy intake, fat intake, height-for-age at 6 years, participation in sports at 6 years, and screen time at 6 years. Models with animal protein intake are additionally adjusted for vegetable protein intake and vice versa.

*Tests for trend were conducted using the tertiles of protein intake as a continuous variable.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; SDS, standard deviation score.

Chapter 2.4

Protein intake in early childhood & kidney health

Manuscript based on this chapter:

Trudy Voortman, Hanneke Bakker, Sanaz Sedaghat, Jessica C. Kiefte-de Jong, Albert Hofman, Vincent W.V. Jaddoe, Oscar H. Franco, Edith H. van den Hooven. Protein intake in early childhood and kidney size and function at school age: the Generation R Study. *Pediatric Nephrology* 2015; 30:1825–33.

ABSTRACT

Background: High protein intake has been linked to kidney growth and function. Whether protein intake is related to kidney outcomes in healthy children is unclear.

Methods: We examined the associations of protein intake in infancy with kidney outcomes at the age of 6 years in 2,965 children participating in a population-based cohort study. Protein intake at the age of 1 year was assessed with a food-frequency questionnaire and was adjusted for total energy intake. At the children's age of 6 years we measured their kidney volume and urinary albumin/creatinine ratio (ACR), and we estimated glomerular filtration rate (eGFR) on the basis of serum creatinine concentrations and on the basis of serum cystatin C concentrations.

Results: In models adjusted for age, sex, body surface area, and sociodemographic factors, a higher protein intake was associated with a lower ACR and a higher eGFR, but not consistently with kidney volume. However, after further adjustment for children's other dietary and lifestyle factors, such as sodium intake, diet quality, and television watching, higher protein intake was no longer associated with kidney function. No differences in associations were observed between animal and vegetable protein intake.

Conclusion: Protein intake in early childhood is not independently associated with kidney size or function at the age of 6 years. Further study is needed on other early life predictors of later kidney size and function.

INTRODUCTION

Kidney function has been shown to track from childhood into adulthood.[1] Subclinical variations in kidney function are already present in childhood and have been shown to relate to kidney disease in later life.[2] This implicates that it is important to study determinants of kidney function already in children. We have recently observed that reduced infant weight growth is associated with smaller kidney volume in childhood,[3] and that longer breastfeeding duration is associated with larger kidney volume and an increased estimated glomerular filtration rate (eGFR).[4] These observations suggest that exposures in infancy may influence later kidney development.

Dietary protein intake during infancy is a key factor for growth and development and may be associated to kidney growth and function.[5] In animal studies, higher protein intake leads to increased kidney growth and function,[6-8] and early postnatal dietary protein affects kidney function.[8-9] Also in healthy adults, a higher protein intake has been associated with increased GFR.[10-12] In patients with chronic kidney disease, high protein intake may further decline kidney function, because the kidneys can no longer handle the excretion of protein metabolites.[13-16] However, randomized controlled trials with low-protein diets in adults or children with renal disease have not consistently been able to slow progression of kidney disease.[17-19]

Not much is known on the effects of protein intake on kidney function in children with a normal kidney function. Trials suggest that infants who receive additional dietary protein have larger kidneys[5] and a higher eGFR[20] in infancy than those who received no additional protein. Whether protein intake in infancy is associated with kidney size and function in later childhood is unknown. Therefore, we examined the associations between protein intake at the age of 1 year and kidney outcomes at the age of 6 years in 2,965 children participating in a population-based prospective cohort. Kidney measures included combined kidney volume, creatinine-based eGFR ($eGFR_{Creat}$), cystatin C-based eGFR ($eGFR_{CysC}$), and urinary albumin/creatinine ratio. In addition, we examined the association between protein intake at the age of 2 years with kidney outcomes at the age of 6 years in a subgroup of the children; and we evaluated whether the associations between protein intake and kidney health differed by protein source, child sex, birthweight, gestational age at birth, kidney volume, or ethnicity.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[21] All children were born between April 2002 and January 2006. The study was conducted in accordance with the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained for all children. A total of 7,893 children were available for follow-up studies in early childhood.[21] A questionnaire on child diet around the age of 1 year was sent to 5,088 mothers who provided consent for follow-up and had sufficient mastery of the Dutch language. In total 3,650 (72%) of these mothers returned

the questionnaire.[22] After exclusion of subjects with invalid dietary data and withdrawn consent, information on infant diet was available for 3,629 children. From these 3,629 children, 2,985 visited the research center around the age of 6 years (Chapter 2.2, Figure 2.2.1), of whom 11 did not have any kidney measurement and 8 were excluded because of congenital kidney abnormalities or an albumin-creatinine ratio >25 mg/mmol.[23] Of the final population for analysis of 2,965 children, 2,755 had data available on kidney volume, 2,868 on ACR, and 2,006 on eGFR.

Dietary assessment

Dietary intake was assessed at a median age of 12.9 months (95% range 12.2 to 19.0) with a semi-quantitative 211-item food-frequency questionnaire (FFQ), as described previously in detail.[22, 24] The FFQ was validated against three 24-h recalls in a representative sample of 32 Dutch children around the age of 1 year living in Rotterdam. The intraclass correlation coefficient was 0.7 for total protein intake (Chapter 5.1).[22] Mothers of a subgroup of 899 Dutch children received an additional FFQ at their child's median age of 24.9 months (95% range 24.3 to 27.6).[24] Of these children, 715 had kidney measures at the age of 6 years available.

Kidney outcome assessments

Children's kidney outcomes were assessed at a median age of 5.9 years (95% range 5.6 to 6.6) in a dedicated research center in the Sophia Children's Hospital in Rotterdam by well-trained staff.[23] Kidney volume was measured with ultrasound, with an ATL-Philips HDI 5000 instrument (Seattle, WA, USA), equipped with a 2.0-5.0 MHz curved array transducer, as described previously in detail.[23, 25] Kidney volume was calculated with the equation for a prolate ellipsoid: volume (cm^3) = 0.523 × length (cm) × width (cm) × depth (cm).[25] Combined kidney volume was calculated by summing right and left kidney volume. We previously reported good intra-observer and inter-observer correlation coefficients.[26]

Non-fasting blood samples were drawn by antecubital venipuncture. Creatinine concentrations were measured with enzymatic methods, and cystatin C concentrations were measured with a particle-enhanced immunoturbidimetric assay (with Cobas 8000 analyzers, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay coefficients of variation of 0.51% for creatinine and 1.65% for cystatin C, and inter-assay coefficients of 1.37% for creatinine and 1.13% for cystatin C.[23] Creatinine-based estimated glomerular filtration rate (eGFR) was calculated according to the revised Schwartz 2009 formula, the most common pediatric equation: $eGFR_{Creat}$ = 36.5 × (height (cm)/ creatinine (μmol/L)).[27] Additionally, we evaluated eGFR calculated with a cystatin C-based and a combined creatinine and cystatin C formula as proposed by Zappitelli in 2006: $eGFR_{CysC} = 75.94 / (\text{cystatin C (mg/L)}^{1.17})$ and $eGFR_{Combined} = 507.76 \times e^{0.003 \times \text{height (cm)}} / (\text{cystatin C (mg/L)}^{0.635} \times \text{creatinine (μmol/L)}^{0.547})$.[28]

Urinary creatinine (mmol/L) and albumin (mg/L) concentrations were measured with a Beckman Coulter AU analyzer, and creatinine concentrations were determined using the Jaffe reaction. We calculated the urinary albumin/creatinine ratio (ACR). In addition to the continuous ACR, we defined microalbuminuria as an ACR ≥ 2.5 mg/mmol for boys, and ≥ 3.5 mg/mmol for girls.[29]

Covariates

Information on maternal age, educational level, and folic acid supplement use was obtained with a questionnaire at enrollment in the study. Maternal height and weight were measured at the research center at enrollment and body mass index (BMI, kg/m^2) was calculated. Maternal smoking during pregnancy was assessed with questionnaires in each trimester and was categorized into never; until pregnancy was known; or continued during pregnancy. Information on child's sex, birthweight and gestational age was available from medical records and hospital registries. Sex- and gestational age-specific SD-scores for birthweight were calculated with the use of reference data.[30] Child's ethnicity was defined according to Statistics Netherlands[31] and classified into eight categories (Western, Cape Verdean, Moroccan, Netherlands Antillean, Turkish, Surinamese Creole, Surinamese Hindustani, and other non-Western).

Information on breastfeeding was obtained from delivery reports and postnatal questionnaires, and breastfeeding was categorized into never; partial in the first 4 months; or exclusively in the first 4 months of life.[22] Total energy, fat and sodium intake from foods were estimated with data from the FFQs, and were adjusted for energy intake with the residual method.[32] A diet score (details in Chapter 5.1) was used to quantify overall diet quality, with the use of data obtained with the FFQ.[24] Information on child's television watching around the age of 2 years was obtained with a questionnaire. At the child's age of 6 years, we measured height and weight at the research center and calculated BMI (kg/m^2) and body surface area (BSA) (with the Du Bois formula: BSA (cm^2) = weight (kg)$^{0.425}$ × height (cm)$^{0.725}$ × 0.007184).[33] Lean body mass was measured with whole body dual-energy X-ray absorptiometry scans (iDXA, GE-Lunar, 2008, Madison, WI, USA).

Statistical analyses

We were interested in the effect of protein independent of its energy content and therefore we adjusted protein intake for total energy intake with the residual method.[32] Briefly, we used the residuals of a linear regression model with energy intake as independent variable and protein intake as dependent variable. These residuals provide a measure of protein intake uncorrelated with total energy intake. To enhance interpretability, predicted protein intake for the mean energy intake (1311 kcal/d) was added to the residuals as a constant.[32] In line with recommendations for dietary exposures, protein intake was analyzed both as a continuous and as a categorical variable.[32] For the latter purpose we categorized protein intake into tertiles and used the lowest tertile as the reference category.

We used multivariable linear regression models to assess the associations of protein intake with combined kidney volume, eGFR, and ACR. We natural log-transformed ACR to obtain a normal distribution. For clinical interpretation, we also assessed the associations of protein intake with the risk of microalbuminuria, using multivariable logistic regression models. Model 1 was adjusted for child's sex, age and BSA at kidney measurement. Model 2 was further controlled for the following prenatal and sociodemographic factors: maternal age, educational level, BMI, smoking during pregnancy, folic acid supplement use, and for child's ethnicity and birthweight Z-score. The final model was additionally adjusted for childhood lifestyle factors: breastfeeding, children's television watching, total energy, fat and sodium intake, and diet quality score (model 3). Covariates were

included in the regression models based on previously shown associations with kidney outcomes[4, 23, 34] or a significant change (≥5%) in effect estimates. Because both protein intake and kidney volume are strongly related to body size[35] and because creatinine levels are associated with muscle mass,[36] we adjusted all models for BSA and we performed sensitivity analyses in which we replaced BSA by height and weight, by BMI, or by lean body mass. In addition, we examined the association between protein intake and the ratio of kidney volume with either body weight, BMI, or BSA.

To assess whether the associations were different by sex, ethnicity, birthweight, gestational age at birth, or kidney volume at age 6 years we evaluated statistical interactions by adding the product term of the covariate and protein intake to model 2. Stratified analyses were conducted in case the interaction term was significant (p<0.05). Because the FFQ was developed for Dutch children, we performed a sensitivity analysis including only children with a Dutch ethnic background. Furthermore, since kidney size and function are different in low birthweight children,[25] we performed a sensitivity analysis among children born with a normal birthweight (≥ 2500g) and among children born at term (≥ 37 weeks).

Missing values of covariates were multiple imputed (n=5 imputations) using the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern.[37] We present results as pooled effect estimates after the multiple imputation procedure. Statistical analyses were performed with SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Subject characteristics

Characteristics of the children and their mothers, stratified by tertiles of protein intake, are presented in Table 2.4.1. Mean (±SD) total protein intake at the age of 1 year was 41.2 g (±12.9), corresponding to 12.9% of total energy intake (E%). This is higher than recommended for this age group,[38] but similar to intakes observed in the general Dutch and other Western pediatric populations.[39-40] At the age of 6 years, mean (SD) combined kidney volume was 121 cm3 (±21) and mean eGFRCreat was 119 ml/min per 1.73m2 (±16). Mean eGFRCysC was lower at 102 ml/min per 1.73m2 (±13). Many children (34%) had urine albumin concentrations at or below the detection limit (≤ 2 mg/L) and microalbuminuria was present in 7.1% of the children.

Table 2.4.1 Subject characteristics

		Tertiles of energy-adjusted total protein intake at 1 year		
	All (n=2,965)	T1 (<37.5 g/d) (n=989)	T2 (37.5-43.9 g/d) (n=990)	T3 (>43.9 g/d) (n=989)
Maternal characteristics				
Maternal age (y)	31.5 (21.7-39.9)	31.8 (22.5-41.4)	31.9 (22.4-39.6)	31.6 (20.6-39.4)
Maternal BMI at enrollment (kg/m^2)	23.4 (18.7-35.2)	23.4 (18.8-34.7)	23.6 (18.9-35.6)	24.5 (18.5-37.1)
Nulliparous (%)	60.4	59.5	59.8	61.6
Educational level (%)				
Primary	3.5	3.7	3.5	3.2
Secondary	33.9	33.2	32.8	35.6
Higher	62.7	63.1	63.7	61.2

Table 2.4.1 (continued) Subject characteristics

	Tertiles of energy-adjusted total protein intake at 1 year			
	All (*n*=2,965)	T1 (<37.5 g/d) (*n*=989)	T2 (37.5-43.9 g/d) (*n*=990)	T3 (>43.9 g/d) (*n*=989)
Folic acid supplement use (%)				
Never	15.8	17.0	14.8	15.4
In the first 10 weeks of pregnancy	30.2	28.5	30.9	31.3
Periconceptional	54.0	54.4	54.3	53.2
Smoking during pregnancy (%)				
Never	78.1	78.7	78.6	77.0
Until pregnancy was known	10.0	9.2	9.4	11.5
Continued	11.8	12.1	11.9	11.5
Child characteristics				
Girls (%)	50.9	51.6	52.6	48.5
Ethnicity (%)				
Western	76.9	75.5	78.1	77.0
Cape Verdean	2.0	1.8	1.8	2.3
Moroccan	3.2	2.8	2.8	4.1
Netherlands Antillean	1.7	2.3	1.9	1.1
Turkish	4.5	4.5	4.0	4.9
Surinamese Creoles	2.3	3.0	2.5	1.3
Surinamese Hindustani	2.2	2.6	1.9	2.2
Other non-Western	7.1	7.5	6.9	7.0
Gestational age at birth (wk)	40.0 (1.7)	39.9 (1.9)	39.9 (1.7)	40.0 (1.6)
Birthweight (g)	3472 (551)	3462 (562)	3466 (557)	3489 (532)
Breastfeeding (%)				
Exclusive ≥ 4 months	29.5	34.8	26.9	27.2
Partial	62.5	58.1	65.5	63.7
Never	8.0	7.1	7.5	9.1
Child characteristics at dietary measurement				
Age at FFQ (mo)	12.9 (12.2-19.0)	12.8 (12.2-18.6)	12.9 (12.2-18.7)	13.0 (12.2-19.4)
Total energy intake (kcal/d)	1266 (678-2212)	1297 (619-2264)	1238 (650-2093)	1253 (765-2237)
Protein intake (g/d)				
Total protein	41.2 (12.9)	34.9 (10.8)	39.7 (10.1)	48.0 (12.1)
Animal protein	25.7 (10.3)	20.8 (8.7)	25.0 (8.1)	33.1 (9.5)
Vegetable protein	14.9 (5.7)	13.5 (5.4)	14.8 (5.2)	16.6 (5.9)
Total fat intake (g/d)	42.3 (17.5)	43.1 (18.8)	40.8 (16.0)	42.7 (16.7)
Sodium intake from foods (g/d)	1.02 (0.35)	0.88 (0.32)	0.98 (0.30)	1.17 (0.35)
Television watching (h/d)	0.9 (0.5)	0.9 (0.5)	0.9 (0.5)	0.9 (0.5)
Diet quality score	4.2 (1.3)	3.3 (1.1)	4.1 (1.1)	5.1 (1.2)
Child characteristics at 6-year visit				
Age (y)	5.9 (5.6-6.6)	5.9 (5.6-6.5)	5.9 (5.6-6.6)	5.9 (5.6-6.6)
Height (cm)	118.2 (5.2)	117.8 (4.9)	118.2 (5.4)	118.5 (5.2)
Weight (kg)	22.4 (3.4)	22.1 (3.1)	22.4 (3.6)	22.7 (3.5)
Body mass index (kg/m^2)	16.0 (1.6)	15.9 (1.5)	16.0 (1.6)	16.1 (1.7)
Body surface area (kg/m^2)	0.86 (0.08)	0.85 (0.07)	0.86 (0.08)	0.86 (0.08)
Combined kidney volume (cm^3)	121 (21)	119 (21)	122 (23)	122 (21)
Creatinine (µmol/L)	37.0 (5.2)	37.2 (5.0)	36.8 (5.4)	36.8 (5.2)
Cystatin C (mg/L)	0.79 (0.08)	0.79 (0.08)	0.78 (0.08)	0.78 (0.08)
$eGFR_{Creat}$ (Schwartz) (ml/min/1.73m^2)	119 (16)	118 (15)	120 (17)	120 (16)
$eGFR_{CysC}$ (Zappitelli) (ml/min/1.73m^2)	102 (13)	100 (13)	102 (14)	102 (13)
Urinary albumin/creatinine ratio	0.79 (0.20-5.70)	0.83 (0.20-7.33)	0.77 (0.190-5.56)	0.77 (0.20-5.00)
Microalbuminuria (%)	7.1	7.6	7.5	6.3

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Abbreviations: eGFR, estimated glomerular filtration rate.

Associations between protein intake and kidney outcomes

Table 2.4.2 presents the associations between protein intake and kidney outcomes. In model 1, adjusted for age, sex, and BSA, a higher protein intake at the age of 1 year was associated with a higher eGFR and lower ACR at the age of 6 years. Protein intake was not consistently associated with kidney volume. Results for $eGFR_{CysC}$ were similar to those for $eGFR_{Creat}$ (Table 2.4.2) and for $eGFR_{Combined}$ (Supplement 2.4.1). After further adjustment for sociodemographic variables and maternal factors (model 2), the effect estimates hardly changed and a higher protein intake remained significantly associated with a higher eGFR and a lower ACR. However, after further adjustment for child lifestyle factor (model 3), all associations attenuated toward null (Table 2.4.2). Important lifestyle confounders in the associations with kidney outcomes were child's television watching, overall diet quality, and sodium intake.

Table 2.4.2 Associations of protein intake at age 1 year with childhood kidney volume and function at the age of 6 years

	Kidney volume (mm^3)	$eGFR_{Creat}$ (Schwartz 2009) (ml/min per 1.73m^2)	$eGFR_{CysC}$ (Zappitelli 2006) (ml/min per 1.73m^2)	ACR (% change)†
Protein intake#	***n*=2,755**	***n*=2,006**	***n*=2,007**	***n*=2,868**
Model 1				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	**2.31 (0.61, 4.02)**	**1.90 (0.17, 3.63)**	**1.84 (0.44, 3.25)**	-6.8 (-14.6, 1.0)
Tertile 3	1.16 (-0.54, 2.87)	**2.46 (0.73, 4.19)**	**1.75 (0.35, 3.15)**	**-7.8 (-15.7, -0.1)**
p for trend*	*0.17*	***<0.01***	***0.01***	***0.04***
Per 10 g/d	0.29 (-0.67, 1.25)	**1.03 (0.04, 1.99)**	0.66 (-0.12, 1.44)	**-5.4 (-9.8, -1.1)**
Model 2				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	**2.33 (0.64, 4.03)**	**1.85 (0.12, 3.58)**	**1.84 (0.43, 3.25)**	-6.7 (-14.5, 1.1)
Tertile 3	1.21 (-0.50, 2.91)	**2.28 (0.56, 4.00)**	**1.70 (0.30, 3.11)**	**-6.9 (-14.8, 0.0)**
p for trend*	*0.16*	***<0.01***	***0.02***	*0.08*
Per 10 g/d	0.31 (-0.65, 1.27)	0.91 (-0.05, 1.86)	0.64 (-0.14, 1.43)	**-4.9 (-9.3, -0.01)**
Model 3				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	**1.96 (0.10, 3.82)**	1.21 (-0.69, 3.11)	1.58 (-0.21, 3.33)	-3.1 (-11.7, 5.4)
Tertile 3	0.36 (-1.91, 2.63)	1.11 (-1.20, 3.42)	1.60 (-0.28, 3.49)	-0.4 (-10.9, 10.0)
p for trend*	*0.74*	*0.35*	*0.10*	*0.93*
Per 10 g/d	-0.55 (-1.86, 0.76)	-0.17 (-1.49, 1.15)	0.37 (-0.71, 1.45)	-2.0 (-8.0, 4.0)

Values are based on multivariable linear regression models and reflect differences or percentage change (95% CI) in kidney outcomes for tertiles of protein intake compared to the lowest tertile, and per 10 g of protein intake per day. Statistically significant values ($p<0.05$) are indicated in **bold**.
Protein intake is energy-adjusted using the nutrient residual method.
Model 1 is adjusted for child's sex, age and body surface area at 6-year visit.
Model 2 is additionally adjusted for and maternal age, educational level, and BMI at enrollment, for smoking and folic acid supplement use during pregnancy, and for children's ethnicity, and gestational-age adjusted birthweight.
Model 3 is additionally adjusted for breastfeeding in the first four months of life, children's television watching, total energy intake, energy-adjusted total fat intake, energy-adjusted sodium intake, and diet quality score.
† Albumin/creatinine ratio is log-transformed, therefore the regression coefficients reflect the percentage change rather than the absolute difference.
**p* for trend over the tertiles of protein intake was obtained by treating tertiles as continuous ordinal variable
Abbreviations: ACR, albumin/creatinine ratio; eGFR, estimated glomerular filtration rate.

Additional analyses

No clear differences were observed for the associations of animal versus vegetable protein intake on kidney outcomes (Supplement 2.4.2). Replacement of BSA for either height and weight, BMI, or lean body mass; or replacement of absolute kidney volume by the ratio of kidney volume with weight, BMI, or BSA did not change the effect estimates (data not shown). Protein intake was not associated with urinary albumin or creatinine concentrations (data not shown). No significant interactions were observed of total protein intake with sex, birthweight, gestational age, ethnicity, or kidney volume on any of the kidney outcomes. We observed similar effect estimates as in the whole group in analyses restricted to children with a Dutch ethnic background (n=1,994), to children born with a normal birthweight (≥ 2500g, n=2,802), or to children born at term (≥ 37 weeks, n=2,781) (data not shown). In line with the results for protein intake at the age of 1 year, higher total protein intake at the age of 2 years was associated with a higher $eGFR_{Creat}$ and a trend toward a lower ACR in crude models, and the associations attenuated to null after adjustment for other lifestyle factors.

DISCUSSION

We examined the associations between protein intake in early childhood with kidney size and function at school age in a large prospective population-based cohort study. We observed that associations between higher protein intake in infancy with higher eGFR and lower ACR at the age of 6 years were explained by other dietary and lifestyle factors of the children, such as sodium intake and television watching. Furthermore, protein intake was not associated with kidney size and no differences in associations were observed for animal versus vegetable protein intake.

Interpretation and comparison with previous studies

Contrary to findings of previous studies in infants and adults, protein intake in infancy was not consistently associated with combined kidney volume in our population-based sample of school-age children. In a multi-center trial in several European countries, healthy infants who received higher protein infant formula had higher kidney volumes at the age of 6 months than infants receiving the lower protein formula.[5] Whether this difference in kidney volume persisted until later age was not studied. A previous observational study in 631 healthy infants in Denmark reported a larger kidney size in 3-month-old infants who received formula feeding than in infants who received breastfeeding and the authors hypothesized that the effect might be attributable to the higher protein content in infant formula.[41] The difference was however no longer present at 18 months of age.[41] In line with this, in a study in young rats that received isocaloric high- or low-protein diets after weaning, a higher protein intake increased kidney size.[6] However, a month after discontinuation of the high-protein diet, kidney size was comparable to that of the rats fed low-protein diets. These studies suggest that the effect of protein intake on kidney growth could be reversible. In our study, kidney outcomes were measured a few years after the assessment of dietary protein intake. Therefore, we could speculate that a potential effect of protein intake in early life on kidney size might be no longer apparent in the children at the age of 6 years. Kidney hypertrophy

in response to high protein intake could be a compensatory response to higher levels of nitrogenous protein metabolites (such as urea), and may be temporary response.[15] Alternatively, hypertrophy of the kidney in response to protein intake may occur via increased insulin-like growth factor 1 secretion, which may lead to permanent changes in kidney size.[42-43]

We observed a higher eGFR in relation to higher protein intake, but this association was explained by other lifestyle factors. Important confounding factors were television watching, overall diet quality, and breastfeeding in early infancy. This is in contrast to finding from a small trial in preterm born infants, which report a higher eGFR with additional dietary protein,[20] and to short-term trials in adults.[11-12, 44] However, in line with our results, the aforementioned large trial in healthy infants did not report an effect of a higher protein infant formula on eGFR at the age of 6 months,[5] and an observational study in healthy infants reported no associations between intake of infant formula and eGFR.[41] Similar to kidney growth, a higher GFR in response to high protein intake is considered to be an adaptive responses to high concentrations of circulating protein metabolites. This will increase the workload of the kidneys, and may lead to hyperfiltration.[15] Similar to effects on kidney growth, this response may be reversible.[6]

In our population, a higher protein intake was associated with a lower albumin/creatinine ratio in crude models, but this was no longer significant after adjustment for other dietary factors, such as sodium intake. This is in contrast to results from a trial in healthy adults which showed that higher protein intake increases urinary albumin levels.[12] However, in line with our results, a few other trials reported no associations between protein intake and urinary albumin excretion.[11, 44]
In contrast to previous observational studies in adults,[45-47] we did not observe clear differences in associations for animal and vegetable protein intake. In contrast to studies in animals,[7, 43] we also did not observe significant interactions between child sex and protein intake on kidney health. Furthermore, we observed no interaction between protein intake and birthweight or gestational age at birth. The results of our study do not indicate that changes in dietary recommendations for healthy infants are required with respect to later kidney health, however, further studies are needed to assess whether protein intake may specifically affect kidney outcomes in preterm or small-for-gestational age born children.

Methodological considerations

An important strength of our study is its prospective design within a large population-based cohort. We had information about protein intake and kidney outcomes for almost 3,000 children and we had data on many potential maternal and child confounders, which were not always considered in previous observational studies.

A limitation of our dietary assessment methods is that an FFQ relies on memory and reported food intakes are subject to measurement error.[48] However, evaluation of our FFQ against three 24-h recalls showed a good intraclass correlation coefficient for protein intake. Another limitation of our FFQ is that it was developed for Dutch children,[22, 24] whereas our study population is multiethnic. However, sensitivity analyses restricted to children with a Dutch ethnic background showed similar results. Strengths of our dietary assessment are that an FFQ measures habitual diet rather than dietary intake at just one or a few days, and that we calculated not only total protein,

but also from animal and vegetable sources. A limitation of our study is that we did not have dietary data at the age of 6 years, therefore we could not assess the association of current diet with kidney health.

We performed detailed kidney measurements, using ultrasound to measure kidney volume, and we had blood and urine samples to estimate kidney function. Unfortunately, we did not measure inulin clearance to calculate actual GFR. To estimate GFR we used a creatinine-based formula that has been validated and is widely used in pediatric populations.[27] A limitation of serum creatinine as marker of kidney function is its strong relation with muscle mass.[36] Cystatin C is a more sensitive marker for kidney function in pediatric populations than serum creatinine, because it is not affected by child age, height, or weight.[49-50] We therefore also evaluated eGFR based on cystatin C levels.[28] This formula has been evaluated against inulin clearance and compared with other eGFR formulas, and was found to be accurate and precise in estimating GFR in addition to the Schwartz 2009 formula.[36]

Conclusions

In this prospective cohort, associations between protein intake in early childhood and kidney function at the age of 6 years were explained by other dietary and lifestyle factors of the children. Furthermore, protein intake was not associated with kidney size and no differences in associations were observed for animal versus vegetable protein intake.

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SUPPLEMENT CHAPTER 2.4

Additional Supplemental Material for this chapter can be found online: http://link.springer.com/article/10.1007/s00467-015-3096-4

Supplement 2.4.1 Associations of protein intake at the age of 1 year with serum creatinine and cystatin C concentrations, $eGFR_{Combined}$ and microalbuminuria at the age of 6 years

	Serum creatinine (µmol/L)	**Serum cystatin C (µg/L)**	**$eGFR_{Combined}$ (Zappitelli 2006) (ml/min per 1.73m²)**	**Microalbuminuria (OR)**
Protein intake#	***n*=2,006**	***n*=2,007**	***n*=2,007**	***n*=2,868**
Model 1				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	-0.51 (-1.05;0.04)	**-10.5 (-18.9;-2.2)**	**2.23 (0.76;3.71)**	1.11 (0.79;1.57)
Tertile 3	**-0.81 (-1.35;-0.27)**	**-11.0 (-19.3;-2.7)**	**2.46 (0.99;3.94)**	0.86 (0.60;1.24)
p for trend*	***<0.01***	***<0.01***	***<0.01***	*0.44*
Per 10 g/d	**-0.35 (-0.65;-0.05)**	-3.9 (-8.6;0.7)	**0.96 (0.14;1.78)**	0.91 (0.75;1.11)
Model 2				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	-0.50 (-1.04;0.04)	**-10.7 (-20.4;-2.3)**	**2.22 (0.74;3.70)**	1.21 (0.85;1.75)
Tertile 3	**-0.74 (-1.28;-0.20)**	**-10.9 (-19.2;-2.5)**	**2.33 (0.86;3.80)**	1.05 (0.8;1.62)
p for trend*	***<0.01***	*0.01*	***<0.01***	*0.49*
Per 10 g/d	**-0.31 (-0.61;-0.01)**	-3.9 (-8.6;0.7)	**0.88 (0.07;1.70)**	0.95 (0.75;1.19)
Model 3				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	-0.27 (-0.86;0.33)	**-10.5 (-19.7;-1.2)**	1.61 (-0.25;3.44)	1.27 (0.87;1.85)
Tertile 3	-0.32 (-1.04;0.40)	-10.5 (-21.7;0.7)	1.60 (-0.38;3.58)	1.10 (0.69;1.78)
p for trend*	*0.39*	*0.07*	*0.12*	*0.77*
Per 10 g/d	0.05 (-0.36;0.47)	-2.1 (-8.5;4.3)	0.08 (-1.06;1.21)	1.04 (0.80;1.36)

Values are based on multivariable linear regression models and reflect differences or odds ratios (95% CI) in kidney outcomes for tertiles of protein intake compared to the lowest tertile, and per 10 g of protein intake per day. Statistically significant values ($p<0.05$) are indicated in **bold**.

#Protein intake is energy-adjusted with the nutrient residual method.

Model 1 is adjusted for child's sex, age and body surface area at 6-year visit.

Model 2 is additionally adjusted for maternal age, educational level, and BMI at enrollment, for smoking and folic acid supplement use during pregnancy, and for children's ethnicity, and gestational-age adjusted birthweight.

Model 3 is additionally adjusted for breastfeeding in the first four months of life, children's television watching, total energy intake, energy-adjusted total fat intake, energy-adjusted sodium intake, and diet quality score.

**p* for trend over the tertiles of protein intake was obtained by treating tertiles as continuous ordinal variable

Abbreviations: eGFR, estimated glomerular filtration rate; OR, odds ratio

Supplement 2.4.2 Associations of animal and vegetable protein intake at the age of 1 year with kidney volume and function at the age of 6 years

	Kidney volume (mm3) *n*=2,755	**$eGFR_{Creat}$ (Schwartz 2009) (ml/min/1.73m²) *n*=2,006**	**$eGFR_{CysC}$ (Zappitelli 2006) (ml/min/1.73m²) *n*=2,007**	**ACR (% change)† *n*=2,868**
Animal protein intake#				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	1.31 (-0.46, 3.07)	-0.43 (-2.21, 1.36)	0.27 (-1.19, 1.72)	3.6 (-4.5, 11.8)
Tertile 3	-0.51 (-2.35, 1.34)	1.11 (-0.75, 2.96)	0.24 (-1.27, 1.76)	4.9 (-3.6, 13.4)
p for trend*	*0.43*	*0.27*	*0.29*	*0.34*
Per 10 g/d	*-0.56 (-1.82, 0.70)*	*0.38 (-0.89, 1.65)*	*0.52 (-0.52, 1.56)*	*-2.0 (-7.7, 3.8)*
Vegetable protein intake#				
Tertile 1	*Reference*	*Reference*	*Reference*	*Reference*
Tertile 2	1.08 (-0.88, 3.03)	0.50 (-1.46, 2.46)	1.09 (-0.52, 2.70)	-2.5 (-11.5, 6.5)
Tertile 3	-1.24 (-3.59, 1.10)	0.96 (-1.41, 3.33)	0.61 (-1.33, 2.55)	0.7 (-10.1, 11.6)
p for trend*	*0.25*	*0.45*	*0.36*	*0.82*
Per 10 g/d	*-1.45 (-4.03, 1.12)*	*1.21 (-1.38, 3.79)*	*0.37 (-1.75, 2.48)*	*-6.8 (-18.5, 5.0)*

Values are based on multivariable linear regression models and reflect differences or percentage change (95% CI) in kidney outcomes for tertiles of animal or vegetable protein intake compared to the lowest tertile, and per 10 g of animal or vegetable protein intake per day.

#Animal and vegetable protein intake are energy-adjusted with the nutrient residual method. Models are adjusted for child's sex, age and body surface area at 6 years; for maternal age, educational level, and BMI at enrollment, for smoking and folic acid supplement use during pregnancy; for children's ethnicity, gestational-age adjusted birthweight, for breastfeeding in the first four months of life, children's television watching, total energy intake, energy-adjusted total fat intake, energy-adjusted sodium intake, and diet quality score (model 3 from main analysis).

Models with animal protein intake are adjusted for vegetable protein intake and vice versa.

†Albumin/creatinine ratio is log-transformed, therefore the regression coefficients reflect the percentage change rather than the absolute difference.

**p* for trend over the tertiles of protein intake was obtained by treating tertiles as continuous ordinal variable

Abbreviations: ACR, albumin/creatinine ratio; eGFR, estimated glomerular filtration rate.

Chapter 3

Fatty acids

Chapter 3.1

Polyunsaturated fatty acids in early life & cardiometabolic health: a systematic review

Manuscript based on this chapter:

Trudy Voortman, Edith H. van den Hooven, Kim V.E. Braun, Marion van den Broek, Wichor M. Bramer, Rajiv Chowdhurry, Oscar H. Franco. Effects of polyunsaturated fatty acid intake and status during pregnancy, lactation, and early childhood on cardiometabolic health: a systematic review. *Progress in Lipid Research* 2015;59:67–87.

ABSTRACT

Background: The importance of polyunsaturated fatty acid (PUFA) intake in fetal life and infancy has been widely studied in relation to child cognitive and visual development, but whether early life PUFA exposure is related to cardiometabolic risk factors is unclear. We conducted a systematic review of the scientific literature to evaluate the effects of PUFA dietary intake and blood levels, during pregnancy, lactation, or early childhood, on cardiometabolic outcomes.

Methods: The databases Embase, Medline and Cochrane Central were searched (up to April 2014) for interventional and observational studies that reported associations between maternal or child (≤ 5 y) PUFA intake or blood levels and cardiometabolic outcomes in the offspring. Fish oil, total PUFA, and *n*-3 and *n*-6 fatty acids were included as exposures; and obesity, blood pressure, blood lipids, and insulin sensitivity as outcomes.

Results: We identified 4,302 abstracts, of which 56 articles, reporting on 45 unique studies, met all selection criteria. Many of the included studies focused on obesity as an outcome (33 studies), whereas studies on insulin sensitivity were relatively scarce (6 studies). Overall, results for obesity, blood pressure, and blood lipids were inconsistent, with a few studies reporting effects in opposite directions and other studies that did not observe any effects of PUFAs on these outcomes. Four studies suggested favorable effects of PUFAs on insulin sensitivity.

Discussion: Despite the substantial number of high-quality studies, there is insufficient evidence to support a beneficial effect of PUFAs in fetal life or early childhood on obesity, blood pressure, or blood lipids. More research is needed to investigate the potential favorable effects of PUFAs on insulin sensitivity, and to examine the role of specific fatty acids in early life on later cardiometabolic health.

INTRODUCTION

Obesity and cardiometabolic risk factors can already develop in childhood and predict cardiovascular disease and type 2 diabetes in later life.[1-2] Therefore, it is important to study early determinants of cardiometabolic risk. Nutritional exposures in critical periods in pregnancy or early childhood may have a lasting influence on later cardiometabolic health.[3-4] Lipids, especially polyunsaturated fatty acids (PUFAs), have received considerable interest in this context because of their diverse roles in cell membrane synthesis, gene expression, and eicosanoid metabolism.[5]

Contrary to saturated and monounsaturated fatty acids, omega-3 (*n*-3) and omega-6 (*n*-6) PUFAs cannot be synthesized by the human body and are therefore considered essential nutrients in the diet.[6] During pregnancy and lactation, PUFAs are transferred from mother to fetus or infant.[7-8] PUFAs are important for growth and development, as they are incorporated into cell membranes in all tissues of the body.[5-6] The importance of PUFA intake during pregnancy and in infancy has been widely studied in relation to child cognitive and visual development.[9-11] In adults, PUFAs have been associated with improved cardiometabolic health,[12-18] but whether early life PUFA exposure affects cardiometabolic health is unclear. The presence of long-chain PUFAs (LCPUFAs) in breast milk has been suggested as a potential mechanism for beneficial effects of breastfeeding on subsequent health outcomes such as a lower blood pressure,[19] but randomized controlled trials with PUFA supplementation to infant formula, or to lactating or pregnant women have reported inconsistent effects on blood pressure in later childhood.[20-24]

Therefore, our aim was to systematically review the current literature on the effects of PUFA intake and blood levels, during pregnancy, lactation, or in early childhood up to the age of 5 y, on cardiometabolic health. Cardiometabolic outcomes included obesity (body mass index (BMI), weight-for-height, or body fat), blood pressure, blood lipids (triacylglycerol (TAG), or total, low-density lipoprotein (LDL), or high-density lipoprotein (HDL) cholesterol), and measures of insulin sensitivity (glucose or insulin concentrations, or homeostatic model assessment (HOMA)).

METHODS

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.[25]

Literature search

The literature search was performed in the electronic databases Medline (OvidSP), Embase (embase.com), and Cochrane Central. All databases were searched from their inception until 1 April 2014. The search strategy (provided in Supplement 3.1.1) consisted of four elements: PUFAs (PUFA, fish oil, *n*-3 and *n*-6 FAs); the population of interest (infants, children, pregnant and lactating women); cardiometabolic outcomes (obesity, blood lipids, blood pressure, insulin sensitivity); and observational or interventional study designs. All elements were searched using both controlled vocabulary terms (MeSH and/or Emtree) and free text words in the title or abstract. Limits were applied to include only human studies and exclude letters or editorials. No limits were set on language or year of publication. In addition to the systematic search, we contacted experts in the field and we screened reference lists of studies that were included in our review.

Selection criteria

Studies were included if they fulfilled the following criteria:

- **Study design**: Intervention, cohort, case-control, or cross-sectional studies.
- **Population**: Exposure measure or intervention in healthy pregnant or lactating women, or in healthy children ≤ 5 years; outcome measures in the offspring at any age.
- **Exposure**: Intake and/or blood levels of PUFAs, including total PUFAs; total *n*-3 FAs; total *n*-6 FAs; ratios between *n*-6 and *n*-3 FAs; fish oil; the *n*-3 FAs alpha-linolenic acid (ALA, C18:3 (*n*-3)); eicosapentaenoic acid (EPA, C20:5 (*n*-3)); docosapentaenoic acid (DPA, C22:5 (*n*-3)); or docosahexaenoic acid (DHA, C22:6 (*n*-3)); or the *n*-6 FAs linoleic acid (LA, C18:2 (*n*-6)); gamma linolenic acid (GLA, C18:3 (*n*-6)); dihomo-gamma-linolenic acid (DGLA, C20:3 (*n*-6); or arachidonic acid (ARA, C20:4 (*n*-6)).
- **Outcomes**: Cardiovascular and metabolic outcomes, including obesity (BMI, weight-for-height, body fat), blood pressure (BP), blood lipids (TAG and total, HDL and LDL cholesterol), or measures of insulin sensitivity (glucose or insulin levels, HOMA, type 2 diabetes mellitus).

Study selection

Working in pairs, two authors independently reviewed each title and abstract to determine whether the study fulfilled the selection criteria. Full text articles were retrieved for the selected titles after initial appraisal and assessed once more by two independent authors to ensure that they satisfied the inclusion criteria. Disagreement with article selection was resolved through discussion or with help of a third independent author.

Data extraction

Data were extracted with a structured data extraction form created prior to the literature search. Detailed study-level characteristics were extracted including study design, study size, study duration, characteristics of the study population, and details on exposure and outcome assessment. We extracted information on the statistical analyses, effect estimates, measures of variability, and covariate adjustments. When data from the same population were used in multiple papers or models, results from the longest follow-up or from the most covariate-adjusted model were included. Results of stratified analyses were included when the statistical interaction was reported to be significant.

Quality assessment

Two reviewers independently evaluated the quality of included studies using a predefined scoring system. As described in Chapter 2.1, this quality score (QS) was developed for use in systematic reviews and meta-analyses to assess the relative quality of studies with various study designs.[26] A score of 0, 1 or 2 points was allocated to each of the following five items: 1) study design (i.e., cross-sectional, longitudinal, or interventional); 2) size of the population for analysis; 3) quality of the methods used for exposure assessment or appropriate blinding of an intervention; 4) quality of the

methods used for outcome assessment; and 5) adjustment for potential confounders or adequate randomization of an intervention. The scores for these five items combined resulted in a total score ranging from 0 to 10 points, with 10 representing the highest quality. Details on the criteria used for the QS are presented in Supplement 3.1.2.

RESULTS

Study selection

We identified 4,302 unique references, of which 4,096 were excluded based on title and abstract (Figure 3.1.1). For the remaining references and six additional references that were obtained via reference lists and contact with authors, full-texts were retrieved and reviewed. Of these 212 papers, 56 articles were included in this systematic review, reporting on 45 unique studies: 28 observational and 19 intervention studies (two studies reported results from both a trial and observational analyses[24, 27]).

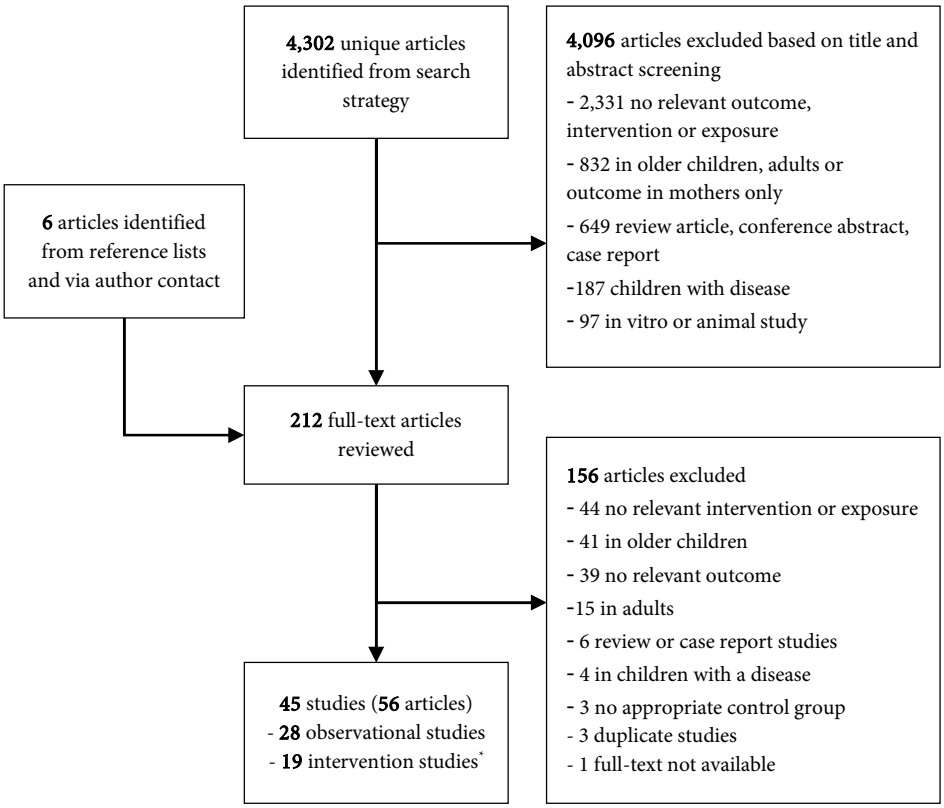

Figure 3.1.1 Flowchart of study selection

*Two studies reported results from both an observational and an intervention study[24,27]

Study characteristics

Table 3.1.1 shows summary characteristics of the 45 included studies and their study populations. A detailed description of each study is provided in Supplements 3.1.2 (intervention studies) and 3.1.3 (observational studies). The studies included a total number of 22,010 participants, ranging from 33 to 6,944 participants in each study. Four of the 45 studies included only children born preterm or with a low birthweight,[21, 28-30] the remaining studies included participants from the general population. Most studies were performed in Europe (28) or North America (11), three studies were performed in Australia,[31-33] two in Latin America,[29, 34] and one in Africa.[35]

Table 3.1.1 Characteristics of the included studies

	All included studies	Intervention studies	Observational studies
General characteristics			
No. of studies	45[*§]	19	28
Total no. of participants	22,010	2,768	19,519
Mean no. of participants per study (range)	489 (33-6,944)	146 (42-739)	697 (33-6,944)
Mean follow-up time in years (range)	3.9 (0-20)	3.8 (0-19)	3.9 (0-20)
Exposure/intervention (no. of studies)			
PUFA dietary intake	17	0	17
PUFA supplementation	19	19	0
PUFA breast milk levels	8	0	8
PUFA blood levels[#]	8	0	8
Exposure population (no. of studies)			
In pregnant women	15	5	10
In lactating women	10	2	8
In children	24	13	11
Outcomes (no. of studies)			
Measures of obesity	33[§]	15	19
Blood pressure	12[§]	7	6
Measures of insulin sensitivity	6	2	4
Blood lipids[†]	14	5	9

* Reported in 56 publications
§Two reported results from both an observational and an intervention study[24,27]
Measured in plasma (7 studies) or erythrocytes (1 study) (Supplement 3.1.4)
† Measured in serum (8 studies) or plasma (4 studies) or not specified (2 studies) (further details in Online Supplement)

In the intervention studies, participants were supplemented with *n*-3 FAs or mixed PUFAs. Details on the intervention supplements are provided in Supplement 3.1.5. No intervention studies were identified that supplemented *n*-6 FAs. Seventeen of the 19 intervention studies were placebo-controlled and blinded. Mean follow-up time of all included trials was 3.8 years (range 28 days to 19 years). Of the 28 observational studies, 24 had a prospective cohort design, one was a retrospective cohort study, and three had a cross-sectional design. Mean follow-up time in the observational studies was 3.9 years (range 0 to 20). Dietary PUFA intake was assessed in 17 studies, breast milk PUFA levels in eight studies, and circulating blood PUFA levels in eight studies. PUFAs in blood were measured in plasma phospholipids (seven studies) or in erythrocytes (one study), mostly in weight percentage of total fatty acids.

The association of PUFAs with obesity as an outcome was investigated in 33 studies, with blood lipids in 14 studies, with blood pressure in 12 studies, and with measures of insulin sensitivity in six studies. Details on outcome measures are provided in online supplemental material. The overall QS of the included studies ranged from 2 to 10 (Supplements 3.1.3 and 3.1.4), with a mean score of 6.3 for the observational studies and 8.5 for the intervention studies.

PUFAs in early life and obesity

Fifteen trials studied the effects of *n*-3 FA or mixed PUFA supplementation in early life on measures of obesity (Table 3.1.2a). Two trials, with a QS of 6 and 9, reported that consuming a PUFA-enriched infant formula led to a lower weight-for-length,[36] or a lower body fat percentage (BF%)[28] at the age of 1 year compared to use of a non-enriched infant formula. A third trial (QS 7) showed that DHA supplementation during pregnancy was associated with a lower ponderal index in infants at birth.[37] The remaining 12 trials reported no significant effects of either maternal[34, 38-42] or child PUFA supplementation[21-22, 30-31, 33, 35, 43] on BMI, weight-for-height, or BF% of the child at ages ranging from 1 to 19 years.

The 19 observational studies that studied PUFAs in relation to obesity reported inconsistent results (Table 3.1.2b). In two studies (QS 8 and 6), maternal blood and breast milk levels of *n*-6, but not *n*-3 FAs, were associated with a lower BMI or BF% at the age of 1 year (QS 8).[27, 44-45] However, in two other studies (QS 8 and 6), *n*-3 but not *n*-6 FAs in maternal blood or breast milk were associated with a lower BMI and BF% at in children at the age of 7 years[46] or a lower risk of obesity and lower skinfold thickness at the age of 3 years.[8] The latter study also reported associations between a higher *n*-6 to *n*-3 FA ratio and higher risk of obesity.[8] Three studies (QS 6 to 7) observed that higher PUFA dietary intake in early childhood or during pregnancy was associated with a lower fetal mid-thigh fat percentage,[32] a lower score on a BF% component at birth,[47] or with a lower obesity risk at 4 years of age.[48]

In contrast to the previous seven studies, four observational studies reported that higher PUFA levels were associated with higher risks of obesity. Total *n*-3 LCPUFA concentrations (i.e., with a carbon chain length of 20 or more) in breast milk were associated with a higher BMI at the age of 6 months in a study (QS 4) in Brazil;[29] and higher levels of ALA (*n*-3) and LA (*n*-6) in breast milk were associated with a higher BMI at the age of 7 years in a study (QS 6) in Norway.[49] In a British

birth cohort study (QS 6), maternal circulating blood concentrations of total *n*-6 FAs and LA (*n*-6), but not ARA (*n*-6) or *n*-3 FAs, were associated with a higher BF% in children at the ages of 4 and 6 years.[50] In line with this, in a recent Dutch study (QS 8) it was observed that higher blood levels of DGLA (*n*-6) during pregnancy were associated with a higher BMI and skinfold thickness in the offspring at the age of 7 years, but no associations were observed for levels of ARA or LA (*n*-6), or for EPA or DHA (*n*-3).[51]

Finally, one study (QS 7) reported an interaction between PUFA levels in cord blood and child's age at follow-up on BMI of the children. In this study, higher *n*-3 FA and lower *n*-6 FA concentrations in cord blood tended to be associated with a higher BMI at the age of 2 years, no difference in BMI at the age of 6 years, and with a higher BMI at the age of 10 years.[52] The remaining seven observational studies (QS ranging from 2 to 7) did not observe any associations between PUFAs in early life and child obesity.[53-59]

PUFAs in early life and blood pressure

The effects of supplementation with PUFAs in early life on subsequent BP were assessed in seven trials (Table 3.1.3a). These studies reported results in different directions. Two trials evaluated the effects of maternal *n*-3 FA supplementation on child BP. One of these studies (QS 10) reported no effect on BP in the offspring at age 19 years.[23] The other trial (QS 8) observed no effects on systolic BP (SBP), and a higher diastolic BP (DBP) in boys, but not in girls at the age of 7 years.[39, 60] The remaining five trials examined PUFA supplementation in early childhood. Two trials (QS 7 and 10) reported a lower DBP or SBP in children who had received PUFA-supplemented formula in infancy,[20, 61] two other trials (QS 8 and 10) found no effects,[22, 31] whereas the fifth trial (QS 9) observed a positive association between PUFA supplementation in infancy and DBP in 11-year-old girls, but no effect in boys.[21]

In addition to the intervention studies, six observational studies (QS 5 to 8) investigated the associations between PUFAs in early life and BP (Table 3.1.3b). One study observed a lower BP in 12-year-old children who had received breast milk with high PUFA content in infancy compared to children who had received no breastfeeding, but no differences in BP were observed between children who had received breast milk with higher versus breast milk with lower PUFA levels.[62] The five remaining studies also reported no associations between PUFA intake either during pregnancy or in early postnatal life on BP in the offspring at birth,[47] in childhood,[24, 63-64] or at the age of 20 years.[56]

PUFAs in early life and insulin sensitivity

Only two trials investigated the effects of early PUFA supplementation on measures of insulin sensitivity (Table 3.1.4a). In one trial (QS 7), lower insulin levels in cord blood were observed for women who had received DHA supplements during pregnancy, as compared to the control group.[37] The other trial (QS 10) showed no effects of *n*-3 supplementation during pregnancy on insulin levels or HOMA-IR in the 19-year-old offspring.[65]

In addition, four observational studies (QS 7 to 8) evaluated associations between PUFA levels in cord blood (2 studies) or PUFA intake during pregnancy (2 studies) and measures of insulin sensitivity the offspring (Table 3.1.4b). In the first study, cord blood concentrations of the *n*-6 FA GLA, but not ARA, LA, DHA, or EPA were inversely associated with fasting insulin and HOMA-IR in children at the age of 7 years.[55] In the second study, cord blood DGLA was inversely associated with the glucose/insulin ratio in cord blood, and DHA and ALA levels were inversely associated with proinsulin levels in cord blood.[66] A higher PUFA/SFA intake ratio during pregnancy was associated with a lower score on a component including insulin levels at birth,[47] while in the fourth study *n*-3 FA intake during pregnancy was not associated with insulin, glucose, or HOMA-IR in the offspring at the age of 20 years.[56]

PUFAs in early life and blood lipids

Five intervention studies investigated PUFA supplementation in early life in relation to cholesterol and/or TAG levels (Table 3.1.5a). One trial (QS 10) studied fish oil supplementation during pregnancy and found no effect on either total, LDL, or HDL cholesterol, or on TAG levels in the offspring at the age of 19 years.[41]

In the four other trials, all with a QS of 7 or 8, infants received PUFA-enriched formula. Results were contradicting: two of the studies reported that children who had received PUFA supplementation had lower TAG levels at the age of 1 month,[67] or lower total, LDL and HDL cholesterol levels at the age of 1 year,[68] whereas in one other trial, total and LDL cholesterol at the age of 1 year were higher in the intervention than the control group.[61] In the last trial, with a longer follow-up, no effects of PUFA supplementation in infancy were observed on cholesterol or TAG levels in the children at the age of 8 years.[31]

Most of the nine observational studies, with a QS of 4 to 7, observed no associations between PUFAs and blood lipids (Table 3.1.5b). For total and LDL cholesterol, eight studies reported no association,[31, 47, 54, 56, 69-72] while one cross-sectional study (QS 4) reported a positive association of PUFA intake with total and LDL cholesterol at the age of 1 year.[73] For HDL cholesterol, one study (QS 7) observed an inverse association between PUFA intake at the age of 18 months and HDL levels at the age of 31 months in girls, but not in boys,[69] while seven other studies observed no association.[31, 47, 54, 56, 70, 73-75] Finally, only one observational study (QS 7) examined TAG levels and found no associations between *n*-3 FA intake during pregnancy and concentrations of TAG in the offspring at the age of 20 years.[56]

Table 3.1.2a Summary of the reported associations between PUFAs and measures of obesity in intervention studies

First author (Publication year)	QS	Intervention	Outcome	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
***n*-3 FAs**								
Andersen (2011)	9	DHA+EPA supplementation at 9-18 mo	BMI Z-score at 18 mo	ANOVA	Mean difference intervention vs. placebo	NR	0.85	++
Ayer (2009)	8	Fish oil supplementation at 6 mo-5 y	BMI at 8 y	T-test	Mean difference intervention vs. placebo	+	NS	0
Bergmann (2007) and (2012)	9	DHA+EPA supplementation during pregnancy and lactation*	BMI Z-score at 6 y§	Mixed models	Mean difference intervention vs. placebo	0.013	NS	++
Carlson (1996)	6	DHA+EPA supplementation at 0-2 mo	Weight-for-length at 12 mo§	ANOVA	Mean difference intervention vs. placebo	**-0.34**	**<0.006**	0
Courville (2011)	7	DHA supplementation 3rd trimester*	PI at birth	Linear regression	Mean difference intervention vs. placebo	**-0.198**	**0.045**	++
Hauner (2012)**	8	DHA+EPA supplementation during pregnancy and lactation*	BMI at 12 mo	Linear regression	Mean difference intervention vs. placebo	0.1 (-0.3, 0.5)	NS	++
			BF% at 12 mo	Linear regression	Mean difference intervention vs. placebo	0.0 (-0.9, 0.9)	NS	++
Asserhoj (2009) and Lauritzen (2005)	8	DHA+EPA supplementation 0-4 mo of lactation*	BMI at 2.5 y	Linear regression	Mean difference intervention vs. placebo	**0.80 (0.25, 1.35)**	**0.006**	++
			BF% at 2.5 y	Linear regression	Mean difference intervention vs. placebo	**1.65 (0.30, 3.00)**	**0.021**	++
			BMI at 7 y§	ANOVA	Mean difference intervention vs. placebo	0.2 (-0.8, 1.2)	NS	++
			BF% at 7 y§	ANCOVA	Median difference intervention vs. placebo	1.0 (0.96, 1.13)§	NS	++
Rytter (2011)	10	DHA+EPA supplementation 3rd trimester*	BMI at 19 y	Linear regression	Mean difference intervention vs. placebo	0.13 (-0.92, 1.17)	NS	++

Table 3.1.2a (continued) Summary of the reported associations between PUFAs and measures of obesity in intervention studies

First author (Publication year)	QS	Intervention	Outcome	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
***n*-3 FAs**								
Stein (2011)	8	DHA supplementation 2nd and 3rd trimester*	BMI Z-score at 18 mo	T-test	Mean difference intervention vs. placebo	0.02	NS	++
van der Merwe (2013)	10	DHA+EPA supplementation at 3-9 mo	BMI Z-score at 12 mo§	Linear regression	Mean difference intervention vs. placebo	0.09 (-0.15, 0.34)	NS	+
Mixed PUFAs								
de Jong (2011)	10	DHA+ARA supplementation at 0-2 mo	BMI>25 at 9 y	T-test	Mean difference intervention vs. placebo	+	NS	0
Gibson (2009)	9	DHA+ARA supplementation at 0-7 mo	BMI change 0 to 7 mo [M]	Mixed model	Mean difference intervention vs. placebo	+	NS	+
			BMI change 0 to 7 mo [F]	Mixed model	Mean difference intervention vs. placebo	+	NS	+
Groh-Wargo (2005)	9	DHA+ARA supplementation 0-40 wk	BF% at 1 y	ANCOVA	Mean difference intervention vs. placebo	-	**< 0.05**	++
Innis (2002)	9	DHA+ARA supplementation at 0-1 mo	Weight-to-length ratio 57 wk§	Linear regression	Mean difference intervention vs. placebo	+	NS	0
Kennedy (2010)	9	DHA+GLA supplementation at 0-9 mo	BMI at 10 y	T-test	Mean difference intervention vs. placebo	+	0.44	0
			BF% at 10 y	T-test	Mean difference intervention vs. placebo	+	0.40	0

*Mother received intervention

**Same population used in observational analyses (Much, 2013a and 2013b, Table 3.1.2b)

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

‡Adjustment level defined as: 0: unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

$ Median and 80% range

§ Also results from shorter follow-up available

Abbreviations: [F], female; [M], male; ARA, arachidonic acid; ANCOVA, analysis of covariance; ANOVA, analysis of variance; BF%, body fat percentage; BMI, body mass index; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LCPUFA, long chain polyunsaturated fatty acid (≥20 C-atoms); mo, months; NR, not reported; NS, not significant; PI, ponderal index; PUFA, polyunsaturated fatty acids; QS, quality score; wk, weeks; y, years; Z-score; age- and sex-specific standard deviation score.

Table 3.1.2b Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
***n*-3 FAs**								
de Vries (2014)	8	EPA (PL)(%FAs) mean throughout pregnancy*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	0.30 (-1.41, 2.01)	0.73	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	-0.01 (-0.33, 0.30)	0.93	++
		DHA (PL)(%FAs) mean throughout pregnancy*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	-0.10 (-1.92, 1.71)	0.91	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	-0.07 (-0.41, 0.26)	0.66	++
		n-3 FAs (PL)(%FAs) mean throughout pregnancy*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	-0.31 (-2.07, 1.47)	0.74	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	-0.16 (-0.48, 0.15)	0.31	++
Donahue (2011)	8	ALA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.85 (0.67, 1.08)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**-0.26 (-0.51, 0.00)**	NR	++
		ALA (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.51 (0.18, 1.44)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.44 (-1.12, 0.24)	NR	++
		ALA (PL)(%FAs)cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.46 (0.19, 1.13)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.46 (-1.12, 0.21)	NR	++
		DHA+EPA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	**0.68 (0.50, 0.92)**	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**-0.31 (-0.58, -0.04)**	NR	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Donahue (2011) (cont'd)		DHA+EPA (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.68 (0.22, 2.16)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.39 (-1.11, 0.33)	NR	++
		DHA+EPA (PL)(%FAs) cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	**0.09 (0.02, 0.52)**	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**-0.91 (-1.63, -0.20)**	NR	++
		n-3 FA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	**0.77 (0.60, 0.99)**	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**-0.34 (-0.59, -0.08)**	NR	++
		n-3 FAs (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.52 (0.16, 1.75)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.50 (-1.21, 0.22)	NR	++
		n-3 FAs (PL)(%FAs) cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	**0.07 (0.01, 0.48)**	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**-0.92 (-1.65, -0.20)**	NR	++
Drouillet (2009)	8	*n*-3 FAs intake (%PUFA) 3rd trimester*	Skinfold thickness at birth	Linear regression	Unit increase	0.11	0.26	++
Helland (2008)	4	ALA (PL) cord blood	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
		DHA (PL) cord blood	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
		EPA (PL) cord blood	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
		ALA (BM) 4 wk postpartum*[$]	BMI at 7 y	Linear regression	Unit increase	**1.01 (0.92, 1.09)**	**0.050**	++
		DHA (BM) 4 wk postpartum*[$]	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
		EPA (BM) 4 wk postpartum*[$]	BMI at 7 y	Linear regression	Unit increase	NR	NS	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Mäkelä (2012)	6	*n*-3 FAs (BM)(%FAs) 3 mo postpartum*	BMI at 13 mo	Linear regression	Unit increase	NR	NS	+
			BMI gain from 0 to 13 mo	Linear regression	Unit increase	NR	NS	+
Moon (2013)	8	*n*-3 FAs (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	0.065	NS	++
		EPA (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	-0.010	NS	++
		DHA (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	0.061	NS	++
Much (2013a & 2013b)	8	DHA (E)(%FAs) 32nd wk of gestation*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	0.08 (-0.1, 0.25)	NS	++
		DHA (BM)(%FAs) 6th wk postpartum*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	0.95 (-0.01, 1.9)	NS	++
		EPA (E)(%FAs) 32nd wk of gestation*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	NR	NS	++
		EPA (BM)(%FAs) 6th wk postpartum*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	4.16 (-0.07, 8.39)	NS	++
		n-3 LCPUFA (E)(%FAs) 32nd wk of gestation*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	0.05 (-0.07,0.18)	NS	++
		n-3 LCPUFAs (BM)(%FAs) 6th wk postpartum*	BF% at 1 y[§]	Linear regression	Unit increase per SD increase	0.59 (-0.03, 1.20)	NS	++
Pedersen (2012)	6	DHA (BM) 1 mo postpartum*	BMI from 2 to 7 y	Generalized linear model	Unit increase per log^2 increase in DHA	**-0.25 (-0.47, -0.04)**	**0.02**	++
			BMI at 7 y	Generalized linear model	Unit increase per log^2 increase in DHA	**-0.47 (-0.79, -0.15)**	**< 0.01**	++
			BF% at 7 y	Generalized linear model	Unit increase per log^2 increase in DHA	**-1.78 (-3.04, -0.53)**	**< 0.01**	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Rump (2002)	8	EPA (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++
		DHA (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++
		n-3 FAs (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++
Rytter (2013)	7	*n*-3 FA intake (E-adj) 2nd trimester*	BMI at 20 y	Linear regression	Highest vs. lowest quintile	-0.02 (-0.99, 0.94)	NS	++
Scholtens (2009)	6	ALA (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	0.007 (-0.004, 0.019)	NS	++
		DHA (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	0.004 (-0.008, 0.016)	NS	++
		EPA (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	-0.004 (-0.015, 0.008)	NS	++
		n-3 FAs (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	0.008 (-0.004, 0.019)	NS	++
Standl (2014)	7	*n*-3 LCPUFA (PL) in cord blood	BMI Z-score at 2, 6, 10 y[§]	Linear mixed models	Regression coefficient	0.02	0.764	++
		n-3 LCPUFA (PL) at 2 y	BMI Z-score at 2, 6, 10 y[§]	Linear mixed models	Regression coefficient	-	NR	++
Tinoco (2009)	4	ALA (BM)(%FAs) 0-6 mo postpartum*	BMI at 0-6 mo	Linear regression	Unit increase	0.96	0.08	0
		EPA (BM)(%FAs) 0-6 mo postpartum*	BMI at 0-6 mo	Linear regression	Unit increase	-0.34	0.54	0
		DHA (BM)(%FAs) 0-6 mo postpartum*	BMI at 0-6 mo	Linear regression	Unit increase	0.76	0.18	0
		n-3 LCPUFA (BM)(%FAs) 0-6 mo postpartum*	BMI at 0-6 mo	Linear regression	Unit increase	**1.06**	**0.05**	0

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
***n*-6 FAs**								
de Vries (2014)	8	LA (PL)(%FAs) mean throughout gestation*	Skinfold thickness (mm) at 7 y	Linear regression	Unit increase per SD increase	1.43 (-0.79, 3.66)	0.21	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	0.25 (-0.16, 0.65)	0.21	++
		DGLA (PL)(%FAs) mean throughout gestation*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	**3.41 (1.88, 4.95)**	**<0.01**	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	**0.44 (0.16, 0.72)**	**<0.01**	++
		ARA (PL)(%FAs) mean throughout gestation*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	1.15 (-0.63, 2.93)	0.20	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	0.17 (-0.15, 0.50)	0.29	++
		n-6 FAs (PL)(%FAs) mean throughout gestation*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	0.02 (-1.77, 1.82)	0.98	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	0.03 (-0.29, 0.35)	0.87	++
Donahue (2011)	8	LA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.88 (0.70, 1.10)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.20 (-0.45, 0.05)	NR	++
		LA (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	1.80 (0.72, 4.48)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	0.16 (-0.49, 0.81)	NR	++
		LA (PL)(%FAs)cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.46 (0.19, 1.13)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.06 (-0.72, 0.60)	NR	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Donahue (2011) (cont'd)		ARA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.82 (0.64, 1.06)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.23 (-0.50, 0.04)	NR	++
		ARA (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	1.49 (0.59, 3.74)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	0.64 (-0.06, 1.33)	NR	++
		ARA (PL)(%FAs) cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.64 (0.35, 1.17)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.46 (-1.12, 0.21)	NR	++
		n-6 FA intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.88 (0.70, 1.10)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.20 (-0.45, 0.05)	NR	++
		n-6 FAs (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	1.89 (0.76, 4.73)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	0.35 (-0.29, 0.99)	NR	++
		n-6 FAs (PL)(%FAs) cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	0.68 (0.39, 1.18)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	-0.23 (-0.90, 0.44)	NR	++
Helland (2008)	6	LA (PL) cord blood	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
		LA (BM) 4 wk postpartum*	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
Mäkelä (2012)	6	*n*-6 FAs (BM)(%FAs) 3 mo postpartum*	BMI at 13 mo	Linear regression	Unit increase	NR	NS	+
			BMI gain from 0 to 13 mo	Linear regression	Unit increase	NR	NS	+

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Moon (2013)	8	Total *n*-6 FAs (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	0.106	NS	++
		LA (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	**0.118**	**<0.05**	++
		ARA (PL)(%FAs) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	0.063	NS	++
Much (2013a) and Much (2013b)	8	ARA (E)(%FAs) 32nd wk of gestation*	BMI at 1 y[§]	Linear regression	Unit increase per SD increase	**-0.07 (-0.13,-0.01)**	**<0.05**	++
			BF% at 1 y[§]	Linear regression	Unit increase per SD increase	-0.00 (-0.13, 0.13)	NS	++
		ARA (BM)(%FAs) 6 wk postpartum*	BMI at 1 y[§]	Linear regression	Unit increase per SD increase	-1.93 (-4.67, 0.82)	NS	++
			BF% at 1 y[§]	Linear regression	Unit increase per SD increase	-0.72 (-2.29, 0.85)	NS	++
		n-6 LCPUFA (E)(%FAs) 32nd wk of gestation*	BMI at 1 y[§]	Linear regression	Unit increase per SD increase	**-0.05 (-0.09, -0.01)**	**<0.05**	++
			BF% at 1 y[§]	Linear regression	Unit increase per SD increase	-0.00 (-0.1, 0.09)	NS	++
		n-6 LCPUFAs (BM)(%FAs) 6 wk postpartum*	BMI at 1 y[§]	Linear regression	Unit increase per SD increase	-0.52 (-1.60, 0.50)	NS	++
			BF% at 1 y[§]	Linear regression	Unit increase per SD increase	-0.07 (-0.68, 0.55)	NS	++
Rump (2002)	8	GLA (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++
		LA (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++
		n-6 FAs (PL)(%FAs) in cord blood	BMI or BF% at 7 y	Linear regression	Unit increase	NR	NS	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Scholtens (2009)	6	ARA (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	**-0.017 (-0.028, -0.005)**	**<0.05**	++
		LA (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	**-0.013 (-0.025, -0.002)**	**<0.05**	++
		n-6 FAs (BM) 3 mo postpartum*	BMI gain birth to 1 y	Linear regression	Highest vs. middle tertile	**-0.014 (-0.025, -0.002)**	**<0.05**	++
Standl (2014)	7	*n*-6 LCPUFA (PL) in cord blood	BMI Z-score at 2, 6, 10 y[§]	Linear mixed models	Regression coefficient	-0.04	0.805	++
		n-6 LCPUFA (PL) at 2 y	BMI Z-score at 2, 6, 10 y[§]	Linear mixed models	Regression coefficient	+	NR	++
Tinoco (2009)	4	LA (BM)(%FAs) 0-6 mo postpartum*	BMI at 6 mo	Linear regression	Unit increase	0.21	0.72	0
		ARA (BM)(%FAs) 0-6 mo postpartum*	BMI at 6 mo	Linear regression	Unit increase	0.16	0.78	0
		n-6 LCPUFAs (BM)(%FAs) 0-6 mo postpartum*	BMI at 6 mo	Linear regression	Unit increase	0.04	0.94	0
Mixed PUFAs								
Blumfield (2012)	6	PUFA intake (%E) wk 19-36 of gestation*	Fetal abdominal fat%	Linear mixed models	Regression coefficient	0.01 (-0.16, 0.18)	0.89	++
			Fetal mid-thigh fat%	Linear mixed models	Regression coefficient	**-0.48 (-0.91, -0.05)**	**0.03**	++
Carruth (2001)	4	PUFA intake (g/d) from 2 to 5 y	BF% at 5.8 y	General linear model	Regression coefficient	NR	NS	0
de Vries (2014)	8	*n*-6/*n*-3 FA ratio (PL) mean throughout gestation*	Skinfold thickness at 7 y	Linear regression	Unit increase per SD increase	0.30 (-1.41, 2.01)	0.73	++
			BMI at 7 y	Linear regression	Unit increase per SD increase	0.20 (-0.04, 0.45)	0.10	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome[#]	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Donahue (2011)	8	*n*-6/*n*-3 FA ratio intake (E-adj g/d) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	1.19 (0.94, 1.50)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**0.32 (0.07, 0.58)**	NR	++
		n-6/*n*-3 FA ratio (PL)(%FAs) mid-pregnancy*	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	2.15 (0.90, 5.15)	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	0.63 (-0.04, 1.30)	NR	++
		n-6/*n*-3 FA ratio (PL)(%FAs) cord blood	Obesity at 3 y (116 cases)	Logistic regression	OR for obesity per SD increase	**3.81 (1.40, 10.36)**	NR	++
			Skinfold thickness (mm) at 3 y	Linear regression	Unit increase per SD increase	**0.76 (0.09, 1.42)**	NR	++
Drouillet (2009)	8	PUFA intake (%fat) 3rd trimester*	Skinfold thickness at birth	Linear regression	Unit increase	-0.00	0.98	++
Helland (2008)	6	*n*-3/*n*-6 FA ratio (PL) cord blood	BMI at 7 y	Linear regression	Unit increase	NR	NS	++
Heppe (2013)	7	PUFA intake (E-adj g/d) at 14 mo	Overweight at 4 y (335 cases)	Logistic regression	OR for obesity per SD increase	**0.77 (0.62, 0.96)**	**<0.05**	++
Mäkelä (2012)	6	PUFA (BM)(%FAs) 3 mo postpartum*	BMI at 13 mo	Linear regression	Unit increase	-0.12	0.261	+
		n-6/*n*-3FA ratio (BM) 3 mo postpartum*	BMI at 13 mo	Linear regression	Unit increase	-0.002	0.982	+
		PUFA (BM)(%FAs) 3 mo postpartum*	BMI gain from 0 to 13 mo	Linear regression	Unit increase	-0.144	0.187	+
		n-6/*n*-3FA ratio (BM) 3 mo postpartum*	BMI gain from 0 to 13 mo	Linear regression	Unit increase	-0.020	0.858	+
Moon (2013)	8	*n*-3/*n*-6 FA ratio (PL) 34 wk of gestation*	BF% at 6 y[§]	Linear regression	SD increase per SD increase	-0.019	NS	++

Table 3.1.2b (continued) Summary of the reported associations between PUFAs and measures of obesity in observational studies

First author (Publication year)	QS	Exposure	Outcome#	Statistical method	Measure of comparison$	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
Morrison (2013)	7	PUFA/SFA ratio intake in mid-pregnancy*	Anthropometry-insulin component at birth¶	Linear regression	SD increase per SD increase	**-0.135**	**0.01**	++
Much (2013b)	8	*n*-3/*n*-6 FA ratio (BM) at 6wk postpartum*	BF% at 1 y§	Linear regression	Unit increase per SD increase	NR	NS	++
Murrin (2013)	7	PUFA intake (%E) 1st trimester*	Overweight at 5 y	Logistic regression	OR highest vs. lowest quartile	1.30 (0.44, 3.86)	0.64	++
Pedersen (2012)	6	*n*-6/*n*-3 FA ratio (BM) 1 mo postpartum*	BMI from 2 to 7 y	Generalized linear model	Unit increase	NR	NS	++
			BMI at 7 y	Generalized linear model	Unit increase	NR	NS	++
			BF% at 7 y	Generalized linear model	Unit increase	+	0.08	++
Scaglioni (2000)	2	PUFA intake (%E) at 1 y	Obesity at 5 y (34 cases)	Logistic regression	Obese vs. normal weight	Null	0.595	0
		PUFA intake (%E) at 5 y	Obesity at 5 y (34 cases)	Logistic regression	Obese vs. normal weight	Null	0.529	0
Standl (2014)	7	*n*-6/*n*-3 FA ratio (PL) in cord blood	BMI Z-score at 2,6, 10 y§	Linear mixed models	Regression coefficient	-0.11	0.802	++
		n-6/*n*-3 FA ratio (PL) at 2 y	BMI Z-score at 2,6, 10 y§	Linear mixed models	Regression coefficient	+	NR	++
Tinoco (2009)	4	Total LCPUFA (BM)(%FAs) 0-6 mo postpartum*	BMI at 6 mo	Linear regression	Unit increase	0.80	0.15	0

*Exposure measured in mothers

¶ Components obtained with principal component analyses on 11 cardiometabolic outcomes

$Unit increase indicates that the measure of comparison is the unit increase in the outcome per unit increase in the exposure

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

**Same population used in an intervention study (Hauner, 2012, Table 3.1.2a)

Skinfold thickness is a measure of body fat. Units for BMI are kg/m^2. Overweight and obesity are defined based on age- and sex- specific cut-offs for BMI. Details on outcome measures per study are provided in online supplemental material and can be accessed via: www.sciencedirect.com/science/article/pii/S0163782715000259

§ Also results from shorter follow-up available

Abbreviations: %E, percentage of total energy; %FAs, percentage of total fatty acids; %fat, percentage of total fat; ARA, arachidonic acid; ALA, alpha-linolenic acid; BF%, body fat percentage; BM, breast milk; BMI, body mass index; CI, confidence interval; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; E, erythrocytes; E-adj, energy-adjusted; EM, erythrocyte membrane; EPA, eicosapentaenoic acid; FA, fatty acids; GLA, gamma-linolenic acid; LA, linoleic acid; LCPUFA, long chain polyunsaturated fatty acid (≥20 C-atoms); mo, months; NR, not reported; NS, not significant; OR, odds ratio; PI, ponderal index; PL, plasma phospholipids; PUFA, polyunsaturated fatty acids; SD, standard deviation; SFA, saturated fatty acids; QS, quality score; wk, weeks; y, years; Z-score; age- and sex-specific standard deviation score.

Table 3.1.3a Summary of the reported associations between PUFAs and blood pressure in intervention studies

First author (Publication year)	QS	Intervention	Outcome#	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
***n*-3 FAs**								
Ulbak (2004), Larnkjaer (2006), & Asserhoj (2009)**	8	DHA+EPA supplementation 0-4 mo of lactation*	SBP at 7 y§	T-test	Mean difference intervention vs. placebo	3.4 (-0.7, 7.5)	NS	++
			DBP at 7 y [M]	T-test	Mean difference intervention vs. placebo	**6.4 (2.8, 10.5)**	**<0.01**	++
			DBP at 7 y [F]	T-test	Mean difference intervention vs. placebo	0.4 (3.1, 3.9)	NS	++
Damsgaard (2006)	7	DHA+EPA supplementation at 9-12 mo	SBP at 1 y	ANCOVA	Mean difference intervention vs. control	**-6.3**	**0.02**	++
			DBP at 1 y	ANCOVA	Mean difference intervention vs. control	-3.0	0.23	++
Rytter (2012)	10	DHA+EPA supplementation in 3rd trimester*	SBP at 19 y	Linear regression	Mean difference intervention vs. placebo	2 (-1, 4)	NS	++
			DBP at 19 y	Linear regression	Mean difference intervention vs. placebo	1 (0, 3)	NS	++
Mixed PUFAs								
Ayer (2009)	8	Fish oil supplementation at 6 mo-5 y	SBP at 8 y	T-test	Mean difference intervention vs. control	NR	0.66	0
			DBP at 8 y	T-test	Mean difference intervention vs. control	NR	0.93	0
de Jong (2011)	10	DHA+ARA supplementation at 0-2 mo	SBP at 9 y	T-test	Mean difference intervention vs. placebo	Null	NS	0
			DBP at 9 y	T-test	Mean difference intervention vs. placebo	-	NS	0
Forsyth (2003)	10	DHA+ARA supplementation at 0-4 mo	SBP at 6 y	T-test	Mean difference intervention vs. placebo	-2.3 (-5.3, 0.7)	0.132	0
			DBP at 6 y	T-test	Mean difference intervention vs. placebo	**-3.6 (-6.5, -0.6)**	**0.018**	0
Kennedy (2010)	9	DHA+GLA supplementation at 0-9 mo	SBP at 10 y	T-test	Mean difference intervention vs. placebo	2.1	0.32	0
			SBP at 10 y [F]	T-test	Mean difference intervention vs. placebo	**5.5**	**0.04**	0
			SBP at 10 y [M]	T-test	Mean difference intervention vs. placebo	-1.9	0.41	0
			DBP at 10 y	T-test	Mean difference intervention vs. placebo	2.3	0.07	0
			DBP at 10 y [F]	T-test	Mean difference intervention vs. placebo	**3.7**	**0.05**	0
			DBP at 10 y [M]	T-test	Mean difference intervention vs. placebo	1.1	0.53	0

*Mother received intervention

**Same population used in an observational study (Ulbak, 2004, Table 3.1.3b)

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

#Unit for BP is mmHg unless noted otherwise

§ Also results from shorter follow-up available

Abbreviations: [F], female; [M] male; ANCOVA, analysis of covariance; CI, confidence interval; DBP, diastolic blood pressure; DHA, docosahexaenoic acid; FA, fatty acids; LCPUFA, long chain polyunsaturated fatty acid; mo, months; NR, not reported; NS, not significant; QS, quality score; SBP, systolic blood pressure; y, years.

Table 3.1.3b Summary of the reported associations between PUFAs and blood pressure in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
***n*-3 FAs**								
Leary (2005)	7	*n*-3 FA intake(g/d) in 3rd trimester*	SBP at 7.5 y	Linear regression	Highest vs. lowest quartile	0.47 (-0.28, 1.22)	0.7	+
Rytter (2013)	7	*n*-3 FA intake (E-adj g/d) 2nd trimester*	SBP at 20 y	Linear regression	Highest vs. lowest quintile	0 (-3, 3)	NS	++
			DBP at 20 y	Linear regression	Highest vs. lowest quintile	0 (-2, 2)	NS	++
Ulbak (2004)**	5	*n* -3 FA intake (%E) at 2.5 y	SBP at 2.5 y	Linear regression	Unit increase	2.71 (-13.0, 18.4)	0.736	++
			DBP at 2.5 y	Linear regression	Unit increase	-6.17 (-20.1, 7.8)	0.390	++
Van Rossem (2012)	8	DHA (BM)(%fat)3 mo postpartum*	SBP at 12 y	Linear regression	DHA ≥ median vs. < median	**-**	NS	++
			DBP at 12 y	Linear regression	DHA ≥ median vs. < median	-	NS	++
		EPA (BM)(%fat) 3 mo postpartum*	SBP at 12 y	Linear regression	EPA ≥ median vs. < median	**-**	NS	++
			DBP at 12 y	Linear regression	EPA ≥ median vs. < median	**-**	NS	++
		n-3 LCPUFA (BM)(%fat) 3 mo postpartum*	SBP at 12 y	Linear regression	*n*-3 LCPUFA ≥ median vs. < median	**-**	NS	++
			DBP at 12 y	Linear regression	*n*-3 LCPUFA ≥ median vs. < median	**-**	NS	++
Mixed PUFAs								
Morrison (2013)	7	PUFA/SFA ratio intake in mid-pregnancy*	SBP-DBP component at birth[¶]	Linear regression	SD increase per SD increase	NR	NS	++
van den Hooven (2013)	8	PUFA intake (E-adj g/d) at 14 mo	SBP at 6 y	Linear regression	Highest vs. lowest tertile	0.26 (-0.41, 0.93)	NS	++
			DBP at 6 y	Linear regression	Highest vs. lowest tertile	0.10 (-0.46, 0.66)	NS	++

1 Same study population

*Exposure measured in mothers

**Same population used in an intervention study (Ulbak, 2004, Table 3.1.3a)

¶ Components obtained with principal component analyses on 11 cardiometabolic outcomes

$Unit increase indicates that the measure of comparison is the per unit increase in the outcome per unit increase in the exposure

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

Abbreviations: %E, percentage of total energy; %FAs, percentage of total fatty acids; %fat, percentage of total fat; BM, breast milk; CI, confidence interval; DBP, diastolic blood pressure; DHA, docosahexaenoic acid; E-adj, energy-adjusted; EPA, eicosapentaenoic acid; FA, fatty acids; LCPUFA, long chain polyunsaturated fatty acid; mo, months; NR, not reported; NS, not significant; PUFA, polyunsaturated fatty acid; QS, quality score; SBP, systolic blood pressure; SFA, saturated fatty acids; y, years.

Table 3.1.4a Summary of the reported associations between PUFAs and measures of insulin sensitivity in intervention studies

First author (Publication year)	QS	Intervention	Outcome	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
***n*-3 FAs**								
Courville (2011)	7	DHA supplementation 3rd trimester*	Insulin in cord blood	Linear regression	Mean difference intervention vs. placebo	**-0.743**	**0.043**	++
Rytter (2011)	10	DHA+EPA supplementation 3rd trimester*	Insulin at 19 y	Linear regression	Mean difference intervention vs. placebo	6% (-10, 23)	NS	++
			HOMA-IR at 19 y	Linear regression	Mean difference intervention vs. placebo	8% (-8, 27)	NS	++

*Mother received intervention

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

Abbreviations: CI, confidence interval; DHA, docosahexaenoic acid; HOMA-IR, homeostasis model assessment of insulin resistance; NR, not reported; NS, not significant; QS, quality score; y, years.

Table 3.1.4b Summary of the reported associations between PUFAs and measures of insulin sensitivity in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison$	Parameter Estimate (95% CI)†	*p*-value	Adjustment level‡
***n*-3 FAs**								
Rump (2002)	8	EPA (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
		DHA (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
		n-3 FAs (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
Rytter (2013)	7	*n*-3 PUFA intake (E-adj g/d) 2nd trimester*	Insulin at 20 y	Linear regression	Highest vs. lowest quintile (% difference)	-9 (-20, 4)	NS	++
			Glucose at 20 y	Linear regression	Highest vs. lowest quintile(% difference)	1 (-2, 3)	NS	++
			HOMA-IR at 20 y	Linear regression	Highest vs. lowest quintile(% difference)	-8 (-20, 6)	NS	++

Table 3.1.4b (continued) Summary of the reported associations between PUFAs and measures of insulin sensitivity in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
Zhao (2014)	8	ALA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	**-2.5 (-4.9, -0.1)**	**0.04**	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		EPA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		DPA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		DHA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	**-3.4 (-5.9, -1.0)**	**0.007**	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		n-3 FA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++

Table 3.1.4b (continued) Summary of the reported associations between PUFAs and measures of insulin sensitivity in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
***n*-6 FAs**								
Rump (2002)	8	LA (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
		ARA (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
		GLA (PL)(%FAs) in cord blood	Fasting insulin at 7 y	Linear regression	Log insulin increase per ‰ increase in GLA	**-4.1 (-7.8, -0.4)**	**0.028**	++
			Fasting glucose at 7 y	Linear regression	Unit increase	Null	NS	++
			HOMA-IR at 7 y	Linear regression	Log HOMA increase per ‰ increase in GLA	**-7.1 (-13.4, -0.8)**	**0.028**	++
			HOMA-β at 7 y	Linear regression	Unit increase	-	NS	++
		n-6 FAs (PL)(%FAs) in cord blood	Fasting insulin, glucose, HOMA-IR, HOMA-β at 7 y	Linear regression	Unit increase	NR	NS	++
Zhao (2014)	8	LA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		DGLA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	**-4.2 (-7.8, -0.7)**	**0.02**	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++

Table 3.1.4b (continued) Summary of the reported associations between PUFAs and measures of insulin sensitivity in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	*p*-value	Adjustment level[‡]
		ARA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
		n-6 PUFA (PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
Mixed PUFAs								
Zhao (2014)	8	*n*-3/*n*-6 FA ratio(PL) in cord plasma	Glucose/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
			Proinsulin/insulin in cord plasma	Generalized linear model	Unit increase per SD increase in FA	NR	NS	++
Morrison (2013)	7	PUFA/SFA ratio intake in mid-pregnancy*	Anthropometry-insulin component at birth[¶]	Linear regression	SD increase per SD increase	**-0.135**	**0.01**	++

*Exposure measured in mothers

¶ Components obtained with principal component analyses on 11 cardiometabolic outcomes

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

$Unit increase indicates that the measure of comparison is the per unit increase in the outcome per unit increase in the exposure

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

Abbreviations: ARA, arachidonic acid; ALA, alpha-linolenic acid; CI, confidence interval; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; E, erythrocytes; E-adj, energy-adjusted; EPA, eicosapentaenoic acid; FA, fatty acids; GLA, gamma linoleic acid; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of beta-cell function; LA, linoleic acid; NR, not reported; NS, not significant; PL, plasma phospholipids; PUFA, polyunsaturated fatty acids; QS, quality score; SFA, saturated fatty acids; SD, standard deviation; y, years

Table 3.1.5a Summary of the reported associations between PUFAs and blood lipids in intervention studies

First author (Publication year)	QS	Intervention	Outcome	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	P-value	Adjustment level‡
***n*-3 FAs**								
Ayer (2009)	8	Fish oil supplementation at 6 mo-5 y	HDL-C at 8 y	T-test	Mean difference intervention vs. placebo	NR	NS	0
			TAG at 8 y	T-test	Mean difference intervention vs. placebo	NR	NS	0
Damsgaard (2006)	7	DHA+EPA supplementation at 9-12 mo	TC at 1 y	ANCOVA	Mean difference intervention vs. control group	**0.51 mmol/L**	**0.02**	++
			HDL-C at 1 y	ANCOVA	Mean difference intervention vs. control group	0.10 mmol/L	0.20	++
			LDL-C at 1 y	ANCOVA	Mean difference intervention vs. control group	**0.51 mmol/L**	**0.04**	++
			TAG at 1 y	ANCOVA	Mean difference intervention vs. control group	-0.21 mmol/L	0.10	++
Rytter (2011)	10	DHA+EPA supplementation 3rd trimester*	TC at 19 y	Linear regression	Mean difference intervention vs. placebo	-1% (-6, 5)	NS	+
			HDL-C at 19 y	Linear regression	Mean difference intervention vs. placebo	+3% (-3, 10)	NS	+
			LDL-C at 19 y	Linear regression	Mean difference intervention vs. placebo	-3% (-11, 7)	NS	+
			TAG at 19 y	Linear regression	Mean difference intervention vs. placebo	-4% (-16, 10)	NS	+

Table 3.1.5a (continued) Summary of the reported associations between PUFAs and blood lipids in intervention studies

First author (Publication year)	QS	Intervention	Outcome	Statistical method	Measure of comparison	Parameter Estimate (95% CI)†	P-value	Adjustment level‡
Mixed PUFAs								
Mize (1995)	7	PUFA supplementation 0-12 mo	TC at 12 mo§	Bonferroni T-test	Mean difference high PUFA vs. high MUFA group	**-28 mg/dL (-48, -8)**	**<0.05**	0
			HDL-C at 12 mo§	Bonferroni T-test	Mean difference high PUFA vs. high MUFA group	**-9 mg/dL (-17, -1)**	**<0.05**	0
			LDL-C at 12 mo§	Bonferroni T-test	Mean difference high PUFA vs. high MUFA group	**-19 mg/dL (-36, -2)**	**<0.05**	0
			TAG at 12 mo§	Bonferroni T-test	Mean difference high PUFA vs. high MUFA group	-59 mg/dL (-125, 7)	NS	0
Siahanidou (2008)	7	DHA+ARA supplementation 0-1 mo	TC at 1 mo	T-test	Mean difference intervention vs. placebo	5.6 mg/dL	NS	0
			HDL-C at 1 mo	T-test	Mean difference intervention vs. placebo	6.5 mg/dL	0.06	0
			LDL-C at 1 mo	T-test	Mean difference intervention vs. placebo	3.7 mg/dL	NS	0
			TAG at 1 mo	T-test	Mean difference intervention vs. placebo	**-23.3 mg/dL**	**0.01**	0

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.
‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)
§ Also results from shorter follow-up available
Abbreviations: ANCOVA, analysis of covariance; CI, confidence interval; HDL-C, high density lipoprotein cholesterol; LCPUFA, long chain polyunsaturated fatty acid; LDL-C, low density lipoprotein cholesterol; mo, months; MUFA, monounsaturated fatty acids; NR, not reported; NS, not significant; PUFA, polyunsaturated fatty acids; QS, quality score; TC, total cholesterol; TAG, triacylglycerol; y, years.

Table 3.1.5b Summary of the reported associations between PUFAs and blood lipids in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	P-value	Adjustment level[‡]
***n*-3 FAs**								
Mäkelä (2012)	6	*n*-3 FAs (BM)(%FAs)3 mo postpartum*	TC at 13 mo	Linear regression	Unit increase	NR	NS	+
			HDL-C at 13 mo	Linear regression	Unit increase	NR	NS	+
Rytter (2013)	7	*n*-3 FA intake (E-adj) 2nd trimester*	TC at 20 y	Linear regression	Highest vs. lowest quintile	0% (-6, 6)	NS	++
			HDL-C at 20 y	Linear regression	Highest vs. lowest quintile	2% (-4, 9)	NS	++
			LDL-C at 20 y	Linear regression	Highest vs. lowest quintile	0% (-9, 9)	NS	++
			TAG at 20 y	Linear regression	Highest vs. lowest quintile	0 %(-12, 14)	NS	++
***n*-6 FAs**								
Mäkelä (2012)	6	*n*-6 FAs (BM) 3 mo postpartum*	TC at 13 mo	Linear regression	Unit increase	NR	NS	+
			HDL-C at 13 mo	Linear regression	Unit increase	NR	NS	+
Mixed PUFAs								
Cowin (2001)	7	PUFA intake (E-adj g/d) at 18 mo	TC at 31 mo [F]	Linear regression	Unit increase per unit increase in ln(PUFA)	NR	NS	++
			TC at 31 mo [M]	Linear regression	Unit increase per unit increase in ln(PUFA)	NR	NS	++
			HDL-C at 31 mo [F]	Linear regression	Unit increase per unit increase in ln(PUFA)	**-0.15 mmol/L (-0.29, -0.01)**	**0.036**	++
			HDL-C at 31 mo [M]	Linear regression	Unit increase per unit increase in ln(PUFA)	NR	NS	++
Hoppu (2013)	5	PUFA intake (%E) at 2 y	TC at 2 y	Spearman correlation	Correlation coefficient	NR	NS	0
			HDL-C at 2 y	Spearman correlation	Correlation coefficient	NR	NS	0
Mäkelä (2012)	6	PUFA (%FAs)(BM) 3 mo postpartum*	TC at 13 mo	Linear regression	Unit increase	NR	NS	+
		n-6/*n*-3 FA ratio (BM) 3 mo postpartum*	TC at 13 mo	Linear regression	Unit increase	NR	NS	+
		PUFA (%FAs)(BM) 3 mo postpartum*	HDL-C at 13 mo	Linear regression	Unit increase	NR	NS	+

Table 3.1.5b (continued) Summary of the reported associations between PUFAs and blood lipids in observational studies

First author (Publication year)	QS	Exposure	Outcome	Statistical method	Measure of comparison[$]	Parameter Estimate (95% CI)[†]	P-value	Adjustment level[‡]
Mäkelä (2012) (cont'd)		*n*-6/*n*-3 FA ratio (BM) 3 mo postpartum*	HDL-C at 13 mo	Linear regression	Unit increase	NR	NS	+
Mellies (1979)	4	PUFA/SFA ratio (BM) at 1-13 mo	TC at 1-13 mo	Pearson correlation	Correlation coefficient	-0.17	NS	0
Morrison (2013)	7	PUFA/SFA ratio intake mid-pregnancy*	HDL-C-ApoA1-LDL-C component at birth[¶]	Linear regression	SD increase per SD increase	-0.089	0.0 8	++
			TAG-LDL-C component at birth[¶]	Linear regression	SD increase per SD increase	NR	NS	++
Ohlund (2008) and (2011)	5	PUFA intake (%fat) 6 mo-4 y	TC at 4 y[§]	Linear regression	Unit increase	NR	NS	++
			HDL-C at 4 y[§]	Linear regression	Unit increase	NR	NS	++
			LDL-C at 4 y[§]	Linear regression	Unit increase	NR	NS	++
Thorsdottir (2003)	6	PUFA intake (g/d) at 9-12 mo**	TC at 12 mo [F]	Linear regression	Unit increase	**0.32 mmol/L (0.04, 0.61)**	**0.031**	++
			TC at 12 mo [M]	Linear regression	Unit increase	**0.31 mmol/L (0.11, 0.51)**	**0.004**	++
			HDL-C at 12 mo [F]	Linear regression	Unit increase	NR	NS	++
			HDL-C at 12 mo [M]	Linear regression	Unit increase	NR	NS	++
			LDL-C at 12 mo [F]	Linear regression	Unit increase	NR	NS	++
			LDL-C at 12 mo [M]	Linear regression	Unit increase	**0.24 mmol/L (0.03, 0.45)**	**0.030**	++
Ward (1980)	4	PUFA/SFA ratio intake at 2.5 y	TC at 2.5 y	Linear regression	Unit increase	-	NS	+

Exposure measured in mothers

**Average intake of PUFAs per child at 9 and 12 mo of age

¶ Components obtained with principal component analyses on 11 cardiometabolic outcomes

†Parameter estimate from mentioned statistical model, +, positive value (increase in outcome); -, negative value (decrease in outcome); null, no association. Statistically significant values are indicated in **bold**.

$Unit increase indicates that the measure of comparison is the per unit increase in the outcome per unit increase in the exposure

‡Adjustment level defined as: 0 unadjusted; + up to age and sex adjusted; ++ further adjusted for at least one more relevant variable (e.g., body mass index, ethnicity, socio-economic status)

§ Also results from shorter follow-up available

Abbreviations: [F], females; [M], males; %E, percentage of total energy; %FAs, percentage of total fatty acids; %fat, percentage of total fat; BM, breast milk; CI, confidence interval; DHA, docosahexaenoic acid; E, erythrocytes; E-adj, energy-adjusted; FA, fatty acids; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; mo, months; NR, not reported; NS, not significant; PL, plasma phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; QS, quality score; TC, total cholesterol; TAG, triacylglycerol.

DISCUSSION

This systematic review summarizes the currently published literature on the effects of PUFA intake and blood levels in early life on cardiometabolic health. After critically reviewing 19 intervention studies and 28 observational studies of overall high quality (mean QS of 7.2), we found no clear detrimental or beneficial effects of PUFA intake or blood levels in pregnancy, during lactation, or in early childhood on obesity, blood pressure, or blood lipids in the children. The relatively scarce literature on insulin sensitivity (6 studies), however, suggests a favorable effect of PUFAs in early life on subsequent measures of insulin sensitivity, but further research is needed.

Summary of main findings and potential mechanisms

The majority of the included studies investigated the association between PUFA intake or circulating blood levels in early life and measures of obesity. Intervention studies in adults suggest that higher intakes of *n*-3 FAs may reduce body fat, but evidence is not conclusive.[76] Reductions in body fat may result from suppression of appetite, apoptosis of adipocytes, or increased fat oxidation and energy expenditure in response to higher circulating *n*-3 FAs.[76-78] In contrast, *n*-6 fatty acids, especially ARA, have been suggested to have an adipogenic effect.[79-80] In our systematic review, we observed no clear effects of *n*-6 fatty acids on obesity. A few intervention studies reported suggestive evidence that *n*-3 FAs supplementation may have a beneficial effect on obesity,[28, 36-37] but overall results across all included trials are somewhat inconsistent. This may partly be explained by the large variation in type and quantity of fatty acids supplemented, as previous studies in adults reported different effects of not only *n*-3 and *n*-6 fatty acids, but also of individual fatty acids.[81] Other explanations for the inconsistencies in results are discussed in section 4.2. Results from observational studies on PUFAs and obesity were very mixed, as studies reported inverse, positive, and null associations between PUFAs and obesity. Our results for body composition are in line with those from three previous reviews on intake of EPA and DHA during the perinatal period on subsequent measures of obesity, which also reported inconsistent results.[82-84] Furthermore, previous meta-analyses on PUFA-enriched infant formula in relation to child growth reported no overall effect on height, weight, or head circumference,[85] or a marginally lower weight at 1 year of age in the supplemented group.[9]

The effect of PUFAs in early life on BP was assessed in seven trials, in which both increases[21, 39] and decreases[20, 61] in BP were reported after PUFA supplementation in pregnancy or early infancy. None of the six observational studies found an association between PUFAs and BP. Our results are not in line with those observed in adults. Trials in adult populations showed that *n*-3 FA supplementation may cause small but relevant reductions in BP in untreated hypertensive individuals.[14-16] Potential antihypertensive effects of *n*-3 FAs may be mediated via inhibitory effects on the renin-angiotensin system, via increased production of vasodilatory eicosanoids, or via better systemic arterial compliance due to incorporation of PUFAs into membrane phospholipids which increases membrane fluidity.[5, 86] However, previous reviews also reported that antihypertensive effects of PUFAs are mainly present in older populations and in populations with hypertension.[14, 16] Therefore, effects of PUFAs on blood pressure may not be present or visible in young healthy individuals.

Only six studies examined the associations between PUFAs in early life and measures of insulin sensitivity. The results of these studies suggest a favorable effect of PUFAs: DHA supplementation in pregnancy led to a lower insulin concentration in cord blood in a trial in 47 women,[37] and observational studies reported inverse associations between DHA, ALA, and DGLA levels with proinsulin in cord blood;[66] between GLA concentrations in cord blood and HOMA-IR at 7 years of age;[55] and between maternal PUFA intake and infant insulin levels.[47] This potential beneficial effect on insulin sensitivity was not observed in a meta-analysis of *n*-3 FA intervention studies in adults.[87] Evidence for effects of PUFAs on type 2 diabetes is inconsistent, with large population-based cohort studies reporting both higher[88-89] and lower[90-91] risks of type 2 diabetes with higher *n*-3 FA intake or blood levels. There is limited data available on potential mechanisms underlying an effect of PUFAs on insulin sensitivity. Suggested pathways include changes in inflammatory markers, changes in phospholipid membrane fluidity, and changes in expression of genes involved in lipogenesis and lipolysis.[5, 78, 81, 86, 92-94] More research is needed in both adults and children to elucidate potential effects of PUFAs on insulin sensitivity and diabetes risk.

Finally, the association PUFA exposure in early life and blood lipid levels was investigated in 14 studies. Results for cholesterol levels were inconclusive, with most studies reporting no significant associations of PUFA intake or blood levels with total, LDL, or HDL cholesterol levels. One trial observed lower TAG concentrations in infants who received PUFA-enriched formula than in infants in the control group,[67] whereas four other trials showed no significant effects, although they all reported inverse trends.[31, 56, 61, 68] A potential decrease in TAG levels with higher PUFA intake is in line with results from meta-analyses of studies in adults, in whom *n*-3 FA supplementation strongly decreases TAG concentrations and slightly increases LDL and HDL cholesterol concentrations.[12-13] Possible ways in which PUFAs may affect cholesterol and TAG levels include a reduction of hepatic synthesis of TAG and VLDL, changes in gene expression of factors involved in lipid metabolism, or an increase lipoprotein lipase activity.[95-97] To better understand the effects of PUFAs on cardiometabolic health, further studies should elucidate the biochemical mechanisms underlying these effects, by further investigating the role of different fatty acids in the production of eicosanoids and inflammatory markers, in cell signaling pathways, and in gene expression.

Differences among the included studies

Overall, the included studies in this systematic review have a relatively high quality and many of the studies are trials. Still, the results of the available studies showed wide variability. The inconsistency in results may be explained by heterogeneity in several study characteristics, for instance the specific types of fatty acids studied, the composition, dose and duration of supplementation, the timing of exposure and outcome measurements, or factors related to the study population, such as baseline fatty acids status.

An important difference between studies is the type of PUFAs investigated. Different fatty acids may have differential effects on cardiometabolic health; however, in many studies they are grouped together as *n*-3 FAs, *n*-6 FAs, or total PUFAs. It has been suggested that *n*-3 and *n*-6 FAs have opposing effects on adipocyte differentiation and inflammatory modulation,[27, 81] which would be

evened out when only total PUFAs are examined. In line with this, effects of certain FAs may depend on levels of other FAs. For example, it has been suggested that not the absolute amount but the ratio of total or certain *n*-3 and *n*-6 FAs may be of importance.[8, 98] Furthermore, individual FAs may have distinct effects. For example, one study included in our review observed a null association between maternal total *n*-6 FA levels and child BMI, but a strong positive association between the *n*-6 FA DGLA and BMI of the child.[51] Future studies are needed to explore the role of individual FAs and potential interactions between FAs in early life on later cardiometabolic health.

Besides the type of FAs examined, studies also varied in whether they investigated dietary intake or blood levels of PUFAs, and whether blood FA levels were measured in erythrocytes, which reflect longer-term dietary intake,[99] or in triacylglycerol, cholesterol esters, phospholipids, or non-esterified fatty acids in plasma, which have a higher turnover.[99] In intervention studies, the dose of fatty acids provided and the duration of supplementation may have influenced the observed cardiometabolic effects. Unfortunately, not all studies included in our review reported the exact amount of PUFA supplied, but in those that did, doses varied from 200 mg to approximately 1 g of *n*-3 FAs per day. Duration of supplementation ranged from 1 month to 5 years. In addition, the form of fatty acids, e.g., as TAG or non-esterified, could have influenced the results. Non-esterified fatty acids, for example, do not require digestive enzymes in order to be absorbed, and may therefore have a higher bioavailability than fatty acids esterified in phospholipids or TAG.[100]

An important potential source of heterogeneity in results is the levels of adjustment for confounders in observational studies. Dietary intake of PUFAs may be confounded by other dietary or lifestyle factors, such as physical activity, total energy intake, intake of other types of fat and nutrients, and overall diet quality. Although blood levels are a more objective measure of PUFA status, and are expected to be mainly modified by dietary intake of PUFAs, other factors can also affect PUFA blood levels.[101] Different FA levels in different blood fractions may depend on baseline FA status of the population, which in many populations is not in line with dietary recommendations,[102] and on individual differences in metabolism. It has for example been proposed that studies need to investigate the role of single-nucleotide polymorphisms in the desaturase encoding genes *FADS1* and *FADS2* of both mother and child to determine how they affect the requirements for PUFAs.[103] These genes encode for delta-5 and delta-6 desaturase enzymes which are involved in the conversion of PUFAs, and variations in these genes are strongly associated with variations in *n*-3 and *n*-6 FA blood levels.[104]

Finally, differences in timing of PUFA exposure may lead to different cardiometabolic effects. For instance, different tissues of the fetus are developed during different stages of pregnancy, and effects of PUFAs may depend on exposure in specific critical periods. Only one of the studies included in our review measured FA levels at different points in time during gestation, but they observed no trimester-specific associations on obesity in the offspring at the age of 7 years.[51] Furthermore, many studies did not take into consideration PUFA status of the offspring at follow-up, which makes it hard to estimate an independent effect of exposure in early life. Besides timing of the exposure, the timing and accuracy of outcome measure may also have led to inconsistencies in the observed results. As suggested by one of the studies included in our review, age at follow-up might interact with the association between PUFAs and obesity, with decreased risk of obesity at a

younger age and increased risk at a later age in response to higher PUFA exposure in early life.[52] Results of two other studies included in this review also suggest differential effects in follow-up time or age at outcome measurement: two trials observed no effects of PUFAs on obesity at the latest follow-up, but with a higher risk of obesity at earlier time points during the follow-up.[30, 39, 105] Of the studies included in our review, outcome measures were mostly assessed in children up to the age of 12 years; with only two studies with longer follow-up, until the age of 19 and 20 years.[23, 56] These latter two studies reported no significant effects of early life PUFAs on cardiometabolic health in adolescence. Among the remaining studies in this review, we did not observe clear differences in results between studies with outcome measurements at different ages. Studies with longer follow-up are needed to investigate whether PUFAs in early life affect not only risk factors, but also occurrence of cardiometabolic diseases in later life. Although systematic reviews of trials in adult populations showed that supplementation with *n*-3 FAs improves blood lipid levels[12-13] and reduces blood pressure,[14-16] the effects of *n*-3 or *n*-6 FAs in adulthood on the incidence and mortality of cardiovascular diseases are still inconclusive.[106-111]

Strengths and limitations of this review

This review provides a comprehensive overview of the currently available evidence on the effects of PUFAs in early life on cardiometabolic health. To our knowledge, this is the first systematic review on this topic. An extensive literature search was performed to identify relevant articles, and we aimed to reduce the problem of publication bias by contacting authors to identify additional studies. We included both PUFA blood levels and PUFA dietary intake; different types of PUFAs; and exposure to PUFAs either during pregnancy, during lactation, or in early childhood. Furthermore, we studied the effects of these exposures to PUFAs on several cardiometabolic health outcomes, including obesity, blood pressure, insulin sensitivity, and blood lipid profile. Because of the diversity in for example study populations, study designs, fatty acids under study, and age at outcome measurement, a meta-analysis was not possible.

We used a quality score to more objectively distinguish between higher and lower quality studies, and to guide the interpretation of the results and qualitative synthesis of the evidence. The overall quality of each of the included studies was assessed with a predefined quality scoring system which was developed for use in systematic reviews. Each study was scored on five aspects: study design, the size of the study population, the quality of the exposure assessment or blinding, the quality of the outcome assessment, and adjustment for confounders or randomization. Most of the studies scored high on the quality of exposure and outcomes measurement. Of the 28 observational studies included in this review, many had a prospective design and most adjusted for an extensive list of confounders. However, in observational studies, even after adjustment for multiple potential confounders, residual confounding may exist. For example, a high intake of fish or nuts is often accompanied by other lifestyle factors which may also be related to cardiometabolic health. Nineteen of the included studies were trials and in most of them allocation to the intervention or control was randomized. However, not all trials were double-blinded, which could have led to for example subject-expectancy or experimenter's bias. Overall, however, the studies included in this review had a relatively high quality (i.e., a mean QS of 7.2 on a scale of 0 to 10).

Conclusions

Despite the substantial number of intervention and observational studies, the currently published literature does not clearly support or refute favorable effects of PUFAs in early life on subsequent obesity, blood lipids, or blood pressure. PUFAs may have a beneficial effect on insulin sensitivity, but the current evidence is insufficient to draw strong conclusions. Further research is warranted to help elucidate whether specific fatty acids at specific time points in early life may influence cardiometabolic health in later life.

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SUPPLEMENT CHAPTER 3.1

Additional Supplemental Material for this chapter can be found online: http://www.sciencedirect.com/science/article/pii/S0163782715000259

Supplement 3.1.1. Search strategy for Medline (OvidSP)

Population	(exp child/ OR exp "Infant, Newborn"/ OR exp pediatrics/ OR "Hospitals, Pediatric"/ OR exp pregnancy/ OR "pregnant woman"/ OR mothers/ OR exp "Maternal Nutritional Physiological Phenomena"/ OR exp "Infant Nutritional Physiological Phenomena"/ OR exp "Child Nutritional Physiological Phenomena"/ OR "Milk, Human"/ OR lactation/ OR (infan* OR newborn* OR (new ADJ born*) OR baby OR babies OR neonat* OR child* OR toddler* OR boy OR girl OR boys OR girls OR minors OR underag* OR juvenil* OR youth* OR kindergar* OR pediatric* OR paediatric* OR schoolchild* OR preschool* OR pregnan* OR mother* OR matern* OR lactati* OR gestation* OR "child bearing" OR childbear* OR (earl* ADJ3 life*) OR preterm* OR (pre ADJ term*) OR ((human OR breast OR artificial) ADJ milk) OR breastfed OR ((breast OR formula*) ADJ3 (feeding OR fed)) OR breastfeeding OR offspring*).ab,ti.)
Exposure	AND (exp "Fatty Acids, Essential"/ OR exp "Fatty Acids, Omega-3"/ OR exp "Fatty Acids, Omega-6"/ OR exp "Fish Oils"/ OR "8,11,14-Eicosatrienoic Acid"/ OR exp "Arachidonic Acids"/ OR (((arachi* OR linol* OR essent* OR eicosatetra* OR eicosapenta* OR epoxyicosatr* OR epoxyeicosatr* OR octadecadien* OR octadecatrien* OR docosahexa* OR docosapenta* OR "n 3" OR "n 6" OR n3 OR n6) ADJ3 (acid* OR fat* OR fa)) OR polyunsaturat* OR (poly ADJ unsaturat*) OR ((omega) ADJ (3 OR 6)) OR omega3 OR omega6 OR "bilantin omega" OR "conchol 36" OR "eicosa e" OR eicosapen OR epaisdin OR "omega forte" OR sakana OR arachidonate* OR linoleate* OR linolenate* OR (fish ADJ oil*) OR ameu OR efamed OR epax OR feniko OR himega OR "k 85" OR "lachs 550" OR lipitac OR maxepa OR olemar OR optimepa OR pikasol OR promega OR superepa OR "tuna oil" OR epa OR docosahexaenoat* OR dha OR dhasco OR dgla OR eicosatrien* OR icosatrien* OR PUFA OR PUFAs OR LCPUFA OR LCPUFAs).ab,ti.)
Outcomes	AND (exp "cardiovascular diseases"/ OR exp "cardiovascular system"/ OR exp "Diabetes Mellitus, Type 2"/ OR exp "Hyperinsulinism"/ OR exp "blood pressure"/ OR cholesterol/bl OR exp "Lipoproteins, HDL"/ OR exp "Lipoproteins, LDL"/ OR exp triacylglycerol/bl OR exp Overweight/ OR "Body Weight"/ OR exp "Body Weight Changes"/ OR exp "Body Composition"/ OR exp lipids/bl OR glucose/bl OR "Blood Glucose"/ OR exp hyperglycemia/ OR exp hypoglycemia/ OR exp Insulin/ OR (cardiovascular OR cardiac OR heart OR vascular OR cardiometabolic OR (diabetes ADJ6 ("type 2" OR "type ii" OR "non insulin" OR noninsulin OR stable OR Ketosis OR "slow onset" OR "adult onset" OR "maturity onset")) OR niddm OR mody OR (glucose ADJ3 (level* OR concentration OR plasma OR blood OR serum OR metabolism OR tolerance OR intolerance OR homeosta*)) OR "blood pressure" OR hypertensi* OR ((cholesterol OR LDL* OR VLDL* OR HDL* OR "density lipoprotein" OR triglyceride* OR triacylglycerol OR lipoprotein* OR lipid* OR fat) ADJ3 (plasma OR blood OR serum OR level* OR profile*)) OR obesity OR obese OR adiposit* OR "metabolic syndrome" OR (body ADJ3 (mass OR weight OR fat)) OR (weight ADJ3 (chang* OR gain* OR fluctuat*)) OR BMI OR insulin OR Hyperinsulin* OR "HOMA-IR" OR "HOMA-B" OR "HOMA-beta" OR "HOMA β").ab,ti.)
Study Design	AND (exp "Cohort Studies"/ OR exp "case control studies"/ OR "Cross-Sectional Studies"/ OR "Clinical Trial".pt. OR ((cross ADJ section*) OR (case ADJ control*) OR cohort* OR trial* OR ((clinical OR prospectiv* OR population* OR observation* OR retrospecti* OR intervention*) ADJ3 stud*) OR 'follow up').ab,ti.)
Limits	NOT (exp animals/ NOT humans/) NOT (congresses OR editorial OR guideline OR letter OR news OR Published Erratum OR review).pt.

Supplement 3.1.2 Quality Score

1. Study design

0 for cross-sectional studies
1 for longitudinal studies (prospective or retrospective) or non-randomized intervention studies
2 for randomized intervention studies

2. Population

Observational studies
0 if $n < 200$
1 if n 200 to <1000
2 if $n \geq 1000$

Intervention studies
0 if $n < 50$
1 is n 50 to <100
2 if $n \geq 100$

3. Exposure

Observational studies
0 if the study used a one-day food record, one 24-h recall or if not reported
1 if the study measured dietary intake of PUFAs with an FFQ, or multiple day food records or 24-h recalls
2 if the study measured PUFA levels in blood or in breast milk

Intervention studies
0 if the intervention diet was not described, consisted of dietary advice only, or was not blinded
1 if the intervention diet was adequately single blinded throughout the study
2 if the intervention diet was adequately double-blinded throughout the study

4. Outcome

0 if the study used no appropriate outcome measurement method (see below) or if not reported
1 if the study used moderate quality outcome measurement methods:
- Obesity: BMI or weight-for-height
- Blood pressure: only one measurement, in resting position, by a trained observer
- Total cholesterol, LDL-cholesterol and TAG: non-fasting blood sampling, otherwise well described
- Insulin sensitivity: only either glucose or insulin measures, blood sampling after 12h or overnight fast

2 if the study used adequate outcome measurement methods*:
- Obesity: body fat percentage with DXA, Bod Pod, bio-impedance, skinfolds, or ultrasound.
- Blood pressure: at least two measurements, in resting position, by a trained observer
- Total cholesterol, LDL-cholesterol and TAG: blood sampling after 12h or overnight fast
- Insulin sensitivity: both glucose and insulin or a composite measure with blood sampling after 12h or overnight fast, or an appropriate glucose or insulin tolerance test

5. Adjustments

0 if findings are not controlled** for at least age and sex
1 if findings are adjusted for: age or Tanner stage and sex
2 if an intervention study is adequately randomized or if findings are additionally controlled for at least two of the following covariates:
- a measure of body weight (body mass index, body weight, or body fat).
- intake of total energy, other macronutrients, or micronutrients,
- blood levels of other fatty acids,
- physical activity,
- birth characteristics (e.g. birthweight, gestational age),
- maternal characteristics (e.g. maternal BMI),
- socioeconomic status, or
- ethnicity.

* Based on guidelines from the American Heart Association (blood pressure and blood lipids) and the American Diabetes Association (insulin sensitivity)

** 'controlled for' here refers to: adjusted for in the statistical analyses (e.g. with multivariable regression); stratified for in the analyses (e.g. boys and girls separately); or narrow selection criteria of study participants on this covariate (e.g. including only 7-year-olds would count as sufficiently controlling for age and including girls only does not require controlling for sex).

Supplement 3.1.3 Baseline characteristics of the 19 intervention studies included in this review (25 articles)

First author (Publication year)	Year of randomi-zation	Location	Baseline population	Age intervention	Male (%)	AC	Blinding		Intervention#	Control#	Intervention group, *n*‡	Placebo Group, *n*‡	Total follow-up	QS
							Partici-pants	Carers						
Mother receiving intervention during pregnancy/lactation														
Bergmann (2007 & 2012)	2000-2002	Germany	Pregnant women	Pregnancy & lactation	48	Y	Y	Y	DHA	Placebo	43	71	21 mo and 6 y	9
Courville (2011)	NR	USA	Pregnant women	3rd trimester	57	Y	Y	Y	DHA	Placebo	22	25	Until birth	7
Hauner (2012)*	2006-2009	Germany	Pregnant women	Pregnancy & lactation	NR	Y	N	N	Fish oil	No oil	104	104	12 mo	8
Rytter (2011, 2011, & 2012)	1990	Denmark	Pregnant women	3rd trimester	44	NR	Y	Y	Fish oil	Olive oil	108	72	19 y	10
Stein (2011)	2005-2007	Mexico	Pregnant women	2nd-3rd trimester	54	NR	Y	Y	DHA	Olive oil	369	370	1.5 y	8
Ulbak (2004), Lauritzen (2005), Larnkjaer (2006), & Asserhoj (2009)*	1999-2000	Denmark	Lactating women§	0-4 mo lactation	53	Y	Y¶	Y¶	Fish oil	Olive oil	36	28	2.5 y and 7 y	8
Infant/child receiving intervention														
Andersen (2011)	NR	Denmark	Healthy infants	9-18 mo	53	N	Y	Y	Fish oil	Sunflower oil	61	72	9 mo	9
Ayer (2009)	NR	Australia	Healthy infants	6 mo-5 y	51	Y	NR	Y	Rapeseed & fish oil	Sunflower oil	51	49	8 y	8
Carlson (1996)	NR	USA	Preterm infants	0-2 mo	49	Y	NR	NR	Fish oil	Placebo	33	26	12 mo	6
Damsgaard (2006)	2003	Denmark	Healthy newborns	9-12 mo	49	N	N	N	Fish oil	No oil	39	44	3 mo	7
de Jong (2011)	1997-1999	Netherlands	Healthy newborns	0-2 mo	53	Y	Y	Y	LCPUFA	Placebo	91	123	9 y	10

Supplement 3.1.3 (continued) Baseline characteristics of the 19 intervention studies included in this review (25 articles)

First author (Publication year)	Year of randomi-zation	Location	Baseline population	Age intervention	Male (%)	AC	Blinding		Intervention#	Control#	Intervention group, *n*‡	Placebo Group, *n*‡	Total follow-up	QS
							Partici-pants	Carers						
Forsyth (2003)	1992	6 European centers	Healthy newborns	0-4 mo	53	Y	Y	Y	LCPUFA	Placebo	65	71	6 y	10
Gibson (2009)	2003-2005	Australia	Healthy newborns	0-7 mo	47	Y	Y	Y	Fish oil	Placebo	72	70	7 mo	9
Groh-Wargo (2005)	1997-1998	USA	Preterm infants	0-40 wk	54	Y	Y	Y	DHA+ARA	Placebo	36	21	1 y	9
Innis (2002)	NR	North America	Low birthweigh t infants	0-1 mo	46	Y	Y	Y	DHA+ARA or DHA	Placebo	58	52	57 wk	9
Kennedy (2010)	1995-1997	UK	Low birthweigh t infants	0-9 mo	26	Y	Y	Y	LCPUFA	Placebo	50	57	10 y	9
Mize (1995)	NR	USA	Healthy infants	0-12 mo	45	Y	Y	Y	PUFA	MUFA	22	20	12 mo	7
Siahanidou (2008)	NR	Greece	Healthy newborns	0-1 mo	53	Y	Y	N	LCPUFA	Placebo	30	30	1 mo	7
van der Merwe (2013)	2007-2008	Gambia	Healthy newborns	3-9 mo	58	NR	Y	Y	Fish oil	Olive oil	87	85	9 mo	10

*Also present in observational studies section

¶For the 1st year of follow-up

§Pregnant women with fish intake below the population median

Details of the intervention and control supplements can be found in Supplement 3.1.5

Abbreviations: ARA, arachidonic acid; AC, allocation concealment; BS, basic supplement (consisting of vitamins and minerals which intervention group took as well); DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; mo, months; NA, not applicable; NR, not reported; QS, quality score.

Supplement 3.1.4 Baseline characteristics of the 28 observational studies included in this review (31 articles)

First author (Publication year)	Country	Study Design	Population source	Study population	Baseline Survey	Age at exposure (y)	Age at follow-up (y)	Male (%)	*n*	Type of exposure[§]	QS
Levels measured in mothers											
Blumfield (2012)	Australia	Prospective cohort	General population	Pregnant women	2006-2007	19-36 wk gestation	19-36 wk gestation	49	156	Diet	6
de Vries (2014)	Netherlands	Prospective cohort	General population	Pregnant women	1989-1995	16, 22, 32, 40 wk gestation	7	55	234	Blood (plasma PL)	8
Donahue (2011)	USA	Prospective cohort	General population	Pregnant women	1999-2002	Mid-pregnancy	3	NR	1250	Diet and blood (plasma PL)	8
Drouillet (2009)	France	Prospective cohort	General population	Pregnant women	2003	3rd trimester	1.8 days	NR	1446	Diet	8
Helland (2008)	Norway	Prospective cohort	Intervention study	Women with singleton birth	1994-1996	4 wk	7	56	143	Breast milk and blood (PL)	6
Leary (2005)	UK	Prospective cohort	General population	Women with singleton birth	1991-1992	3rd trimester	7.5	NR	6944	Diet	7
Mäkelä (2012)	Finland	Prospective cohort	General population	Pregnant women	2007-2010	3 mo	13 mo	51	100	Breast milk	6
Mellies (1979)	USA	Prospective cohort	General population	Pregnant women	NR	1-13 mo	1-13 mo	NR	33	Breast milk	4
Moon (2013)	UK	Prospective cohort	General population	Pregnant women	1998-2002	34 wk gestation	6	52	293	Blood (plasma PL)	8
Morrison (2013)	Canada	Prospective cohort	General population	Pregnant women	NR	Mid-pregnancy	Birth	50	901	Diet	7
Much (2013a)*[1]**	Germany	Prospective cohort	Intervention study	Healthy pregnant women	NR	32 wk gestation	1	NR	152	Breast milk	8
Much (2013b)*[1]**	Germany	Prospective cohort	Intervention study	Healthy pregnant women	NR	6 wk	1	NR	205	Blood (E)	
Murrin (2013)	Ireland	Prospective cohort	General population	Women with singleton birth	2001-2003	1st trimester	5	NR	585	Diet	7

Supplement 3.1.4 (continued) Baseline characteristics of the 28 observational studies included in this review (31 articles)

First author (Publication year)	Country	Study Design	Population source	Study population	Baseline Survey	Age at exposure (y)	Age at follow-up (y)	Male (%)	*n*	Type of exposure[§]	QS
Pedersen (2012)	Denmark	Prospective cohort	General population	Asthmatic mothers	NR	1 mo	7	51	222	Breast milk	6
Rytter (2013)	Denmark	Prospective cohort	General population	Pregnant women	1988-1989	2nd trimester	20	48	915	Diet	7
Scholtens (2009)*[2]	Netherlands	Prospective cohort	General population	Mothers from general population	1996-1997	3 mo	1	52	177	Breast milk	6
Van Rossem (2012)*[2]	Netherlands	Prospective cohort	General population	Mothers from general population	1996-1997	3 mo	12	49	314	Breast milk	8
Tinoco (2009)	Brazil	Prospective cohort	Maternity wards	Mothers of premature born infants	NR	Birth	0.5	57	37	Breast milk	4
Levels measured in infants/children											
Carruth (2001)	USA	Prospective cohort	General population	Healthy children	NR	24-60 mo	5.8	55	53	Diet	4
Cowin (2001)	UK	Prospective cohort	Hospital records	All infants born in Avon	1992	Birth	2.6	55	496	Diet	7
Heppe (2013)*[3]	Netherlands	Prospective cohort	General population	Healthy children	2001-2005	12-19 mo	4	50	3610	Diet	7
van den Hooven (2013)*[3]	Netherlands	Prospective cohort	General population	Healthy children	2001-2005	12-19 mo	6	49	2882	Diet	8
Hoppu (2013)	Finland	Prospective cohort	Intervention study	Healthy children	NR	Birth	4	NR	208	Diet	5
Ohlund (2008)*[4]	Sweden	Cross-sectional	General population	Healthy infants	1995	1	1	54	223	Diet	5
Ohlund (2011)*[4]	Sweden	Prospective cohort	General population	Healthy infants	1995	6-18 mo	4	50	127	Diet	5
Rump (2002)	Netherlands	Retrospective cohort	General population	Singleton babies	1997-2000	Birth	6.7-8.1	55	259	Blood (plasma PL)	8

Supplement 3.1.4 (continued) Baseline characteristics of the 28 observational studies included in this review (31 articles)

First author (Publication year)	Country	Study Design	Population source	Study population	Baseline Survey	Age at exposure (y)	Age at follow-up (y)	Male (%)	*n*	Type of exposure§	QS
Scaglioni (2000)	Italy	Prospective cohort	Maternity ward	Healthy, singleton newborns	1991	1	5	46	147	Diet	2
Standl (2014)	Germany	Prospective cohort	Maternity wards	Healthy babies	1997-1999	Birth	10	56	388	Blood (plasma PL)	7
Thorsdottir (2003)	Iceland	Prospective cohort	Maternity wards	Healthy, singleton newborns	NR	Birth	1	50	103	Diet	6
Ulbak (2004)**	Denmark	Cross-sectional	Intervention study	Healthy children	2001-2002	2.5	2.5	NR	72	Diet	5
Ward (1980)	USA	Cross-sectional	Previous study	Volunteer families	NR	2.5	2.5	NR	74	Diet	4
Zhao (2014)	Canada	Prospective cohort	General population	Singleton babies	NR	Birth	Birth	NR	108	Blood (plasma PL)	8

*Same study

**Also present in intervention studies section

§Type of exposure being blood levels, dietary intake or both

Abbreviations: E, erythrocytes; mo, months; NA, not applicable; NR, not reported; PL, phospholipids; QS, quality score; y, years.

Supplement 3.1.5 Details on the intervention and control supplements used in the 19 intervention studies included in this review (25 articles)

First author (Publication year)	Intervention						Control
	Supplement form	Source	Fatty acids	Fatty acid form	Daily dose	Duration	
Mother receiving intervention during pregnancy/lactation							
Bergmann (2007 & 2012)	NR	Fish oil	DHA +EPA	TAG	200 mg DHA +60 mg EPA	7 months	Corn oil
Courville (2011)	Cereal bars	NR	DHA	NR	300 mg DHA	4 months	Maize oil
Hauner (2012)	Capsules	Fish oil	DHA + EPA	NR	1020 mg DHA+ 180 mg EPA	10 months	No oil
Rytter (2011, 2011, & 2012)	Capsules	Fish oil	DHA + EPA	TAG	2.7 g *n*-3 PUFA	3 months	Olive oil
Stein (2011)	Capsules	Algae	DHA	NR	400mg DHA	4 months	Olive oil
Ulbak (2004), Lauritzen (2005), Larnkjaer (2006), Asserhoj (2009)	Muesli bars	Fish oil	DHA + EPA	TAG	4.5 g fish oil containing 1.5g DHA + EPA	4 months	Olive oil
Infant/child receiving intervention							
Andersen (2011)	Oil	Fish oil	DHA + EPA	NR	1.6 g fish oil	9 months	Sunflower oil
Ayer (2009)	Oils, spreads, and infant formula	Rapeseed & fish oil	NR	NR	500 mg	5 years	Sunflower oil
Carlson (1996)	Infant formula	Fish oil	DHA + EPA	NR	NR	2 months	Sunflower oil
Damsgaard (2006)	Oil	Fish oil	DHA + EPA	TAG	924 mg *n*-3 PUFA	3 months	No supplement
de Jong (2011)	Infant formula	Egg yolk, tuna oil, fungal oil	DHA + ARA	15% as PL and 85% as TAG	NR	2 months	Standard formula
Forsyth (2003)	Infant formula	Egg yolk	DHA + ARA	70% as PL	NR	4 months	Standard formula
Gibson (2009)	Infant formula	Fish oil	DHA + ARA	NR	NR	7 months	Standard formula
Groh-Wargo (2005)	Infant formula	Fish/fungal oil; or egg/fish oil	DHA + ARA	TAG	24 kcal/ fl.oz.	10 months	Standard formula
Innis (2002)	Infant formula	Fungal oil (ARA), algae oil (DHA)	DHA+ARA or DHA	>95% TAG	NR	9 months	Standard formula
Kennedy (2010)	Infant formula	Tuna & borage oil	DHA + GLA	TAG	NR	7 months	Standard formula
Mize (1995)	Infant formula	Vegetable oil (NR)	PUFA	NR	NR	1 year	Standard formula
Siahanidou (2008)	Infant formula	NR	DHA + ARA	NR	NR	1 month	Standard formula
van der Merwe (2013)	Oil	Fish oil	DHA + EPA	TAG	200 mg DHA + 300 mg EPA	6 months	Olive oil

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids; mo, months; NR, not reported; PL, phospholipid; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol.

Chapter 3.2

Fatty acid patterns in fetal life & cardiometabolic health

Manuscript based on this chapter:

Trudy Voortman, Myrte J. Tielemans, Wendy Stroobant, Josje D. Schoufour, Jessica C. Kiefte-de Jong, Jolien Steenweg-de Graaff, Edith H. van den Hooven, Henning Tiemeier, Vincent W.V. Jaddoe, Oscar H. Franco. Plasma fatty acid patterns during pregnancy and child body composition and cardiometabolic health: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Exposure to different concentrations of fatty acids during fetal life may have persistent effects on growth and metabolism. However, most previous studies only examined individual fatty acids and did not consider their overall patterns. We aimed to evaluate whether plasma fatty acid patterns during pregnancy are associated with body composition and cardiometabolic health of the offspring at 6 years of age.

Methods: This study was performed in 4,830 mother-child pairs participating in a population-based cohort study in Rotterdam, the Netherlands. At approximately 20 weeks of gestation, we measured plasma concentrations of 22 individual fatty acids, in which we identified three fatty acid patterns using principal component analysis: a *'high n-6 polyunsaturated fatty acid (PUFA)'* pattern, a *'monounsaturated (MUFA) and saturated fatty acid (SFA)'* pattern, and a *'high n-3 PUFA'* pattern. When the children were 6 years old, we measured their anthropometrics and body fat (with dual-energy X-ray absorptiometry), and calculated their body mass index (BMI), fat mass index (FMI), and fat-free mass index (FFMI). At the same age, children's blood pressure, and serum insulin, HDL cholesterol, and triacylglycerol concentrations were measured. For all outcomes we calculated age- and sex-specific SD-scores.

Results: After adjustment for confounders, a higher *'high n-6 PUFA'* pattern score during pregnancy was associated with a higher BMI and FFMI in the offspring at the age of 6 years, but not independently with cardiometabolic outcomes. The *'MUFA and SFA'* pattern was not consistently associated with body composition or cardiometabolic health of the children. A higher score for the *'high n-3 PUFA'* pattern was associated with a lower FMI and a higher FFMI, and with higher HDL cholesterol and lower triacylglycerol concentrations.

Conclusion: Our results suggest that plasma fatty acid patterns during pregnancy may affect the body composition and cardiometabolic health of the offspring. Specifically, a pattern characterized by high *n*-3 PUFA concentrations was associated with a more favorable body composition and blood lipid profile. Future studies should examine whether these associations are causal and what underlying mechanisms are involved.

INTRODUCTION

Nutritional exposures in critical periods in fetal life or early childhood may have a lasting influence on subsequent growth and cardiometabolic health.[1-2] Lipids have received considerable interest in this context because of their diverse regulatory functions. Fatty acids play an important role in cell membrane synthesis, gene expression, and inflammatory processes.[3] During pregnancy, fatty acids are transferred from mother to fetus,[4-5] and may persistently influence growth and metabolism of the child from early life onward.[3, 6]

Previous research in this area mainly focused on polyunsaturated fatty acids (PUFAs). However, as described in Chapter 3.1, although a few studies suggest favorable effects of omega-3 (*n*-3) PUFAs in early life on later body composition and insulin concentrations,[5,7-8] overall evidence for PUFAs in relation to subsequent cardiometabolic health is inconclusive.[9] Studies on saturated (SFA) or monounsaturated fatty acid (MUFA) concentrations in early life in relation to later cardiometabolic health in humans are scarce. Furthermore, most previous studies only targeted a few selected individual fatty acids or groups of fatty acids, whereas there are numerous circulating fatty acids, that are highly correlated and may interact.[10] A relatively novel approach in fatty acid analysis, that takes these interrelations into account, is to study patterns of fatty acids.[10] These patterns can be identified with the use of principal component analysis (PCA) or other data reduction techniques. The study of fatty acid patterns is a complementary approach to examine individual fatty acids or groups of fatty acids and may provide new insights into the relation between fatty acid concentrations and health.[10]

Studies in adults have shown associations of different types of circulating or adipose tissue fatty acid patterns with weight changes,[11] metabolic syndrome,[12] arterial stiffness, and atherosclerosis.[13] Furthermore, we have previously shown that certain fatty acid patterns during pregnancy are associated with gestational weight gain.[14] However, fatty acid patterns during pregnancy have not yet been studied in relation to health of the offspring. The aim of this study was to assess whether women's plasma fatty acid patterns during pregnancy are associated with body composition and measures of cardiometabolic health of their children. In addition, we examined whether these associations differ by maternal body mass index, maternal ethnicity, child sex, or birthweight.

METHODS

Study design and population

This study was embedded in the Generation R Study, an ongoing population-based prospective cohort in Rotterdam, the Netherlands.[15-16] Pregnant women living in Rotterdam with an expected delivery date between April 2002 and January 2006 were invited to participate in the study. Of all women contacted, 61% provided written informed consent.[15] The study was approved by the Medical Ethics Committee of Erasmus Medical Center and was conducted in accordance with the Declaration of Helsinki. A total of 8,663 women were enrolled before 25 weeks of gestation. For 6,999 of these women we had information on complete plasma fatty acid status. Of this group, 6,925 women gave birth to singleton live-born children, of whom 4,830 visited our research center around the age of 6 years for measurements of body composition and cardiometabolic health (Figure 3.2.1).

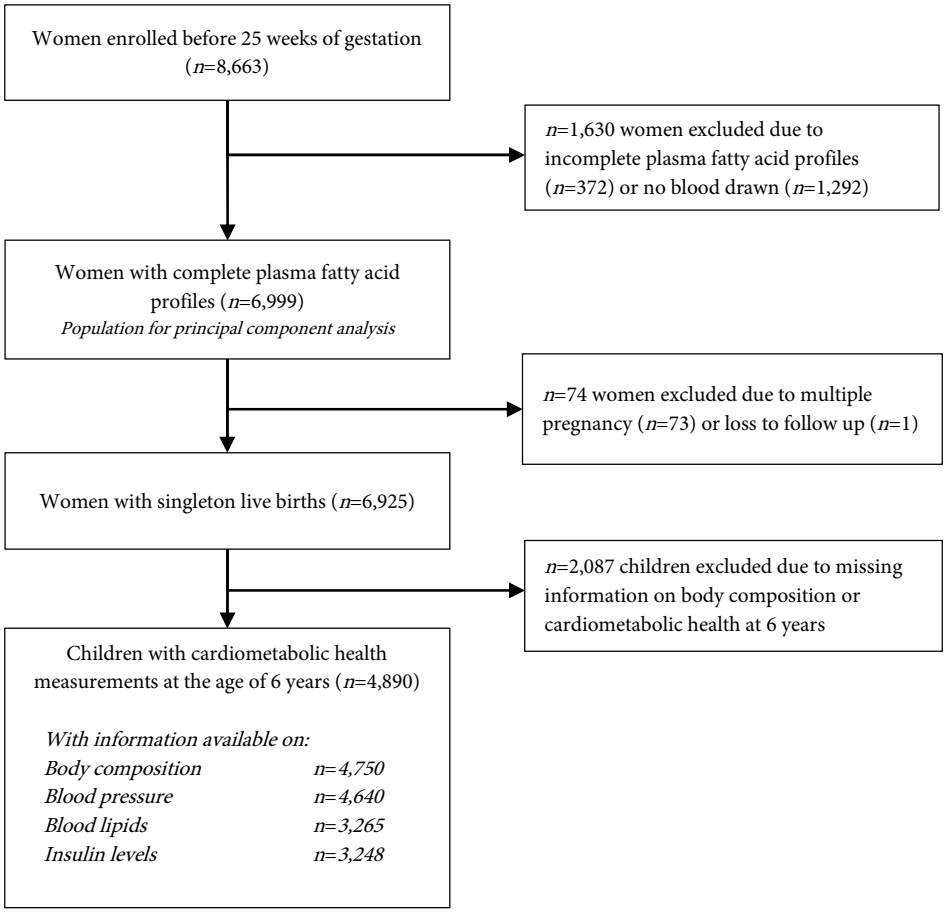

Figure 3.2.1 Flowchart of study participants included in the analysis

Identification of maternal fatty acid patterns

Non-fasting venous blood samples were collected in mid-pregnancy at a median gestational age of 20.5 weeks (95% range 16.5 to 24.9) and transported and stored as described previously.[16] For the fatty acid analyses, plasma samples were transported to the Division of Metabolic Diseases and Nutritional Medicine, Dr. von Hauner Children's Hospital, Ludwig-Maximilians-University of Munich, Germany. The fatty acid composition of plasma phosphoglycerides was analyzed using gas chromatography as described by Glaser *et al.*[17] The average coefficient of variation was 15.7%.[18]

We had information on the concentrations of 22 individual fatty acids, which were expressed as weight percentage (wt%) of total fatty acids present in the chromatogram (Table 3.2.1). Most fatty acid concentrations were correlated with each other.[14] As described in detail previously,[14] we performed a PCA on the concentrations of the 22 fatty acids among all women in our study with complete information on fatty acid profiles (*n*=6,999). Based on the scree-plot, an Eigenvalue ≥2

and the distinctive character of the principal components, we identified three plasma fatty acid patterns. On the basis of high factor loadings (≥0.20) for the respective fatty acids (Table 3.2.1), we named these patterns: 1) '*high n-6 PUFA'* pattern; 2) '*MUFA and SFA'* pattern; and 3) '*high n-3 PUFA'* pattern.[14]

The '*high n-6 PUFA'* pattern had high positive factor loadings for almost all *n*-6 fatty acids, except for a negative loading for linoleic acid (LA, C18:2*n*-6). This pattern also had negative loadings for eicosenoic acid (C20:1*n*-9), eicosapentaenoic acid (EPA, C20:5*n*-3), and docosahexaenoic acid (DHA, C22:6*n*-3) and positive loadings for myristic acid (C14:0) and palmitoleic acid (C16:1*n*-7). The '*MUFA and SFA'* pattern was characterized by positive factor loadings for all of the MUFAs, except for eicosenoic acid (C20:1*n*-9). In addition, this pattern had positive loadings for the SFAs myristic (C14:0) and palmitic acid (C16:0), but negative loadings for margaric (C17:0) and stearic acid (C18:0). Finally, the '*high n-3 PUFA'* pattern was characterized by positive factor loadings for all *n*-3 fatty acids, with the highest loadings (>0.5) for EPA (C20:5*n*-3), docasapentaenoic acid (DPA, C22:5*n*-3), and DHA (C22:6*n*-3). This pattern was also characterized by negative loadings for LA (C18:2*n*-6) and eicosadienoic acid (C20:3*n*-6), but positive loadings for arachidonic acid (ARA, C20:4*n*-6). The three fatty acid patterns together explained 39.2% of the variance in fatty acids (Table 3.2.1).[14]

Assessment of body composition and cardiometabolic health

Children's body composition and cardiometabolic health factors were measured at a median age of 5.9 years (95% range 5.6 to 6.6) in our dedicated research center at Erasmus Medical Center.[16] Height was determined with a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.) and weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). Total and regional body fat, lean, and bone mass were measured with a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA), using enCORE software version 13.6. We calculated body mass index (BMI) (weight (kg)/height $(m)^2$), fat mass index (FMI) (fat mass (kg)/height $(m)^2$), and fat-free mass index (FFMI) (fat-free mass (kg)/height $(m)^2$).[19] Body fat percentage (BF%) was calculated by expressing total fat mass as percentage of total body weight. Android fat mass was divided by gynoid fat mass to obtain the ratio (A/G ratio). We defined child's weight status as underweight, normal weight, or overweight on the basis of international age- and sex-specific BMI cut-offs.[20]

Non-fasting blood samples were obtained and concentrations of insulin, C-peptide, triacylglycerol (TAG), and total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) were measured with enzymatic methods (Cobas 8000, Roche, Almere, the Netherlands).[16] While the children were lying, systolic (SBP) and diastolic blood pressure (DBP) were measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmomanometer Datascope Accutorr Plus™ (Paramus, NJ, USA). We used mean SBP and mean DBP of the last three measurements. For all outcomes we calculated age- and sex-specific SD-scores (SDS) on the basis of data from the total Generation R Study population with cardiometabolic data available at the age of 6 years (*n* ranging from 4,414 to 6,491).[15]

In line with previous studies that defined scores for a metabolic syndrome-like phenotype in children[21] and as described in Chapter 2.3, we created a continuous score including five components: BF%, blood pressure, HDL-C, TAG, and insulin concentrations. The cardiometabolic score was calculated as: BF% SDS + 0.5 × SBP SDS + 0.5 × DBP SDS + TAG SDS + (-1 × HDL-C SDS) + insulin SDS, with a higher score reflecting less optimal cardiometabolic health.[22]

Table 3.2.1 Factor loadings of the individual fatty acids in fatty acid patterns

Fatty acids		Percentage of total FAs*	Fatty acid patterns		
			'high n-6 PUFA' pattern	*'MUFA and SFA'* pattern	*'high n-3 PUFA'* pattern
Saturated fatty acids					
Myristic acid	14:0	0.64	0.23	0.30	0.06
Palmitic acid	16:0	30.68	0.06	0.88	0.14
Margaric acid	17:0	0.36	-0.03	-0.31	0.03
Stearic acid	18:0	11.49	0.08	-0.84	-0.02
Monounsaturated fatty acids (cis)					
Pentadecenoic acid	15:1*n*-5	0.06	0.01	0.16	0.14
Palmitoleic acid	16:1*n*-7	0.68	0.43	0.63	0.16
Oleic acid	18:1*n*-9	10.30	0.19	0.29	0.21
Vaccenic acid	18:1*n*-7	1.46	0.00	0.28	0.08
Eicosenoic acid	20:1*n*-9	0.19	-0.25	-0.31	-0.09
Polyunsaturated fatty acids *n*-3					
α-Linolenic acid (ALA)	18:3*n*-3	0.30	-0.13	-0.03	0.09
Eicosatrienoic acid	20:3*n*-3	0.10	0.13	-0.07	0.16
Eicosapentaenoic acid (EPA)	20:5*n*-3	0.44	-0.33	0.06	0.69
Docasapentaenoic acid (DPA)	22:5*n*-3	0.70	0.09	-0.02	0.59
Docosahexaenoic acid (DHA)	22:6*n*-3	4.67	-0.39	0.06	0.67
n-6					
Linoleic acid (LA)	18:2*n*-6	22.37	-0.45	-0.45	-0.67
γ-linolenic acid (GLA)	18:3*n*-6	0.08	0.53	0.10	0.19
Eicosadienoic acid	20:2*n*-6	0.52	-0.06	-0.04	-0.69
Dihomo-γ-linolenic acid (DGLA)	20:3*n*-6	3.68	0.44	0.42	-0.19
Arachidonic acid (ARA)	20:4*n*-6	9.72	0.40	-0.15	0.30
Adrenic acid	22:4*n*-6	0.42	0.85	0.03	-0.08
Osbond acid	22:5*n*-6	0.47	0.82	0.04	-0.17
n-9					
Mead acid	20:3*n*-9	0.12	0.53	0.12	0.37
Explained variance (%)			14.4	12.7	12.2

* Median percentage in all 6,999 study participants with information available on fatty acid concentrations.
Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Covariates

We used questionnaires at enrollment to obtain information on maternal age, educational level (low; mid; or high), ethnicity (Western or non-Western), parity (0 or ≥1 child), pre-pregnancy weight, start of folic acid supplement use (periconceptionally; within the first 10 weeks of pregnancy; or not before 10 weeks of pregnancy), and on net household income (<1400; 1400 to <2200; or ≥2200 Euros per month). Maternal smoking and alcohol consumption during pregnancy were assessed using questionnaires in each trimester (both categorized into: never; quit in the first trimester; or continued). A food-frequency questionnaire (FFQ) was administered around 13.5 weeks of gestation to calculate daily food intake, energy intake and overall diet quality in accordance to the Dutch Health Diet-index.[23] Gestational weight gain was calculated as the difference in weight between 13 and 30 weeks of gestation, as measured in our research center.[14] Information on hypertensive disorders during pregnancy comorbidities (preeclampsia or pregnancy-induced hypertension) was obtained from medical records.[24]

Information on child sex, gestational age at birth, and birthweight was collected from delivery reports.[15] We calculated sex- and gestational age-specific Z-scores for birthweight on the basis of reference data.[25] Information on breastfeeding (never; partial; or exclusive in the first 4 months) was obtained from delivery reports and postnatal questionnaires. Information on children's food intake around the age of 1 year was obtained using an FFQ. We used a diet score (described in Chapter 5.1) to assess overall diet quality of these children.[26] Screen time (time spent watching television or using a computer) and participation in sports (yes/no) at the age of 6 years were assessed with a questionnaire.

Statistical analyses

Population characteristics were described as means, medians, or percentages. Fatty acid pattern scores are examined as continuous variables; these scores are standardized and can be interpreted as SD-scores. Among women with information on both fatty acid concentrations and diet in first trimester, we explored the variance in the fatty acid patterns explained by food intake in multivariable regression analyses.

We used multivariable linear regression analyses to assess the associations of maternal plasma fatty acid patterns with cardiometabolic health outcomes of the children. Because the fatty acid patterns are statistically uncorrelated, all patterns were included in the same model. In the basic model we additionally included gestational age at fatty acid measurement, and child's sex and age at outcome measurement (model 1). Potential confounders were selected based on theory and were included in model 2 on the basis of a significant change in effect estimates (≥10%) when added to model 1. Following these criteria, the confounder model was additionally adjusted for maternal age, ethnicity, educational level, pre-pregnancy BMI, folic acid supplement use, smoking and alcohol use during pregnancy, household income, gestational age-adjusted birthweight, and child's participation in sports at 6 years of age (model 2). The following covariates were not included because they did not meet the 10%-change criterion: maternal parity, maternal diet score, maternal caloric intake, pregnancy complications, breastfeeding, child dietary intake of SFA, MUFA or PUFA, child diet quality score, and child screen time.

Table 3.2.2 Characteristics of the mothers and their children (*n*=4,890)

Maternal characteristics	Mean ± SD, median (95% range), or %
Age (years)	30.4 ± 5.0
Western ethnic background	62.1%
Educational level	
Low	10.6%
Mid	43.5%
High	45.9%
Net household income	
<€1400/mo	26.5%
€1400 to <€2200/mo	20.2%
≥€2200/mo	53.3%
Nulliparous	57.2%
Pre-pregnancy body mass index (kg/m^2)	22.9 (18.3-34.1)
Gestational age at fatty acid measurement (weeks)	20.7 ± 1.2
Alcohol consumption during pregnancy	
Never	45.7%
Until pregnancy was known	14.0%
Continued	40.3%
Smoking during pregnancy	
Never	73.5%
Until pregnancy was known	9.4%
Continued	17.1%
Folic acid supplementation in early pregnancy	
Started periconceptional	41.3%
Started first 10 weeks	32.2%
No supplementation	26.5%
Total energy intake (kcal)	2051 ± 497
Gestational weight gain (kg)	7.3 ± 3.9
Hypertensive compilations during pregnancy	5.7%
Child characteristics	
Girls	50.2%
Birthweight (g)	3415 ± 568
Gestational age at birth (weeks)	40.1 (35.4-42.3)
Age at follow up (years)	6.0 (5.7-7.9)
Participation in sports (%)	44.2%
Screen time (h/d)	1.5 (0.2-4.8)
Overweight or obese (%)	17.5%
Body mass index (kg/m^2)	16.2 ± 1.9
Fat mass index (kg/m^2) (*n*=4,750)	4.1 ± 1.4
Fat-free mass index (kg/m^2) (*n*=4,750)	12.0 ± 0.9
Systolic blood pressure (mmHg) (*n*=4,640)	103 ± 8
Diastolic blood pressure (mmHg) (*n*=4,640)	61 ± 7
Insulin (pmol/L) (*n*=3,238)	114 (17-407)
HDL cholesterol (mmol/L) (*n*=3,265)	1.35 ± 0.31
Triacylglycerol (mmol/L) (*n*=3,252)	0.95 (0.39-2.35)

Values are percentages for categorical variables, means ± SD for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution; on the basis of unimputed data.

As potential mediators we evaluated gestational weight gain, birthweight, and – for the cardiometabolic outcomes only – height and body composition (FMI and FFMI) of the children, by adding them separately as covariates to model 2. Results after additional adjustment for the potential mediators FMI and FFMI are presented as model 3. Potential effect modification was evaluated for pre-pregnancy BMI, maternal ethnicity, and child sex and birthweight by adding an interaction term to model 2. We applied natural cubic splines to test for non-linearity of the associations of fatty acid patterns with the body composition and cardiometabolic outcomes in model 2.[27] Finally, in sensitivity analyses, we evaluated if results were altered if women who had experienced pregnancy complications (*n*=350) were excluded, because these complications may influence plasma fatty acid patterns.[14]

To reduce bias caused by missing information on confounders, missing values of covariates were multiple imputed (*n*=10 imputations) with the Fully Conditional Specification method (predictive mean matching).[28] We report the pooled regression coefficients after the multiple imputation procedure. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Subject characteristics and fatty acid patterns

Baseline characteristics of the participating women are presented in Table 3.2.2. Most women were of Western ethnic backgrounds (58.5%) and were nulliparous (57.2%) at enrollment in our study. Many of the women in our study population had a high educational level (45.9%) and a high household income (53.3%). Mean (±SD) BMI of their children at the age of 6 years was 16.2 (±1.9) kg/m^2, with 17.5% classified as overweight or obese. As described previously,[14] women with fatty acid pattern data available were slightly older, more highly educated and were more often of Western ethnicity than women without fatty acid data. Moreover, of those women with fatty acid pattern data, mothers of children who visited the research center at the age of 6 years were also slightly more highly educated, more often had a Western ethnicity, and had a higher household income than mothers of children who were not included in the current analyses. Among 5,238 women with information on both fatty acid patterns and diet, we examined the association between food intake and the patterns. On top of sociodemographic and other lifestyle factors, food intake explained an additional 7.5% of the variance in the *'high n-6 PUFA'* pattern, 2.3% in the 'MUFA and SFA' pattern, and 5.1% in the *'high n-3 PUFA'* pattern (Supplement 3.2.1). Fish intake was inversely associated with the *'high n-6 PUFA'* and positively with the *'high n-3 PUFA'* pattern.

Associations between maternal fatty acid patterns and child body composition

Associations of fatty acid patterns during fetal life with body composition at school age are described in Table 3.2.3. In model 1, we observed a positive association of the 'high *n*-6 PUFA' pattern with BMI, FMI and FFMI. These effect estimates attenuated after adjustment for confounders (model 2), and remained statistically significant only for BMI and FFMI (0.03 SD (95% CI 0.00, 0.06) for both). However, using cubic splines (*df*=3), we found a significantly improved model fit for a non-linear model versus a linear model for the association between the 'high *n*-6

PUFA' pattern and FMI (X^2=10.16, p<0.001) (Figure 3.2.2). Especially the children of mothers with lowest scores on this pattern during pregnancy had a lower FMI at the age of 6 years than the other children (Figure 3.2.2 and Supplement 3.2.2). The 'MUFA and SFA' pattern was not associated with body composition of the children. For the 'high *n*-3 PUFA' pattern, we observed that, after adjustment for socio-demographic and lifestyle factors (model 2), a 1 SD higher score on this pattern was associated with a 0.03 SD lower FMI (95% CI -0.06, -0.01) and a 0.04 SD higher FFMI (95% CI 0.01, 0.07) of the children, resulting in a null association with BMI. A higher score on this pattern was also associated with a lower android/gynoid fat mass ratio (Supplement 3.2.3).

Table 3.2.3 Associations of maternal fatty acid patterns with child body composition

Fatty acid pattern (per SD)	BMI (SDS) *n=4890*	FMI(SDS) *n=4750*	FFMI (SDS) *n=4750*
'high n-6 PUFA' pattern			
Model 1	**0.07 (0.04, 0.09)**	**0.07 (0.05, 0.10)**	**0.03 (0.01, 0.06)**
Model 2	**0.03 (0.00, 0.06)**	0.03 (-0.00, 0.05)	**0.03 (0.00, 0.06)**
'MUFA and SFA' pattern			
Model 1	-0.02 (-0.05, 0.01)	-0.03 (-0.06, 0.00)	-0.00 (-0.03, 0.02)
Model 2	-0.01 (-0.04, 0.01)	-0.01 (-0.03, 0.02)	-0.03 (-0.06, 0.00)
'high n-3 PUFA' pattern			
Model 1	**-0.06 (-0.09, -0.03)**	**-0.10 (-0.13, -0.08)**	**0.03 (0.01, 0.06)**
Model 2	-0.00 (-0.03, 0.03)	**-0.03 (-0.06, -0.01)**	**0.04 (0.01, 0.07)**

Values are based on multivariable linear regression models and reflect differences (95% CI) in child body composition outcomes (age- and sex-specific SDS) per SD higher fatty acid pattern score. Statistically significant effect estimates are indicated in **bold**.
Model 1 is adjusted for gestational age at fatty acid measurement, child sex, and child's age at body composition measurement.
Model 2 is additionally adjusted for maternal age, ethnicity, pre-pregnancy BMI, educational level, folic acid supplement use, smoking and alcohol consumption during pregnancy, household income, birthweight Z-score, and participation in sports at 6 years.
Abbreviations: BMI, body mass index; FMI, fat mass index; FFMI, fat-free mass index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SDS, standard deviation score; SFA, saturated fatty acid.

Associations between maternal fatty acid patterns and child cardiometabolic health

As shown in Table 3.2.4, after adjustment for confounders, a 1 SD higher score on the *'high n-6 PUFA'* pattern was associated with a lower HDL-C concentration (-0.04 SD (95% CI -0.08, -0.01)), but not with any of the other cardiometabolic outcomes (model 2). Furthermore, the inverse association with HDL-C was no longer statistically significant after adjustment for body composition (model 3). The *'MUFA and SFA'* pattern during pregnancy was not associated with cardiometabolic health of the offspring. A 1 SD higher score on the *'high n-3 PUFA'* pattern during fetal life was associated with 0.06 SD higher HDL-C (95% CI 0.03, 0.10) and with 0.04 SD lower TAG concentrations (95% CI -0.07, -0.00) in childhood. These two effect estimates did not change after additional adjustment for FMI and FFMI (model 3). We also observed a positive association with insulin concentrations in model 2, but this association was no longer significant after adjustment for body composition (model 3). Initial associations with a lower blood pressure and a lower cardiometabolic score in model 1 were explained by confounders. None of the fatty acid patterns were associated with total cholesterol, LDL-C, or C-peptide concentrations (Supplement 3.2.3). With respect to cardiometabolic outcomes, we found no indications of non-linear associations.

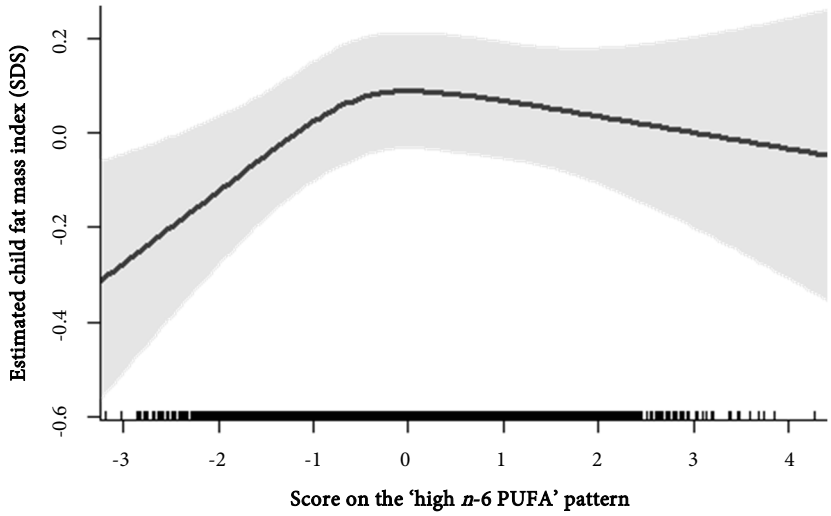

Figure 3.2.2 Estimated child fat mass index (SDS) given different scores on the 'high *n*-6 PUFA' pattern during pregnancy, while keeping all confounders in model 2 constant (Table 3.2.3).

Additional analyses

Additional adjustment for gestational weight gain or for child height did not change the results. Exclusion of birthweight from the multivariable model did not change the results for fatty acid patterns suggesting that the associations were not mediated by birth weight. We observed no interactions of the fatty acid patterns with fetal sex or with birthweight on any of the outcomes. There were significant as well as borderline significant interactions of maternal ethnicity with the *'high n-6 PUFA'* pattern on child FMI and FFMI ($p<0.01$) and with the *high n-6* pattern on child BMI ($p=0.02$), FMI ($p=0.06$), and FFMI ($p=0.05$). Results of analyses stratified for maternal ethnic background showed that the associations observed in the whole group were generally stronger (larger effect estimates) among women with a non-Western background than among those with a Western ethnic background (Supplement 3.2.4). We also observed an interaction between maternal prepregnancy BMI and the *'high n-6 PUFA'* pattern on DBP ($p=0.02$). Analyses stratified for maternal overweight status revealed that a higher score on this pattern tended to be associated with a higher DBP (0.06 SD (95% CI -0.01, 0.12)) in offspring of women who were overweight before pregnancy (BMI≥25), but not among children of women who were not overweight (BMI<25) (0.00 SD (95% CI -0.03, 0.04)). The exclusion of women with pregnancy complications did not meaningfully change the observed effect estimates.

Table 3.2.4 Associations of maternal fatty acid patterns with child cardiometabolic health

Fatty acid pattern (per SD)	BF% (SDS) *n=4750*	Insulin (SDS) *n=3238*	SBP (SDS) *n=4640*	DBP (SDS) *n=4640*	HDL-C (SDS) *n=3265*	Triacylglycerol (SDS) *n=3252*	Cardiometabolic risk factor score *n=3046*
'high n-6 PUFA' pattern							
Model 1	**0.07 (0.04, 0.10)**	-0.02 (-0.05, 0.02)	**0.05 (0.02, 0.08)**	**0.04 (0.01, 0.07)**	**-0.04 (-0.07, -0.01)**	0.00 (-0.03, 0.04)	**0.12 (0.03, 0.21)**
Model 2	0.02 (-0.01, 0.05)	-0.02 (-0.05, 0.02)	0.03 (-0.00, 0.06)	0.02 (-0.02, 0.05)	**-0.04 (-0.08, -0.01)**	0.00 (-0.03, 0.04)	0.05 (-0.05, 0.15)
Model 3	-	-0.02 (-0.06, 0.02)	0.02 (-0.01, 0.07)	0.02 (-0.02, 0.05)	-0.03 (-0.07, 0.01)	0.00 (-0.04, 0.03)	0.03 (-0.05, 0.11)
'MUFA and SFA' pattern							
Model 1	**-0.03 (-0.06, -0.00)**	0.00 (-0.03, 0.03)	-0.02 (-0.05, 0.01)	-0.01 (-0.04, 0.02)	**-0.05 (-0.08, -0.02)**	0.03 (-0.01, 0.06)	0.04 (-0.05, 0.13)
Model 2	0.00 (-0.03, 0.03)	-0.01 (-0.04, 0.03)	-0.02 (-0.05, 0.01)	0.01 (-0.02, 0.04)	-0.03 (-0.06, 0.01)	0.02 (-0.02, 0.06)	0.05 (-0.05, 0.15)
Model 3	-	-0.01 (-0.04, 0.03)	-0.01 (-0.04, 0.02)	0.01 (-0.02, 0.04)	-0.03 (-0.07, 0.01)	0.02 (-0.02, 0.06)	0.06 (-0.02, 0.15)
'high n-3 PUFA' pattern							
Model 1	**-0.12 (-0.15, -0.09)**	**0.06 (0.02, 0.09)**	**-0.04 (-0.06, -0.01)**	**-0.03 (-0.06, -0.01)**	**0.04 (0.00, 0.07)**	-0.03 (-0.06, 0.02)	**-0.12 (-0.22, -0.03)**
Model 2	**-0.05 (-0.07, -0.02)**	**0.05 (0.01, 0.09)**	-0.01 (-0.05, 0.02)	-0.00 (-0.03, 0.03)	**0.06 (0.03, 0.10)**	**-0.04 (-0.07, -0.00)**	-0.07 (-0.17, 0.02)
Model 3	-	0.04 (-0.00, 0.09)	-0.01 (-0.05, 0.02)	-0.00 (-0.03, 0.03)	**0.06 (0.02, 0.10)**	**-0.04 (-0.07, -0.00)**	-0.03 (-0.11, 0.06)

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex- adjusted SD-scores) and in cardiometabolic risk factor score per SD higher fatty acid pattern score. Statistically significant effect estimates are indicated in **bold**.

Model 1 is adjusted for gestational age at fatty acid measurement, child sex, and child's age at body composition measurement.

Model 2 is additionally adjusted for maternal age, ethnicity, pre-pregnancy BMI, educational level, folic acid supplement use, smoking and alcohol consumption during pregnancy, household income, birthweight Z-score, and participation in sports at 6 years.

Model 3 is additionally adjusted for child's FMI and FFMI SDS at 6 years.

Abbreviations: BF%, body fat percentage; BMI, body mass index; DBP, diastolic blood pressure; FFMI, fat-free mass index; FMI, fat mass index; HDL-C, high-density lipoprotein cholesterol; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SBP, systolic blood pressure; SDS, standard deviation score; SFA, saturated fatty acid.

DISCUSSION

Overall, in this population-based prospective cohort, we observed that patterns of fatty acid concentrations during pregnancy are associated with body composition and cardiometabolic health in the offspring at the age of 6 years. After adjustment for maternal and child sociodemographic and lifestyle factors, a pattern characterized by high concentrations of *n*-3 PUFAs was associated with a lower FMI and higher FFMI. Independent of its association with body composition, this pattern was also associated with higher HDL-C and lower TAG concentrations of the children. A pattern characterized by high *n*-6 PUFA concentrations was associated with a higher BMI and FFMI in the offspring, but not independently with cardiometabolic outcomes. Patterns characterized by high MUFA concentrations were not consistently associated with child cardiometabolic health.

Interpretation and comparison with previous studies

Our observation that a *'high n-3 PUFA'* pattern is associated with less adiposity in the offspring is in line with results of a few previous studies, including previous analyses in our own cohort, that indicate inverse associations between *n*-3 PUFAs in early life and later measures of adiposity.[5, 8, 29-30] However, several other studies observed a null or even a positive association[31-34] and as discussed in Chapter 3.1, overall evidence for an effect of *n*-3 PUFAs in early life on obesity is inconsistent.[9] Intervention studies in adults suggest that higher intakes of *n*-3 PUFAs may reduce body fat.[35] Several potential mechanisms have been proposed via which *n*-3 PUFAs may lower body fat mass, including a reduction in leptin concentrations[5] and thereby suppression of appetite; or an increase in fat oxidation and thereby an increase in energy expenditure.[35] In the prenatal period specifically, *n*-3 PUFAs may inhibit adipose tissue growth and adipocyte development of the fetus.[33, 36] This may have lasting effects on reducing later obesity risk as the acquisition of adipocytes has been suggested to be irreversible.[37]

In the current analyses, we observed an association of the *'high n-3 PUFA'* pattern with not only a lower FMI, but also with a higher FFMI. The pattern that we named *'high n-3 PUFA'* pattern was characterized by high levels of *n*-3 fatty acids, but also by high levels of ARA. The high ARA concentrations may have contributed to the positive associations of this pattern with FFMI, as a previous study showed that supplementation of preterm born infants with DHA and ARA led to a higher lean mass at the age of 1 year,[38] whereas another similar trial in which preterm infants received DHA and EPA-enriched formula observed a lower fat-free mass in the intervention group.[39] Further studies are needed to evaluate whether this effect can be attributed to ARA or to interactions of ARA with other fatty acids.

We also show an association of a higher score for the *'high n-3 PUFA'* pattern with more favorable blood lipid levels in the children: higher HDL-C and lower TAG concentrations. Previous studies also observed inverse associations between *n*-3 or total PUFAs in early life and TAG concentrations, but not with HDL-C.[9] Possible ways in which PUFAs may affect concentrations of cholesterol and TAG include a reduction of hepatic synthesis of TAG and very-low-density-lipoprotein (VLDL), changes in gene expression of factors involved in lipid metabolism, and an increase lipoprotein lipase activity.[40-42] We found no associations between any of the patterns and

BP and no consistent associations with insulin concentrations. A few previous studies reported associations between PUFAs in early life and measures of better insulin sensitivity,[7, 43] but overall evidence is scarce (Chapter 3.1).[9] Studies in adults reported antihypertensive effects of *n*-3 PUFAs, but primarily in older subject and in subjects with hypertension,[44-45] thus effects may not be present already among children.[9]

Most of the associations we initially observed between the *'high n-6 PUFA'* pattern and cardiometabolic health were explained by confounders. Previous studies suggested higher *n*-6 PUFAs concentrations are associated with more obesity,[30, 32] which may be mediated via increased preadipocyte differentiation.[36] However, several studies did not observe any associations.[5, 46] We observed a significant positive association of the *n*-6 *PUFA* pattern with a higher child BMI, however, the effect estimate was small and this higher BMI was caused by both a higher FMI and FFMI and thus not specifically with adiposity. However, we did observe non-linear associations: lowest prenatal scores on this pattern might be associated with lower FMI. In minimally adjusted models we observed associations of the *'high n-6 PUFA'* pattern with higher BP, lower HDL-C and high cardiometabolic risk factor score, but all associations were explained by confounding. Hence, our results do not support strong associations of this *'high n-6 PUFA'* pattern with adverse cardiometabolic health, but suggest that lower scores may be associated with a lower FMI. Future studies should explore the potential non-linear association of fatty acids and FMI.

The *'MUFA and SFA'* pattern that we identified was not consistently associated with cardiometabolic health of the offspring. This pattern was characterized by high levels of most of the MUFAs, but also by positive loadings for the SFAs myristic (C14:0) and palmitic acid (C16:0), and negative loadings for margaric (C17:0) and stearic acid (C18:0). In adults, higher levels of MUFAs have been associated with improved cardiometabolic health,[44-45, 47-51] whereas higher SFAs have been linked to higher cardiovascular risk.[10] The potentially opposing effects of the individual fatty acids may explain the null findings for the overall pattern. However, variations in MUFAs and SFAs during pregnancy may also be less important for child health because these variations may not reach the fetus.[52] Because of differences in placental transfer of different fatty acids, levels of maternal fatty acids may not fully reflect the levels of fatty acids that the fetus was exposed to.[53]

Overall, our results suggest that variations in fatty acid patterns, particularly patterns characterized by *n*-3 PUFAs, may be associated with offspring body composition and cardiometabolic health. Future studies should examine whether changing fatty acid patterns during pregnancy could improve body composition and cardiometabolic health of the offspring. If proven causal, further studies should focus on how fatty acid patterns during pregnancy can be optimized. Although variations in fatty acids will be to a large extent attributable to differences in genes and metabolism, diet can also play a role. We observed in our study population that up to 7.5% of the variation in the patterns was explained by differences in food intake, particularly in fish intake.

Methodological considerations

Major strengths of this study are the availability of plasma fatty acids and the application of a novel approach to evaluate patterns of these fatty acids, which takes into account that individual fatty acids are correlated.[10] A limitation is that we measured plasma fatty acids only once, whereas fatty

acid concentrations change over the course of pregnancy.[54-55] For this reason we adjusted for the gestational age of fatty acid measurement, but we were not able to examine whether changes in fatty acid patterns would be associated with cardiometabolic health of the offspring. However, a previous study in which PUFA concentrations were measured at multiple time points during pregnancy observed similar associations of PUFAs in different trimesters with child adiposity at the age of 7 years.[32] Another limitation is that we had no information on mothers' *FADS1* or *FADS2* genes, which encode for desaturase enzymes involved in the conversion of PUFAs.[56] Variations in these genes may influence the association between especially the two PUFA patterns and cardiometabolic health.

Other strengths of our study are its prospective population-based design and the large sample size. There was loss to follow-up with a selection toward an overall slightly higher educated and more affluent population with a Western ethnic background.[14] If the associations between maternal fatty acid patterns and child cardiometabolic health would be different between subjects included and subjects not included in the analyses, this may have led to bias in our observed effect estimates. For example, within our population for analysis we observed that associations of both the PUFA patterns with body composition were stronger in women with non-Western ethnic background than among those with a Western ethnicity. The selection toward a population with a higher proportion of Western women may thus have led to an underestimation of the observed effect estimates for body composition in the original study population.

Another strength of our study is that we performed detailed outcome measurements in the children. For body composition assessment we used DXA, which can precisely and accurately measure body fat and fat-free mass.[57] We had child blood samples to measure several metabolic markers. A limitation is that these blood samples were not collected in a fasting state. According to a large study in adults, fasting time had little influence on HDL-C levels, but variations for TAG levels were larger.[58] If we assume that fasting time of the children when they visited the research center was randomly distributed and not related to maternal fatty acid patterns, this measurement error of the outcome may have led to non-differential misclassification of TAG concentrations and therefore an underestimation of our effect estimates for TAG concentrations.

Finally, we had information available on many potential confounders. However, residual confounding may still occur, for example from unmeasured maternal lifestyle factors or from child fatty acid patterns. However, in the aforementioned study by de Vries *et al.*, adjustment for child fatty acid concentrations did not change the association of maternal PUFAs with child body composition,[32] suggesting that this may be an independent effect of fatty acid exposure in early life.

Conclusions

Results from this large cohort of children followed since pregnancy suggest that certain plasma fatty acid patterns during fetal life may be associated with later body composition and cardiometabolic health. Specifically, a pattern characterized by high concentrations of *n*-3 PUFAs was associated with a more favorable body composition and a more favorable lipid profile during childhood. Future studies could examine whether these associations are causal and what the underlying mechanisms are.

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SUPPLEMENT CHAPTER 3.2

Supplement 3.2.1 Associations of food group intake in first trimester and fatty acid patterns (*n*=5,389)

Food groups* (intake per 10 g/d)	***'high n-6 PUFA'* pattern**	***'MUFA and SFA'* pattern**	***'high n-3 PUFA'* pattern**
Fatty fish	-0.25 (-0.29,-0.21)	-*	0.17 (0.13,0.21)
Lean fish	-0.08 (-0.12,-0.05)	-*	0.10 (0.06, 0.14)
Fish products	0.11 (0.05, 0.16)	-*	-0.10 (-0.15, -0.04)
Mushrooms	-0.09 (-0.17,-0.02)	-*	-*
Cream	-0.14 (-0.26,-0.02)	-0.05 (-0.15, 0.05)	-*
Margarines	-*	-0.05 (-0.07,-0.03)	-*
Nuts and seeds	-*	-0.05 (-0.07,-0.02)	-*
Salty snacks	-*	-*	-0.15 (-0.19, -0.10)
Adjusted R^2 for the model without food intake	6.0	7.4	11.9
Adjusted R^2 for the model with food intake	13.5	9.7	17.0
Additional explained variance by food intake	7.5	2.3	5.1

* 48 food groups were included in the model, only food groups with regression coefficients >|0.05| are shown in the table

Values are based on multivariable linear regression models and reflect differences (95% CI) in fatty acid pattern scores per 100 g/d higher food group intake.

Models are adjusted for gestational age at fatty acid measurement, maternal age, pre-pregnancy BMI, educational level, folic acid supplement use, gestational weight gain, pregnancy complications, smoking and alcohol consumption during pregnancy, household income and all other food groups.

Supplement 3.2.2 Associations of quartiles of the 'high *n*-6 PUFA' pattern during pregnancy with child body composition

Fatty acid pattern (per SD)	**BMI (SDS)** ***n=4890***	**FMI (SDS)** ***n=4750***	**FFMI (SDS)** ***n=4750***	**BF% (SDS)** ***n=4750***
'high n-6 PUFA' pattern				
Quartile 1	**-0.08 (-0.16, -0.00)**	-0.07 (-0.15, 0.01)	**-0.08 (-0.16, -0.00)**	-0.05 (-0.14, 0.03)
Quartile 2	0.01 (-0.06, 0.08)	0.02 (-0.06, 0.10)	-0.02 (-0.10, 0.06)	0.02 (-0.06, 0.10)
Quartile 3	-0.00 (-0.08, 0.07)	0.01 (-0.06, 0.09)	-0.03 (-0.11, 0.05)	-0.01 (-0.09, 0.07)
Quartile 4	Reference	Reference	Reference	Reference

Values are based on multivariable linear regression models and reflect differences (95% CI) in child body composition outcomes (age- and sex-specific SD-scores) in different quartiles of the *n*-6 PUFA pattern compared to the highest quartile. Statistically significant effect estimates are indicated in **bold**.

Model is adjusted for gestational age at fatty acid measurement, child sex, and child's age at body composition measurement; and for maternal age, pre-pregnancy BMI, educational level, folic acid supplement use, smoking and alcohol consumption during pregnancy, household income, birthweight Z-score, and participation in sports at 6 years (model 2).

Abbreviations: BMI, body mass index; FFMI, fat-free mass index; FMI, fat mass index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SDS, standard deviation score; SFA, saturated fatty acid.

Supplement 3.2.3 Associations of maternal fatty acid patterns with cardiometabolic outcomes not included in the risk factor score

Fatty acid pattern (per SD)	A/G ratio (SDS) *n=4,750*	Total-C (SDS) *n=3,261*	LDL-C (SDS) *n=3,261*	C-peptide (SDS) *n=3,240*
'high n-6 PUFA' pattern				
Model 1	**0.05 (0.02, 0.08)**	-0.02 (-0.05, 0.02)	0.01 (-0.02, 0.04)	0.02 (-0.02, 0.05)
Model 2	0.01(-0.02, 0.04)	-0.02 (-0.05, 0.02)	0.00 (-0.03, 0.04)	-0.02 (-0.06, 0.01)
Model 3	-0.01 (-0.3, 0.02)	-0.02 (-0.06, 0.02)	0.00 (-0.04, 0.03)	-0.02 (-0.06, 0.01)
'MUFA and SFA' pattern				
Model 1	0.03 (-0.00, 0.06)	-0.03 (-0.06, 0.01)	-0.02 (-0.05, 0.02)	0.04 (-0.00, 0.07)
Model 2	**0.04 (0.01, 0.07)**	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	0.02 (-0.02, 0.05)
Model 3	**0.04 (0.02, 0.06)**	-0.01 (-0.05, 0.03)	-0.00 (-0.04, 0.04)	0.02 (-0.02, 0.05)
'high n-3 PUFA' pattern				
Model 1	**-0.10 (-0.13, -0.07)**	0.02 (-0.01, 0.06)	0.01 (-0.02, 0.05)	**0.06 (0.02, 0.09)**
Model 2	**-0.06 (-0.09, -0.03)**	0.04 (-0.00, 0.07)	0.02 (-0.02, 0.05)	0.03 (-0.01, 0.07)
Model 3	**-0.03 (-0.05, -0.01)**	0.04 (-0.00, 0.08)	0.02 (-0.01, 0.06)	0.03 (-0.00, 0.07)

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex-adjusted SDS) and in cardiometabolic risk factor score per SD higher fatty acid pattern score. Statistically significant effect estimates are indicated in **bold**.
Model 1 is adjusted for gestational age at fatty acid measurement, child sex, and child's age at body composition measurement.
Model 2 is additionally adjusted for maternal age, ethnicity, pre-pregnancy BMI, educational level, folic acid supplement use, smoking and alcohol consumption during pregnancy, household income, birthweight Z-score, and participation in sports at 6 years.
Model 3 is additionally adjusted for child FMI and FFMI SDS at 6 years.
Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SBP, systolic blood pressure; SDS, standard deviation score; SFA, saturated fatty acid.

Supplement 3.2.4 Associations of maternal fatty acid patterns with child body composition, stratified by maternal ethnicity

Fatty acid pattern (per SD)	BMI (SDS)	FMI (SDS)	FFMI (SDS)
Non-Western ethnic background	*n=1,850*	*n=1,792*	*n=1,792*
'high n-6 PUFA' pattern	**0.06 (0.00, 0.11)**	0.05 (-0.00, 0.10)	**0.05 (0.00, 0.10)**
'MUFA and SFA' pattern	-0.01 (-0.06, 0.05)	0.01 (-0.05, 0.06)	-0.04 (-0.09, 0.01)
'high n-3 PUFA' pattern	-0.01 (-0.06, 0.04)	**-0.07 (-0.12, -0.01)**	**0.09 (0.04, 0.14)**
Western ethnic background	*n=3,040*	*n=2,958*	*n=2,958*
'high n-6 PUFA' pattern	0.01 (-0.02, 0.04)	0.01 (-0.02, 0.04)	0.03 (-0.00, 0.06)
'MUFA and SFA' pattern	-0.01 (-0.04, 0.02)	-0.01 (-0.04, 0.02)	-0.03 (-0.06, 0.00)
'high n-3 PUFA' pattern	0.01 (-0.02, 0.04)	-0.01 (-0.04, 0.03)	**0.04 (0.01, 0.07)**

Values are based on multivariable linear regression models and reflect differences (95% CI) in child body composition outcomes (age- and sex-specific SDS) per SD higher fatty acid pattern score. Statistically significant effect estimates are indicated in **bold**.
Women with a non-Western ethnic background had slightly lower scores on the 'MUFA and SFA' and on the 'high *n*-3 PUFA' patterns than women with a Western ethnic background, and similar scores on the 'high *n*-6' pattern.
Model is adjusted for gestational age at fatty acid measurement, child sex, and child's age at body composition measurement; and for maternal age, pre-pregnancy BMI, educational level, folic acid supplement use, smoking and alcohol consumption during pregnancy, household income, birthweight Z-score, and participation in sports at 6 years (model 2).
Abbreviations: BMI, body mass index; FFMI, fat-free mass index; FMI, fat mass index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SDS, standard deviation score; SFA, saturated fatty acid.

Chapter 3.3

Fatty acid intake in early childhood & cardiometabolic health

Manuscript based on this chapter:

Wendy Stroobant*, Kim V.E. Braun*, Jessica C. Kiefte-de Jong, Vincent W.V. Jaddoe, Oscar H. Franco, Trudy Voortman. Fatty acid intake in early childhood and cardiometabolic health at school age: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Studies in adults have indicated that a higher saturated fatty acid and a lower unsaturated fatty acid intake are associated with increased cardiovascular disease risk. However, studies on fat intake in relation to cardiometabolic health during childhood are scarce. We aimed to study the associations between the intake of different types of fatty acids at the age of 1 year and cardiometabolic health at 6 years of age.

Methods: This study was performed in 2,927 children participating in the Generation R Study, a prospective population-based cohort in Rotterdam, the Netherlands. When the children were 1 year old, we measured their dietary intake of total fat, saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) using a semi-quantitative food-frequency questionnaire which was filled out by the parents. At 6 years of age, children's body fat percentage, diastolic and systolic blood pressure, and insulin, triacylglycerol and HDL cholesterol blood concentrations were measured. These outcomes were combined into a cardiometabolic risk factor score.

Results: In multivariable macronutrient substitution models, we observed no association of a higher total fat intake or of a higher intake of SFA, MUFA, or PUFA with cardiometabolic health, when fat was consumed at the expense of carbohydrates. In subsequent models, also no associations were observed for MUFA or PUFA intake at the expense of SFA with any of the individual cardiometabolic outcomes or the combined cardiometabolic risk factor score.

Conclusion: The results of this study indicate that there is no association between the intake of different types of fatty acids in early childhood and body composition or cardiometabolic health at 6 years of age. Additional studies in early childhood, using more detailed dietary assessment methods, are needed to further investigate the associations between fatty acid intake and cardiometabolic health in children.

INTRODUCTION

Well-known risk factors for cardiometabolic diseases include obesity, dyslipidemia, elevated blood pressure, and insulin resistance. A clustering of these risk factors is often referred to as the metabolic syndrome.[1] Several studies have suggested that the development of cardiometabolic risk factors already begins in early life and that these risk factors track during further life.[2-4] Therefore, gaining knowledge about factors that may influence cardiometabolic health among children is highly relevant for early prevention of later cardiometabolic diseases such as cardiovascular disease and type 2 diabetes.

Several studies among adults have reported associations for dietary intake of different types of fatty acids with cardiometabolic risk factors and cardiometabolic disease risk.[5-8] Overall, these studies suggest that a lower saturated fatty acid (SFA) intake, when replaced by a higher polyunsaturated fatty acid (PUFA) or monounsaturated fatty acid (MUFA) intake, may reduce coronary heart disease risk and provide cardiometabolic benefits.[8-9] For PUFA intake some studies further suggest differential effects of omega-3 (*n*-3) and omega-6 (*n*-6) PUFAs on cardiometabolic health.[9-11] Based on these studies, dietary guidelines for adults generally advise to lower SFA intake and increase PUFA intake.[9]

In contrast to dietary recommendations for adults, no clear dietary guidelines are available regarding fat intake for young children, because evidence on the health effects of fatty acid intake in early life is scarce.[12] A few studies among school-aged children have indicated that the intake of different types of fatty acids may be associated with certain cardiometabolic risk factors,[13-15] but studies on fat intake in early childhood are scarce. Therefore, the aim of this study was to examine the association between the intake of different types of fatty acids in children at the age of 1 year and their body composition and cardiometabolic health at 6 years of age, in a prospective cohort study.

METHODS

Study design and population

This study was embedded in the Generation R Study, a prospective cohort conducted in Rotterdam, the Netherlands. In this study, participants have been followed from fetal life onward.[16] The study was approved by the Medical Ethics Committee of Erasmus Medical Center in Rotterdam and parents of all children provided written informed consent. In total, 7,893 children were available for postnatal follow-up studies.[16] To determine the child's dietary intake, mothers of 5,088 children received a food-frequency questionnaire (FFQ) and for 3,629 of them we received complete and valid food intake data.[17-18] Of these children, 2,967 visited the research center around the age of 6 years and had data about at least one of the cardiometabolic outcomes (Figure 3.3.1).

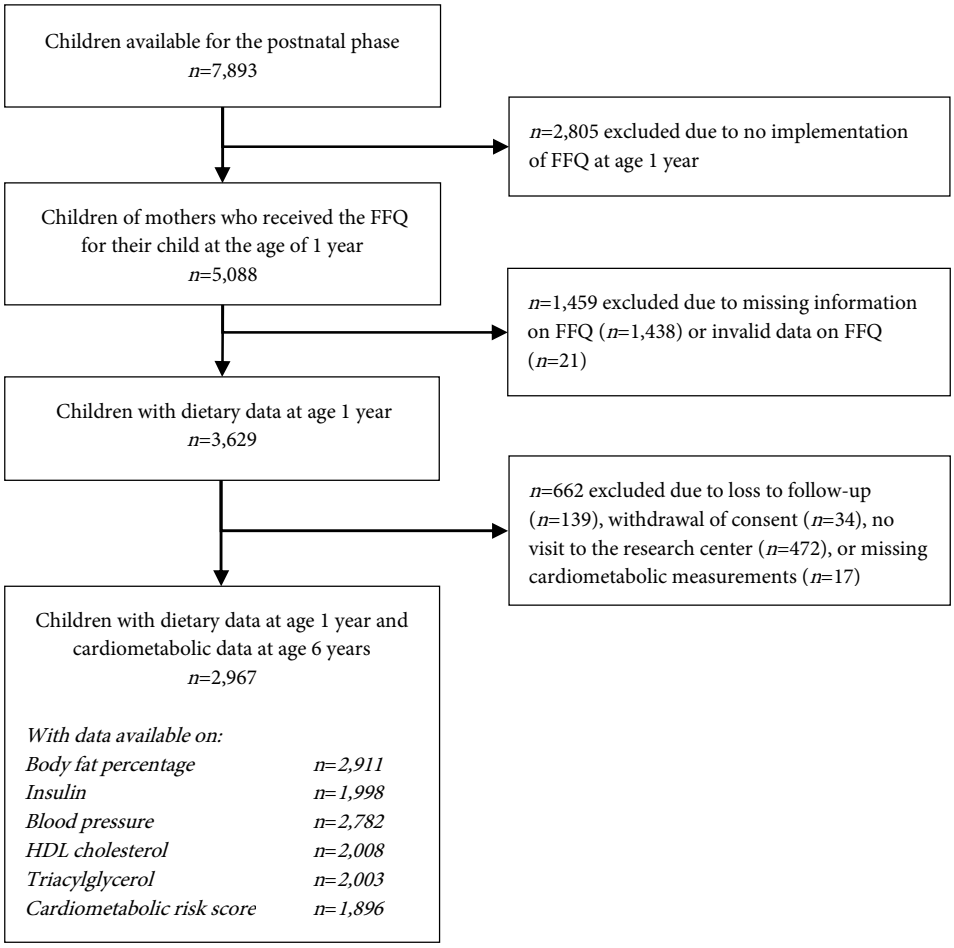

Figure 3.3.1 Flowchart of participants included for analysis

Dietary assessment

Food intake was assessed at a median age of 12.9 months (95% range: 12.2 to 18.9), using a semi-quantitative FFQ. This questionnaire was completed by the parents or caregivers of the children. The FFQ was specifically designed for this age group and contained 211 food items.[17] The FFQ was evaluated against three 24-hour recalls and the intraclass correlation coefficient for total fat intake was 0.4 (further details in Chapter 5.1).[17-18] With the use of the Dutch Food Composition Database (2006), we calculated the intake of total fat, SFA, MUFA, PUFA, *n*-3 PUFA, and *n*-6 PUFA.[19] For our macronutrient substitution models, we also calculated intake of other macronutrients and we calculated energy from total fat minus energy from the fatty acids (i.e., from glycerol, sterols, and phospholipids) and energy from total PUFA minus *n*-3 and *n*-6 PUFA.[20] The intake of macronutrients was expressed as percentage of total energy intake.

Cardiometabolic health assessment

Children's anthropometrics and cardiometabolic outcomes were assessed at a median age of 6.0 years (95% range: 5.7-6.6) in a dedicated research center in the Sophia Children's Hospital at Erasmus Medical Center in Rotterdam. Body height and weight were measured and BMI was calculated (kg/m^2). Body fat mass was measured with a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA) and was analyzed with enCORE software (version 13.6).[21] Body fat percentage (BF%) was calculated by expressing total fat mass as percentage of total body weight. Blood pressure was measured four times at the right brachial artery using a validated automatic sphygmomanometer (Datascope Accutorr Plus^{TM}, Paramus, NJ, USA). The mean of the last three measurements was calculated and used for analyses. Non-fasting venous blood samples were obtained and concentrations of insulin, triacylglycerol (TAG), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), total cholesterol, and C-peptide were determined. For all cardiometabolic outcomes age- and sex- specific SD-scores were calculated.[16]

A cardiometabolic risk factor score was created as the sum as SD-scores from BF%, SBP, SBP, HDL cholesterol concentrations, insulin concentrations, and TAG concentrations (Chapter 2.3).[22-23] The SD-scores for HDL-C were multiplied by -1, because higher HDL-C concentrations reflect lower cardiometabolic risk. The SD-scores for SBP and DBP were halved to represent one cardiometabolic risk factor for blood pressure. A higher cardiometabolic risk factor score is indicative of a higher cardiometabolic risk. For our analyses, the cardiometabolic risk factor score and the individual components of this score were selected as primary cardiometabolic outcomes of interest.

Covariates

Information about maternal age, maternal educational level, net household income, and parity was collected via self-administered questionnaires at enrollment. Maternal highest finished educational level was classified into no or primary education, middle school or less than 4 years of high school, or higher education. Net household income was categorized into <1400, 1400 to 2200, or ≥2200 Euros per month. Parity was categorized into 0, 1, or 2 or more. Information on smoking and consuming alcohol during pregnancy was obtained through questionnaires in each trimester and both variables were categorized into never, until pregnancy was known, or continued during pregnancy.

Information on children's sex, birthweight, and gestational age was available from obstetric records.[16] Sex- and gestational age-specific z-scores for birthweight were determined in accordance with reference data.[24] The child's ethnicity was defined as Dutch or non-Dutch on the basis of countries of birth of the parents.[16] Information on breastfeeding was available from postnatal questionnaires and was categorized into never, partial, or exclusively for at least 4 months. Screen time, including watching television or using a computer, and participation in sports were assessed with parental questionnaires when the children were 6 years old. Screen time was calculated into hours per day and participation in sports was defined as 'yes' or 'no'.

Statistical analyses

The cardiometabolic outcomes were checked for normality with histogram normality plots. Insulin and TAG concentrations had skewed distributions and therefore TAG levels were log-transformed and insulin levels were square-root transformed before calculating SD-scores. The associations between the intake of fatty acids and cardiometabolic outcomes were analyzed with linear regression analyses. To test for non-linearity, we applied natural cubic splines with 3 degrees of freedom.[25] We found indications for a non-linear association only between PUFA intake and the cardiometabolic score ($p<0.05$). Therefore we examined PUFA intake categorized in quartiles with the lowest quartile as reference, in addition to analyzing PUFA intake as continuous variable.

To account for the effect of energy intake and substitution effects of macronutrients in the diet, two different multivariable nutrient density substitution models were used.[26] All nutrients were entered in the model per 5 energy percent (E%). Firstly, we examined the association of fat intake at the expense of carbohydrates with cardiometabolic outcomes. In order to study this we included total energy, total fat and protein intake in the same model. Similarly, in order to examine the associations of the different types of fatty acids, we included SFA, MUFA, and PUFA in one model, together with energy intake and intake of all other macronutrients except for carbohydrates. Because total carbohydrate intake is the only macronutrient left out of these models, coefficients for the fat components can be interpreted as the effect of a 5 E% higher intake of total fat, SFA, MUFA or PUFA when consumed at the expense of carbohydrate intake. Secondly, we examined the association of MUFA and PUFA intake with cardiometabolic health at the expense of SFA. In these models, energy, carbohydrate, protein, total fat minus fatty acids, MUFA and PUFA intake were included in one model, i.e., all energy sources except for SFA. As additional analysis, we also examined *n*-3 and *n*-6 PUFA intake separately, by replacing total PUFA with *n*-3 PUFA, *n*-6 PUFAs, and energy from remaining PUFAs in the models described above. To be able to compare effects estimates of different models, these nutrients were also expressed per 5 E%, even though the average intake of *n*-3 PUFAs in our population was less than 0.5 E%.

The crude substitution models were adjusted for sex, ethnicity, and age at filling out the FFQ. In this model sex, ethnicity, age at FFQ, and birthweight were tested as possible effect modifiers, but none of the interaction terms were statistically significant. Other covariates were included when adding them to the crude model resulted in changes in effect estimates of ≥10%. Based on this criterion, multivariable models were additionally adjusted for maternal BMI, maternal age, maternal alcohol consumption during pregnancy, maternal smoking during pregnancy, maternal educational level, household income, parity, breastfeeding, birthweight Z-score, child's screen time, and playing sports at 6 years of age. As sensitivity analyses, we repeated analyses among children with a Dutch ethnic background only, and we repeated analyses of the first model in which we replaced protein intake by carbohydrate intake in the first model.

To increase statistical power and to reduce bias due to missing data, missing values of covariates (ranging from 0 to 19%) were multiple imputed (10 imputations).[27] We report the pooled regression coefficients after the multiple imputation procedure. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Table 3.3.1 Population characteristics (*n*=2,967)

	Mean ± SD, median (95% range) or %
Maternal characteristics	
Maternal age (years)	31.5 ±4.5
Maternal BMI at intake (kg/m^2)	23.5 (18.8-35.3)
Nulliparous	60.5%
Educational level	
No or primary education	5.5%
Middle school or < 4 years of high school	36.0%
Higher education	58.6%
Net household income	
<€1400	13.9%
€1400 to <€2200/mo	19.4%
≥€2200/mo	66.6%
Smoking during pregnancy	
Never	77.3%
Until pregnancy was known	10.7%
Continued	11.9%
Alcohol consumption during pregnancy	
Never	37.7%
Until pregnancy was known	16.4%
Continued	45.9%
Child characteristics	
Sex (% boys)	48.7%
Dutch ethnicity	68.6%
Gestational age at birth (weeks)	40.1 (35.6-42.3)
Birthweight (g)	3,452 ± 569
Breastfeeding in the first 4 months	
Exclusive	29.8%
Partial	59.9%
Never	10.3%
Child characteristics at dietary intake assessment	
Age at FFQ (months)	12.9 (12.2-18.9)
Total energy intake (kcal)	1,310 ± 388
Total fat intake (g/d)	39.0 (17.1-85.8)
SFA intake (g/d)	14.2 (4.32-33.9)
MUFA intake (g/d)	11.8 (3.68-29.9)
PUFA intake (g/d)	7.31 (2.64-15.8)
n-3 PUFA intake (g/d)	0.5 (0.1-1.6)
n-6 PUFA intake (g/d)	4.1 (0.9-12.2)
Child characteristics at 6-year visit	
Age (years)	5.9 (5.6-6.6)
BMI (kg/m^2)	16.0 ±1.6
Screen time (h/d)	1.25 (0.25-4.71)
Participation in sports	44.9%

Values are percentages for categorical variables, means ± SD for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution; on the basis of unimputed data.

Subject characteristics

Maternal characteristics, child characteristics, dietary intake and cardiometabolic outcomes are presented in Table 3.3.1. More than half of the children were girls (51.3%) and most children had a Dutch ethnic background (68.8%). Mothers were on average 31.6 (±4.5) years old at enrollment and most mothers had a high educational level (59.4%). Mean (± SD) total energy intake of the children was 1,310 (±388) kcal per day and 28.6 (±5.6) E% was derived from fat. This is in line with WHO recommendations, in which a total fat intake of maximal 35 E% is advised depending on the level of physical activity of the child.[28] The mean intake of SFA in our population was 10.4 E% (±3.1), of MUFA 8.8 E% (±2.8), and of PUFA 5.3 E% (±1.5).

Associations between fatty acid intake and cardiometabolic health

A higher total fat intake or a higher intake of SFA, MUFA, or PUFA at the age of 1 year at the expense of carbohydrates was not associated with any of the cardiometabolic outcomes at 6 years of age. For the analysis with PUFA intake in quartiles also no significant associations were observed (data not shown). When we subsequently examined intake of *n*-3 and *n*-6 PUFA separately, we observed that – at the expense of carbohydrates – a higher intake of *n*-3 PUFA was associated with higher insulin and lower triacylglycerol concentrations, and that higher *n*-6 PUFA intake was associated with lower insulin levels (Table 3.3.2).

Substitution models in which we examined the substitution of SFA by unsaturated fatty acids showed similar results as observed for carbohydrate substitution models: Neither MUFA nor total PUFA intake were associated with any of the cardiometabolic outcomes at the age of 6 years, but a higher intake of *n*-3 PUFAs was associated with higher insulin and lower triacylglycerol concentrations (Table 3.3.3).

The results of the crude associations between different types of fat intake and cardiometabolic health are shown in Supplement 3.3.1. In these models, a 5 E% higher SFA intake, at the expense of carbohydrates, was associated with 0.189 SD (95% CI -0.349, -0.029) lower insulin concentrations. Other results were similar to the results of the adjusted model.

Additional analyses

Sensitivity analyses in the children with a Dutch ethnic background only showed similar effect estimates as observed in the whole population. A higher total fat, PUFA, or MUFA intake at the expense of protein was associated with a lower body fat percentage, but not with any of the other outcomes, which is in line with results for protein intake we described previously (Chapter 2.3).[23] Analyses with secondary cardiometabolic health outcomes showed that a 5 E% higher SFA intake at the expense of carbohydrates was associated with a 0.172 (95% CI -0.334, -0.010) lower C-peptide concentration, whereas a 5 E% higher MUFA intake at the expense of carbohydrates was associated with a 0.209 (95% CI 0.003, 0.416) higher C-peptide concentration. No significant associations were found between different types of fatty acid intake, at the expense of carbohydrates, and BMI, total cholesterol, or LDL cholesterol concentrations (Supplement 3.3.2).

Table 3.3.2 Adjusted associations between fat intake, at the expense of carbohydrates at the age of 1 year with cardiometabolic health at 6 years of age

Fat intake per 5 E%	BF% (SDS) *n=2911*	SBP (SDS) *n=2843*	DBP (SDS) *n=2843*	HDL (SDS) *n=2008*	Insulin (SDS) *n=1998*	TAG (SDS) *n=2003*	Cardiometabolic score *n=1896*
Total fat	-0.01	0.02	-0.01	0.03	-0.01	-0.02	-0.08
	(-0.04, 0.014	(-0.01, 0.05)	(-0.04, 0.03)	(-0.02, 0.07)	(-0.05, 0.03)	(-0.07, 0.02)	(-0.18, 0.03)
SFA	-0.03	0.07	0.07	0.04	-0.16	-0.07	-0.21
	(-0.14, 0.09)	(-0.06, 0.21)	(-0.06, 0.21)	(-0.12, 0.21)	(-0.32, 0.01)	(-0.24, 0.09)	(-0.62, 0.20
MUFA	0.02	-0.07	-0.13	-0.01	0.16	0.03	0.04
	(-0.12, 0.16)	(-0.24, 0.11)	(-0.30, 0.04)	(-0.22, 0.20)	(-0.05, 0.37)	(-0.18, 0.24)	(-0.48, 0.56)
PUFA	-0.079	0.11	0.12	0.05	-0.06	-0.04	-0.02
	(-0.23, 0.05)	(-0.05, 0.272)	(-0.04, 0.28)	(-0.14, 0.25)	(-0.26, 0.13)	(-0.23, 0.15)	(-0.50, 0.47)
n-3 PUFA	0.13	-0.23	0.14	1.13	**2.14**	**-3.10**	-0.16
	(-1.10, 1.36)	(-1.63, 1.16)	(-1.21, 1.48)	(-0.67, 2.94)	**(0.30, 3.98)**	**(-4.90, -1.30)**	(-4.85, 4.53
n-6 PUFA	-0.10	0.13	0.05	-0.048	**-0.26**	0.23	0.02
	(-0.27, 0.07)	(-0.06, 0.32)	(-0.14, 0.23)	(-0.30, 0.20)	**(-0.52, -0.01)**	(-0.02, 0.48)	(-0.57, 0.61)

Values are based on covariate-adjusted linear regression models and reflect differences (95% CI) in cardiometabolic outcomes (age- and sex specific SD-scores) per 5 E% higher intake of a specific fat, at the expense of carbohydrates. See footnote Table 3.3.3

Table 3.3.3 Adjusted associations for MUFA and PUFA intake at the expense of SFA at the age of 1 year with cardiometabolic health at 6 years of age

Fat intake per 5 E%	BF% (SDS) *n*=2911	SBP (SDS) *n*=2843	DBP (SDS) *n*=2843	HDL (SDS) *n*=2008	Insulin (SDS) *n*=1998	TAG (SDS) *n*=2003	Cardiometabolic score *n*=1896
MUFA	-0.11	-0.21	-0.02	-0.05	0.32	0.10	0.25
	(-0.24, 0.08)	(-0.50, 0.09)	(-0.14, 0.10)	(-0.41, 0.30)	(-0.04, 0.68)	(-0.25, 0.46)	(-0.65, 1.15
PUFA	-0.21	0.05	0.09	0.01	0.09	0.04	0.20
	(-0.46, 0.24)	(-0.11, 0.20)	(-0.05, 0.23)	(-0.18, 0.20)	(-0.10, 0.28)	(-0.15, 0.22)	(-0.28, 0.68)
n-3 PUFA	0.16	-0.28	0.33	1.06	**2.28**	**-3.02**	0.06
	(-1.06, 1.38)	(-1.75, 1.19)	(-1.11, 1.77)	(-0.73, 2.85)	**(0.45, 4.11)**	**(-4.81, -1.23)**	(-4.60, 4.72)
n-6 PUFA	-0.07	0.09	0.03	-0.12	-0.12	0.31	0.22
	(-0.25, 0.11)	(-0.13, 0.31)	(-0.18, 0.24)	(-0.38, 0.14)	(-0.38, 0.14)	(-0.05, 0.67)	(-0.46, 0.89)

Values are based on covariate-adjusted linear regression models and reflect differences (95% CI) in cardiometabolic outcomes (age- and sex specific SD-scores) per 5 E% higher intake of a specific fat, at the expense of saturated fat. **Bold** values indicate statistically significant results ($p<0.05$).

To use the nutrient density substitution model, models were adjusted for total energy intake, intake of protein, intake of carbohydrates and intake of other components of fat, and the different types of fat intake (MUFA and PUFA) were adjusted for each other. Models were additionally adjusted for age, sex, ethnicity, maternal education, net household income, parity, maternal BMI at intake, maternal age at intake, smoking during pregnancy, drinking alcohol during pregnancy, breastfeeding, birthweight adjusted for sex and gestational age, screen time at 6 years of age and playing sports at 6 years of age.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL, high-density-lipoprotein; E%, energy percent; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SBP, systolic blood pressure; SDS, Standard deviation score; TAG, triacylglycerol.

DISCUSSION

The aim of this study was to examine the associations between different types of fatty acid intake in children at the age of 1 year and their cardiometabolic health at the age of 6 years. Overall, our results show no consistent association between intake of total fat, or different types of fatty acids, at the expense of either carbohydrates or saturated fatty acids with cardiometabolic outcomes age 6 years.

Interpretation and comparison with previous studies

One large intervention study focused on similar outcome variables as our study. The Special Turku coronary Risk factor Intervention Project (STRIP) study investigated the influence of low-saturated fat counseling versus no counseling in over one thousand children from 7 months of age on their cardiometabolic outcomes in later life. Outcomes in the STRIP study included metabolic syndrome, DBP and SBP, insulin sensitivity and cholesterol levels at different ages. In several follow-up studies in later childhood or adolescence, a beneficial effect of the intervention was observed for many of these outcomes.[15, 29-31] In contrast to the findings of the STRIP study, in our study we did not find associations between SFA intake and cardiometabolic health. However, because the intervention in the STRIP study consisted of dietary counseling it is not certain whether the effects were caused by the low-saturated fat diet or other effects of the long-term lifestyle advice. For example, in one of the analyses it was shown that the intervention group had a lower HOMA-IR, but actual SFA intake was not significantly associated with HOMA-IR in multivariable analysis.[31] Besides, dietary counseling in the STRIP study remained up to 20 years of age, and observed effects may therefore also be caused by dietary changes in later childhood rather than in early childhood.

A few other observational studies focused on dietary fat intake in young children in relation to their BMI. In previous analyses in our study population, Heppe et al. (2013) observed that higher PUFA intake at the age of 1 year was associated with a lower BMI at 4 years of age – an earlier follow-up measurement in our cohort.[32] Considering that we did not find this association with BF% or with BMI at 6 years of age might suggest that the potential effects of fat intake fade away after a longer follow-up period, as we have previously seen for protein intake in early childhood in relation to repeatedly measured BMI.[33] In line with our results, Williams *et al.* (2008), who performed a prospective study in 519 children, did not find any significant associations between SFA or MUFA intake at 3-4 years of age and BMI at 7-10 years.[34] Also Agostoni *et al.* (2000), who measured dietary intake at the ages of 1 and 5 years and BMI at age 5 years in 147 children, observed that total fat, SFA, MUFA or PUFA intake at neither 1 nor 5 years were associated with BMI at 5 years of age.[35]

A few other studies investigated the association between fatty acid intake with cholesterol or TAG concentrations in children. Contrary to our findings, Cowin *et al.* (2000) observed that a higher intake of PUFA or SFA at the age of 18 months was associated with a lower HDL cholesterol concentration at the age of 31 months.[36] However, this association was only present among girls and not among boys. Moreover, among boys, but not girls, a higher total fat intake or SFA intake was associated with a higher total cholesterol concentration.[36] In our population, we found no significant interaction between child sex and fatty acid intake on cardiometabolic health. In line with our results, the study of Williams *et al.* (2008) did not find any association between SFA or MUFA intake at 3-4 years of age and total cholesterol, HDL cholesterol or TAG concentrations at

7-10 years.[34] However, cross-sectional data from the same study population showed that MUFA, but not SFA intake, at age 7-10 years were inversely associated with cholesterol and TAG levels.[34]

In our study no association was found for PUFA intake and cardiometabolic health. One possible explanation for the fact that we did not find associations with PUFA might be that the different PUFA subtypes have different effects on cardiometabolic health. Therefore, we additionally examined the association for *n*-3 and *n*-6 fatty acid intake at the age of 1 year in relation to cardiometabolic outcomes at the age of 6 years. We found that a higher *n*-3 PUFA intake was associated with lower TAG and higher insulin concentrations, and a higher *n*-6 intake with lower insulin levels, irrespective of whether the PUFAs were replacing carbohydrates or SFAs. The result for TAG is in line with several previous studies which suggests that higher *n*-3 PUFA intake is associated with lower TAG concentrations.[37-38] However, evidence on potential opposing effects of *n*-3 and *n*-6 PUFAs on insulin levels or other cardiometabolic outcomes is inconsistent.[10-11, 39-40]

Methodological considerations

Our study had several strengths and limitations. An important strength is the large sample size. Moreover, compared with other studies, a strength of our study is that we accounted for the effect of energy intake and substitution effects by using multivariable nutrient density substitution models. Another strength is the large number of variables measured in this cohort, which made it possible to test for many potential confounders and effect modifiers. A limitation of this study is the dietary assessment method. Although many food items were included in the FFQ and it was therefore was expected to make a good estimation of fat intake, the intraclass correlation coefficient for total fat intake measured with our FFQ against three-day 24-h recalls as reference method was only 0.4. This indicates measurement errors in our assessment of fat intake in early childhood. However, the reference instrument of three 24-hour recalls is also not an optimal way to measure dietary fat intake, because only three days do not provide a good estimate of fat intake.[41] Therefore, it is difficult to judge the quality of the estimates of fat intake in our study. Another limitation was that the FFQ was constructed for Dutch children, whereas our study included many children with a non-Dutch ethnic background. Nevertheless, we observed almost similar effect estimates when we restricted our analyses to the Dutch population. Another limitation was that no data was obtained of glucose levels and that insulin levels were measured non-fasting. Therefore it was not possible to determine insulin resistance, which is most commonly used as predictor for a metabolic syndrome-like phenotype.[22] Finally, no information was available about the children's cardiometabolic health at the age of 1 or their dietary intake at the age of 6 years, and therefore it was not possible to perform longitudinal analyses.

Conclusions

Results from this prospective cohort show no consistent associations between intake of total, saturated, monounsaturated, or polyunsaturated fat in early childhood and cardiometabolic health outcomes at school age. Because the number of studies examining these associations is scarce, future studies are needed to further investigate the associations between different types of fatty acid intake in early childhood and cardiometabolic health, preferably with the use of more detailed dietary assessment methods and combined with fatty acid biomarkers.

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Supplement 3.3.1 Crude associations between fat intake, at the expense of carbohydrates at the age of 1 year and cardiometabolic health at 6 years of age

	BF (SDS) *n=2,911*	SBP (SDS) *n=2,843*	DBP (SDS) *n=2,843*	HDL (SDS) *n=2,008*	Insulin (SDS) *n=1,998*	Triacylglycerol (SDS) *n=2,003*	Cardiometabolic risk factor score *n=1,896*
Total fat (per 5 E%)	-0.007	0.019	-0.002	0.026	-0.015	-0.024	-0.073
	(-0.036, 0.022)	(-0.015, 0.053)	(-0.035, 0.030)	(-0.014, 0.066)	(-0.055, 0.026)	(-0.064, 0.016)	(-0.176, 0.030)
SFA (per 5 E%)	-0.028	0.074	0.073	0.082	**-0.189**	-0.062	-0.287
	(-0.147, 0.092)	(-0.064, 0.212)	(-0.061, 0.207)	(-0.076, 0.240)	**(-0.349, -0.029)**	(-0.221, 0.098)	(-0.687, 0.112)
MUFA (per 5 E%)	0.009	-0.078	-0.133	-0.057	0.190	0.023	0.118
	(-0.142, 0.161)	(-0.253, 0.096)	(-0.303, 0.036)	(-0.259, 0.145)	(-0.015, 0.394)	(-0.181, 0.226)	(-0.149, 0.385)
PUFA (per 5 E%)	-0.003	0.134	0.145	0.075	-0.082	-0.057	0.007
	(-0.142, 0.136)	(-0.025, 0.294)	(-0.010, 0.299)	(-0.111, 0.261)	(-0.270, 0.106)	(-0.245, 0.130)	(-0.471, 0.485)

Values are based on crude linear regression models and reflect differences (95% CI) in cardiometabolic outcomes (age- and sex-specific SD-scores) and cardiometabolic risk factor score per 5 E% higher intake of a specific fat, replacing carbohydrates. For the nutrient density substitution model, models were adjusted for total energy intake, intake of protein and intake of other components of fat, and the different types of fatty acids (SFA, MUFA and PUFA) were adjusted for each other. Models were additionally adjusted for child's age at FFQ, sex and ethnicity.

Abbreviations: BF%, body fat percentage; DBP, diastolic blood pressure; HDL, high-density-lipoprotein; E%, energy percent; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SBP, systolic blood pressure; SDS, standard deviation score; TAG, triacylglycerol.

Supplement 3.3.2 Adjusted associations between fat intake, at the expense of carbohydrates, at the age of 1 year and BMI, and LDL cholesterol, total cholesterol, and C-peptide concentrations at 6 years of age

	Total-C (SDS) *n=1947*	LDL-C (SDS) *n=1948*	C-peptide (SDS) *n=1939*	BMI (SDS) *n=2889*
Total fat (per 5 E%)	-0.007 (-0.048, 0.034)	-0.006 (-0.047, 0.036)	0.006 (-0.035, 0.048)	-0.017 (-0.045, 0.010)
SFA (per 5 E%)	-0.052 (-0.214, 0.109)	-0.060 (-0.222, 0.103)	**-0.172 (-0.334, -0.010)**	0.005 (-0.106, 0.116)
MUFA (per 5 E%)	0.019 (-0.187, 0.225)	0.058 (-0.149, 0.266)	**0.209 (0.003, 0.416)**	-0.005 (-0.145, 0.135)
PUFA (per 5 E%)	0.038 (-0.153, 0.230)	-0.021 (-0.213, 0.171)	-0.045 (-0.237, 0.146)	-0.119 (-0.248, 0.011)

Values are based on adjusted linear regression models and reflect differences (95% CI) in cardiometabolic outcomes (age- and sex-specific SD-scores) and cardiometabolic risk factor score per 5 E% higher intake of a specific fat, at the expense of carbohydrates.

For the nutrient density substitution model, models were adjusted for total energy intake, intake of protein and intake of other components of fat, and the different types of fatty acids (SFA, MUFA and PUFA) were adjusted for each other. Models were additionally adjusted for age at FFQ, sex, ethnicity, maternal education, net household income, parity, maternal BMI at intake, maternal age at intake, smoking during pregnancy, drinking alcohol during pregnancy, breastfeeding, birthweight adjusted for sex and gestational age, screen time at 6 years of age and playing sports at 6 years of age.

Abbreviations: E%, energy percent; LDL, low density lipoprotein; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SDS, standard deviation score; SFA, saturated fatty acids; TAG, triacylglycerol.

Chapter 4

Vitamin D status

Chapter 4.1

Sodiodemographic & lifestyle determinants of vitamin D deficiency in childhood

Manuscript based on this chapter:

Trudy Voortman*, Edith H. van den Hooven*, Annemieke C. Heijboer, Albert Hofman, Vincent W.V. Jaddoe, Oscar H. Franco. Vitamin D deficiency in school-age children is associated with sociodemographic and lifestyle factors. *Journal of Nutrition* 2015;145:781–8.

ABSTRACT

Background: There is concern about a reemergence of vitamin D deficiency in children in developed countries. Our aims were to describe vitamin D status of 6-year-old children in the Generation R Study, a large multiethnic cohort in the Netherlands; and to examine sociodemographic, lifestyle, and dietary determinants of vitamin D deficiency.

Methods: We measured serum 25-hydroxyvitamin D (25 (OH)D) levels in 4,167 children aged 6 years and defined deficiency following recommended cut-offs. We examined the associations of subject characteristics with vitamin D deficiency with multivariable logistic regression analyses.

Results: Serum 25 (OH)D levels ranged from 4 to 211 nmol/L (median 64 nmol/L); with 6.2% having severely deficient 25 (OH)D (<25 nmol/L), 23.6% deficient (25 to <50 nmol/L), 36.5% sufficient (50 to <75 nmol/L), and 33.7% optimal levels (≥75 nmol/L). Prevalence of vitamin D deficiency (25 (OH)D<50 nmol/L) was higher in winter (51.3%) than in summer (10.3%); and in African, Asian, Turkish and Moroccan children (54.5%) compared to those with a Dutch or other Western ethnic background (17.6%). In multivariable models, several factors were associated with vitamin D deficiency, including household income (odds ratio (OR) 1.74 (95% CI 1.34, 2.27) for low vs. high income), child age (OR 1.39 (95% CI 1.20, 1.62) per year), child television watching (OR 1.32 (95% CI 1.06, 1.64 for ≥2 vs. <2 h/d)), and playing outside (OR 0.71 (95% CI 0.57, 0.89) for ≥1 vs. <1 h/d). In a subgroup with dietary data (*n*=1,915), vitamin D deficiency was associated with a lower diet quality, but not with vitamin D intake or supplement use in early childhood.

Conclusion: Suboptimal vitamin D status is common among 6-year-old children in the Netherlands, especially among non-Western children and in winter and spring. Important modifiable factors associated with vitamin D deficiency were overall diet quality, sedentary behavior, and playing outside.

INTRODUCTION

In recent years, vitamin D status and its potential health effects have received much attention in research.[1-3] Vitamin D is important for bone health as it is required for calcium absorption from the gut, hence vitamin D deficiency is associated with rickets in children and osteomalacia in adults.[3] In addition to skeletal health, studies have suggested that vitamin D deficiency is associated with several other health risks in adults, including cognitive decline, auto-immune disease, cancer, cardiovascular disease, and mortality,[4-6] although evidence is not consistent.[7] In children, vitamin D has been shown to be important for maturation of the immune system, and higher vitamin D concentrations have been linked to a lower prevalence of asthma and a reduced risk of respiratory infections.[8-9]

There has been concern about a reemergence of vitamin D deficiency in children living in developed countries, with insufficient vitamin D levels reported in up to 70% of children and adolescents in general populations (1 to 21 years).[10-12] Vitamin D is a nutrient, but vitamin D status depends mainly on cutaneous production.[3] Because vitamin D synthesis depends on exposure to sunlight, changes in lifestyle such as spending much time indoors and using sun protection, are considered to be important causes of vitamin D insufficiency.[1, 3] The increase in prevalence of vitamin D deficiency, in combination with the widespread report of the involvement of vitamin D in various health aspects, makes vitamin D status of important public health interest.[2] To better identify children at high risk and to define strategies to improve vitamin D status, it is important to recognize sociodemographic and lifestyle determinants of vitamin D status. A number of previous studies have evaluated vitamin D status and its determinants in young children, but most of these had limited sample sizes,[10, 13-15] and were performed in children with a Western ethnic background only, while immigrants may be at increased risk of vitamin D deficiency.[16] Only two previous studies have examined determinants of vitamin D status in large groups of children (aged 1-17 and 1-21 years), but both studies had a cross-sectional design and measured a limited number of potential determinants.[11-12]

Therefore, our aims were to describe vitamin D status in a large multiethnic population of 6-year-old children born in Rotterdam, the Netherlands, to determine the prevalence of vitamin D deficiency, and to identify parental and child sociodemographic and lifestyle factors related to child vitamin D status. In a subgroup of children with dietary data, we also evaluated the associations between dietary characteristics in early childhood and subsequent vitamin D status at the age of 6 years.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from pregnancy onward in the city of Rotterdam [52°north latitude], the Netherlands.[17] The study protocol was approved by the Medical Ethics Committee of Erasmus University Medical Center, Rotterdam and written informed consent was obtained by caregivers of all children. Mothers living

in the study area with an expected delivery date between April 2002 and January 2006 were eligible for inclusion. In total, 9,778 mothers were enrolled in the study and gave birth to 9,749 live born children. At the age of 6 years, 8,305 of these children were participating in the study,[17] of whom 6,690 visited the research center for detailed follow-up measurements. During this visit, we collected a blood sample from 4,473 children who provided consent for venous puncture and we successfully assessed 25-hydroxyvitamin D (25 (OH)D) concentrations in the blood samples of 4,167 children (Figure 4.1.1).

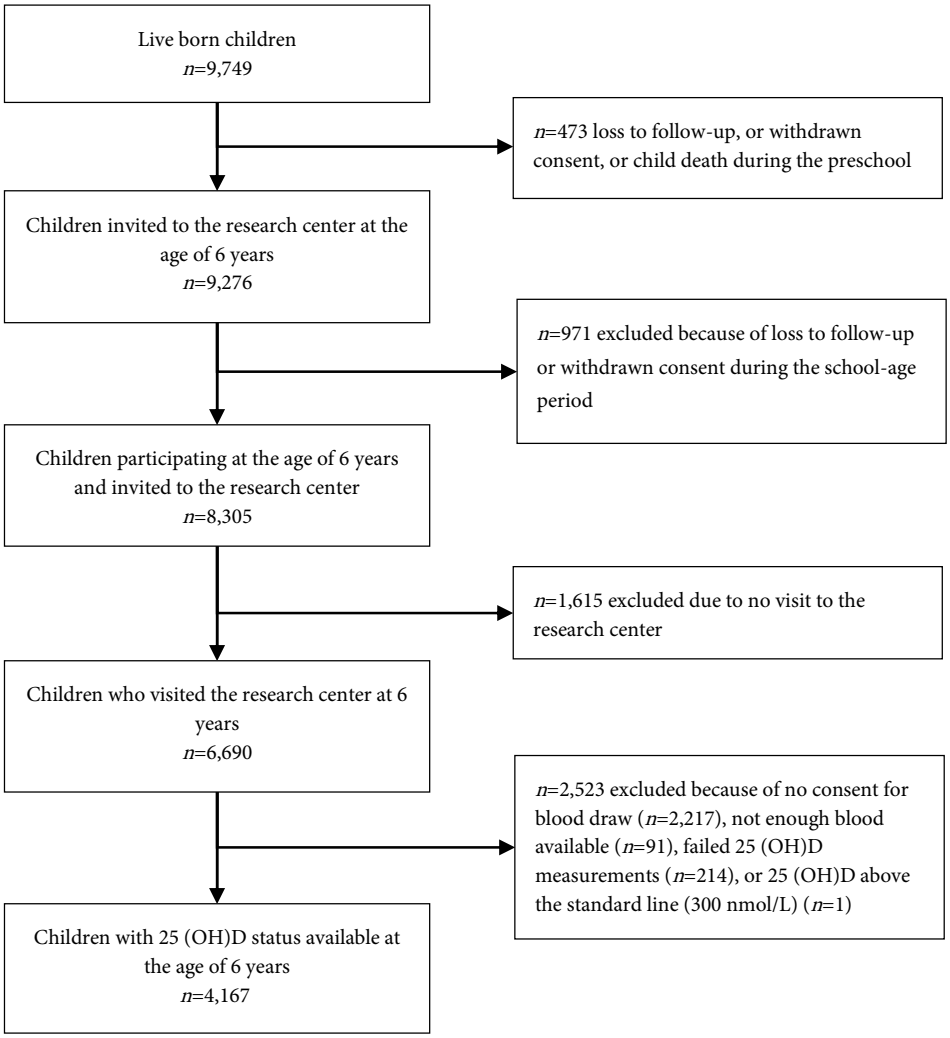

Figure 4.1.1 Flowchart of study participants

Vitamin D assessment

At a median age of 6.0 years (95% range 5.6 to 7.9), non-fasting blood samples were drawn by antecubital venipuncture and stored at -80 °C until analysis.[18] Measurements of 25 (OH)D were conducted at the Endocrine Laboratory of the VU University Medical Center, Amsterdam, as described before.[19] Serum 25 (OH)D was measured with the use of isotope dilution on-line solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS),[19-21] a highly sensitive and specific method for the quantification of 25 (OH)D,[19-21] and the recommended method for vitamin D assessment in epidemiological studies.[21] The limit of quantitation was 4.0 nmol/L; intra-assay coefficient of variation (CV) was <6%, and inter-assay CV was <8% for concentrations between 25 and 180 nmol/L. Optimal vitamin D levels for health remain a subject of debate.[3] On the basis of recommendations and previous studies in pediatric populations we defined the following categories: <25 nmol/L (<10 ng/mL) as *severely deficient*; 25 to <50 nmol/L (10 to <20 ng/mL) as *deficient*; 50 to <75 nmol/L (20 to <30 ng/mL) as *sufficient*; and ≥75 nmol/L (≥30 ng/mL) as *optimal*.[1, 11, 14-15, 22-24]

Parental characteristics

We identified potential determinants of vitamin D deficiency in children on the basis of previous literature.[10-15] Information on parity and on maternal smoking, alcohol use, and folic acid supplementation during pregnancy was obtained from questionnaires. Information on maternal age, marital status, educational level, parental employment status, net household income, and smoking in the household was obtained from a questionnaire at the child's age of 6 years. Maternal height and weight were measured around the child's age of 6 years, and body mass index (BMI) (kg/m^2) was calculated.

Infant characteristics

Information on child's sex, birthweight, and gestational age at birth was available from medical records and hospital registries. Gestational age- and sex- specific SD-scores for birthweight were calculated according to reference data.[25] Information on child ethnicity was obtained from a questionnaire and was defined based on country of birth of the parents.[26] We categorized ethnicity into Dutch and other Western (European, American, Oceanian); Turkish and Moroccan; African (Cape Verdean, other African, Surinamese-Creole, Dutch Antillean); and Asian (Indonesian, other Asian, Surinamese-Hindu) according to the largest ethnic groups in our study population, and similarities in skin color and cultural background (Supplement 4.1.1). Information on breastfeeding was obtained from delivery reports and postnatal questionnaires.

Child characteristics

At the age of 6 years, child weight and height were measured without shoes and heavy clothing at the research center, and BMI (kg/m^2) was calculated. Age- and sex-specific SD-scores for weight and height were calculated on the basis of Dutch growth charts.[27] Child weight status (underweight; normal weight; overweight; obese) was defined in accordance with international age- and sex-specific BMI cut-offs.[28] Information on the amount of sunlight in Rotterdam was obtained from

the Royal Netherlands Meteorological Institute, and average amount of sunlight in the 28 days before blood sampling was estimated for each child.[29] Information on amount of television watching and computer use, participation in sports, means of transportation to school, and playing outside during daytime at age 6 years was obtained using a questionnaire filled out by the caregivers.

Dietary characteristics

Dietary data was available from a subgroup of children (n=1,915). Mother of these children had received a semi-quantitative food-frequency questionnaire (FFQ) at a median child's age of 12.9 months (95% range 12.2 to 19.0).[30] We calculated mean daily intakes of vitamin D and of the following food groups: margarines and cooking fats (fortified with vitamin D); dairy (not fortified in the Netherlands); infant formula (fortified with vitamin D); and fish and shellfish (naturally rich in vitamin D). Information on vitamin supplement use was available from the same FFQ. Vitamin D supplement use was defined (yes/no), reporting yes if using vitamin D, vitamin A and D, or multivitamin supplements. We used a child diet quality score with a theoretical range of 0 to 10 as a measure of overall diet quality (Chapter 5.1).[31]

Statistical analyses

Differences in parental or child characteristics between vitamin D categories were assessed with one-way ANOVA or Kruskal Wallis tests for continuous variables and Chi-square tests for categorical variables. We performed logistic regression models to examine the associations of sociodemographic and lifestyle factors with 25 (OH)D deficiency (<50 nmol/L). All variables with p<0.10 in univariable models were included in one multivariable model. To retain only the strongest determinants, we performed a stepwise backward elimination procedure on the full multivariable model, with p<0.10 as endpoint. We performed separate logistic regression models in the subgroup of children with dietary data (n=1,915). These models were adjusted for the most important sociodemographic and lifestyle determinants of vitamin D deficiency that were identified in the stepwise regression analysis in the full group (i.e., factors with p<0.001 in the final multivariable model). We performed sensitivity analyses in which we used 25 nmol/L and 75 nmol/L as cut-offs to define 25 (OH)D deficiency. Because non-Western populations may have other factors associated with vitamin D status, we performed sensitivity analyses including Dutch and other Western children only. Furthermore, we evaluated statistical interaction by adding the product terms of the different ethnic groups and other covariates (i.e., season, child's weight status, maternal educational level, and household income) to the models with vitamin D deficiency as an outcome. Stratified analyses were conducted if the interaction term was significant (p<0.05).

To reduce potential bias associated with missing data, we performed multiple imputations of missing covariates based on the correlation between the variable with missing values and other subject characteristics.[32] Data were imputed (n=10 imputations) using the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. Analyses were performed in the original dataset and in the imputed datasets. Because we observed similar effect estimates, we only present the results based on imputed datasets. Statistical analyses were performed with SPSS version 21.0 for Windows (IBM Corp., Armonk, NY, USA).

RESULTS

Table 4.1.1 Characteristics of the children and their parents (*n*=4,167)

	n	Mean, median, or %
Child characteristics		
Child age (y)	4,167	6.0 (5.7-8.0)
Boy (%)	4,167	51.5
Ethnicity (%)	4,011	
Dutch and other Western		69.0
Moroccan and Turkish		13.4
African		11.4
Asian		6.3
Weight (kg)	4,159	22.6 (17.6-34.6)
Height (cm)	4,159	119.8 ± 6.0
Participation in sports (% yes)	3,526	45.6
TV watching ≥2h/d (%)	3,257	19.5
Computer use ≥1h/d (%)	3,249	7.3
Amount of sunlight in the month before blood draw (h/d)	4,167	5.0 (1.3-9.5)
Season of blood sampling (%)	4,167	
Winter		21.5
Spring		28.3
Summer		25.4
Fall		24.7
25 (OH)D level (nmol/L)	4,167	64 (18-131)
25 (OH)D status (%)	4,167	
Severely deficient (<25 nmol/)		6.2
Deficient (25 to <50 nmol/L)		23.6
Sufficient (50 to <75 nmol/L)		36.5
Optimal (≥75 nmol/L)		33.7
Parental characteristics		
Maternal age (y)	4,090	37.3 ± 4.9
Maternal educational level (%)	3,550	
Primary		4.0
Secondary		38.7
Higher		57.3
Net household income (%)	3,370	
<€2000/mo		24.0
€2000 to <€3200/mo		25.0
≥€3200/mo		51.1

Values are percentages for categorical variables, means ± SD for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution; on the basis of unimputed data.

Subject characteristics and 25 (OH)D status

Serum 25 (OH)D concentrations ranged from 4 to 211 nmol/L, with a median of 64 nmol/L (Table 4.1.1). Of all children, 6.2% was severely vitamin D deficient (25 (OH)D <25 nmol/L), 23.6% was vitamin D deficient (25 (OH)D 25 to <50 nmol/L), 36.5% had sufficient levels (25 (OH)D 50 to <75 nmol/L), and only 33.7% had optimal levels (25 (OH)D ≥75 nmol/L). The prevalence of vitamin D deficiency (25 (OH)D <50 nmol/L) was highest in winter (51.3%) and lowest in summer (10.3%) (Figure 4.1.2). Vitamin D deficiency was also highly prevalent in non-Western children: 54.5% of Turkish and Moroccan, 55.5% of African, and 51.9% of Asian children were vitamin D deficient, as compared to only 17.6% of the Dutch and other Western children (Figure 4.1.2).

The amount of sunlight in the month before the blood draw was higher in those with sufficient or optimal vitamin D status than in those with deficiency (Supplement 4.1.2). Non-response analysis showed that children who visited the research center but did not have blood samples taken (n=2,523) were more often girls, were slightly younger and shorter, had a lower weight, and more often had mothers with a lower educational background or with a lower household income, as compared to children with blood samples (results not shown), although these differences were very small.

Sociodemographic and lifestyle determinants of vitamin D deficiency

In the full multivariable model after the backward elimination procedure, several child and parental sociodemographic and lifestyle factors were associated with vitamin D deficiency (<50 nmol/L) in children (Table 4.1.2). Risk of vitamin D deficiency was higher in winter and spring (OR 3.38 (95% CI 2.31, 4.93) and OR 4.50 (95% CI 3.42, 5.94), respectively, as compared to summer) and in children with a non-Western ethnicity (ORs ranging from 3.07 (95% CI 2.23, 4.23) for Turkish and Moroccan to 4.00 (95% CI 2.85, 5.62) for Asian children, as compared to children with a Dutch or other Western background). Also, children's higher age, lower birthweight, current underweight, more television watching, playing less outside, and biking less to school were associated with higher risks of vitamin D deficiency. In contrast, child's sex, height, participation in sports, or breastfeeding history were not associated with vitamin D deficiency. Furthermore, the risk of vitamin D deficiency was higher in children of mothers who were younger, multiparous, had a higher BMI, did not use folic acid supplements during pregnancy, or with a lower household income. Maternal educational level, marital status, or smoking or alcohol use during pregnancy were not associated with child vitamin D deficiency.

Multivariable models with continuous 25 (OH)D concentrations or with different cut-offs to define vitamin D deficiency as an outcome revealed similar determinants (data not shown). We observed no significant interactions for ethnicity with season, child's weight status, maternal educational level, or household income on vitamin D deficiency. Results from the sensitivity analysis including Dutch and other Western children only were similar to those for the whole group (Online Supplement).

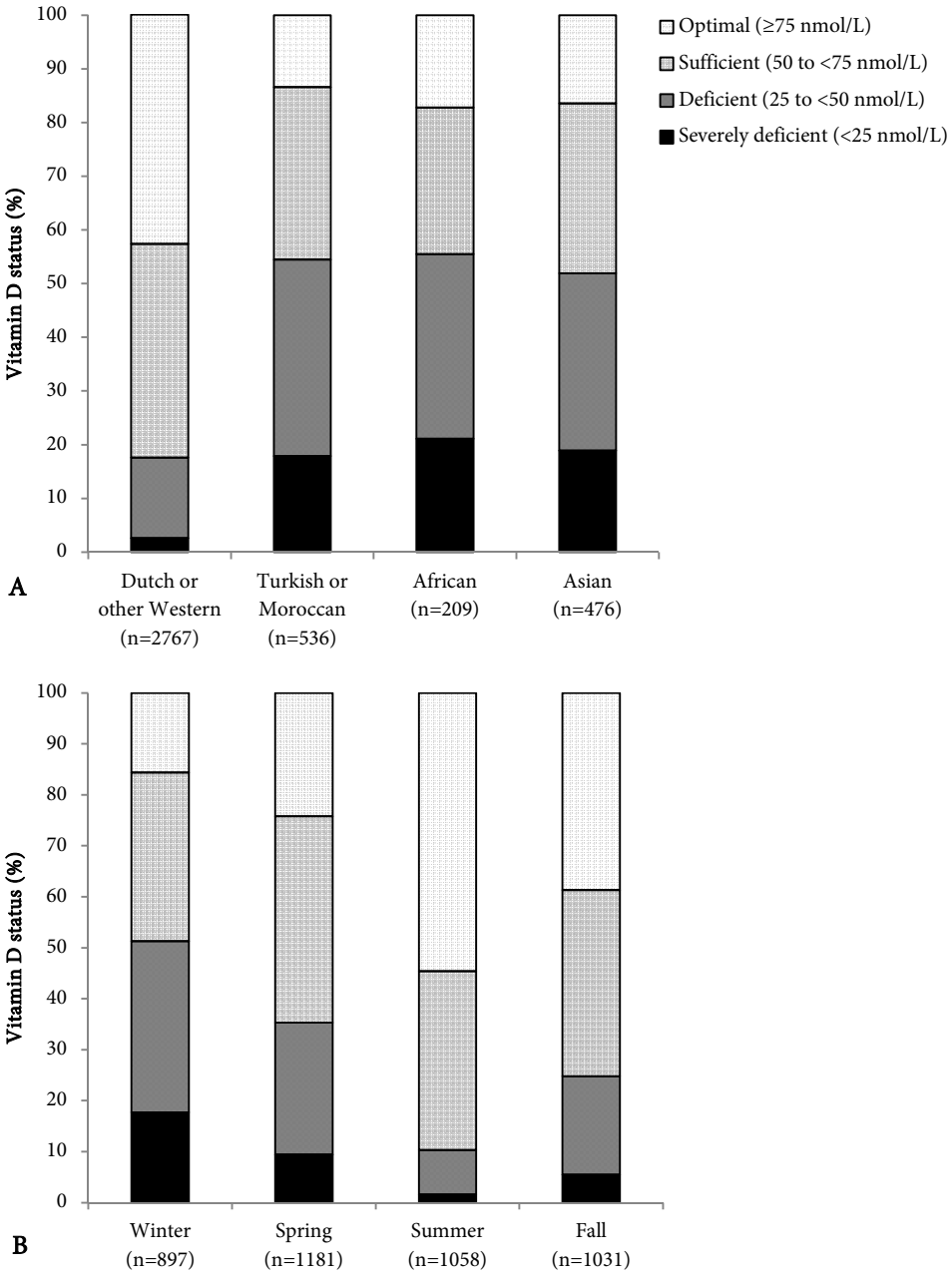

Figure 4.1.2 Serum 25 (OH)D concentrations in different ethnic group (A) and in different seasons (B) in children aged 6 years (*n*=4,167).

Table 4.1.2 Associations between sociodemographic and lifestyle factors and vitamin D deficiency (25 (OH)D <50nmol/L) in children aged 6 years (*n*=4,167)

	Multivariable model†		**Multivariable model after stepwise backward selection‡**	
	Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value
Child characteristics				
Age (y)	1.41 (1.21, 1.64)	*<0.001*	1.39 (1.20, 1.62)	*<0.001*
Sex§	-		-	
Ethnicity				
Dutch or other Western	Reference		Reference	
Turkish or Moroccan	2.90 (2.10, 4.01)	*<0.001*	3.07 (2.23, 4.23)	*<0.001*
African	3.74 (2.79, 5.01)	*<0.001*	3.88 (2.86, 5.27)	*<0.001*
Asian	3.93 (2.75, 5.61)	*<0.001*	4.00 (2.85, 5.62)	*<0.001*
Birthweight Z-score	0.90 (0.82, 0.98)	*0.02*	0.91 (0.84, 1.00)	*0.04*
Breastfeeding§				
Never	-		-	
Partial	-		-	
Exclusive ≥ 4 months	Reference		Reference	
Amount of sunlight in the month before blood draw (h/d)	0.75 (0.71, 0.79)	*<0.001*	0.75 (0.71, 0.79)	*<0.001*
Season of blood sampling				
Winter	3.42 (2.34, 5.02)	*<0.001*	3.38 (2.31, 4.93)	*<0.001*
Spring	4.53 (3.42, 5.99)	*<0.001*	4.50 (3.42, 5.94)	*<0.001*
Summer	Reference		Reference	
Fall	1.49 (1.08, 2.06)	*0.02*	1.47 (1.07, 2.03)	*0.02*
Height Z-score	1.07 (0.98, 1.16)	*0.15*	-	
Weight status				
Underweight	2.26 (1.55, 3.28)	*<0.001*	2.20 (1.51, 3.21)	*<0.001*
Normal weight	Reference		Reference	
Overweight	1.00 (0.78, 1.29)	*0.99*	1.03 (0.80, 1.31)	*0.84*
Obese	1.37 (0.90, 2.10)	*0.14*	1.45 (0.97, 2.18)	*0.07*
Participation in sports				
No	Reference		Reference	
Yes	0.83 (0.67, 1.02)	*0.07*	-	
Television watching				
<2h/d	Reference		Reference	
≥2h/d	1.29 (1.04, 1.60)	*0.02*	1.32 (1.06, 1.64)	*0.01*
Computer use				
<1h/d	Reference		Reference	
≥1h/d	1.13 (0.84, 1.53)	*0.41*	-	
Playing outside during daytime				
<1h/d	Reference		Reference	
≥1h/d	0.70 (0.56, 0.88)	*0.01*	0.71 (0.57, 0.89)	*0.01*
Walking to school				
<10 min/d	Reference		Reference	
≥10 min/d	0.99 (0.80, 1.22)	*0.91*	-	
Biking to school				
<10 min/d	Reference		Reference	
≥10 min/d	0.65 (0.48, 0.86)	*0.01*	0.65 (0.49, 0.86)	*0.01*

Table 4.1.2 (continued) Associations between sociodemographic and lifestyle factors and vitamin D deficiency (25 (OH)D <50nmol/L) in children aged 6 years (*n*=4,167)

	Multivariable model†		**Multivariable model after stepwise backward selection‡**	
	Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value
Parental characteristics				
Maternal folic acid use during pregnancy				
Never	Reference		Reference	
Start in first ten weeks	0.79 (0.59, 1.06)	*0.11*	0.77 (0.58, 1.04)	*0.09*
Start periconceptional	0.57 (0.43, 0.74)	*<0.001*	0.55 (0.41, 0.73)	*<0.001*
Maternal smoking during pregnancy				
Never	Reference		Reference	
In first trimester	1.01 (0.71, 1.45)	*0.94*	-	
Continued	1.04 (0.77, 1.40)	*0.79*	-	
Maternal alcohol consumption during pregnancy				
Never	Reference		Reference	
In first trimester	1.03 (0.69, 1.53)	*0.90*	-	
Continued	0.94 (0.74, 1.20)	*0.62*	-	
Parity (at enrollment)				
Nulliparous	Reference		Reference	
Multiparous	1.33 (1.09, 1.62)	*0.01*	1.33 (1.10, 1.61)	*0.01*
Maternal age (y)	0.95 (0.93, 0.97)	*<0.001*	0.95 (0.93, 0.97)	*<0.001*
Maternal BMI (kg/m^2)	1.03 (1.01, 1.05)	*0.01*	1.03 (1.01, 1.05)	*0.01*
Maternal educational level				
Primary	1.35 (0.82, 2.21)	*0.23*	-	
Secondary	0.97 (0.77, 1.21)	*0.76*	-	
Higher	Reference		Reference	
Net household income				
<€2000/mo	1.46 (1.04, 2.05)	*0.03*	1.74 (1.34, 2.27)	*<0.001*
€2000 to €3200/mo	1.06 (0.83, 1.37)	*0.63*	1.11 (0.86, 1.44)	*0.40*
≥€3200mo	Reference		Reference	
Marital status				
Married/living together	Reference		Reference	
No partner/not living together	1.14 (0.87, 1.50)	*0.34*	-	
Maternal employment status				
Paid job	Reference		Reference	
No paid job	1.09 (0.87, 1.36)	*0.44*	-	
Paternal employment status				
Paid job	Reference		Reference	
No paid job	1.20 (0.76, 1.89)	*0.42*	-	
Smoking in the household				
No	Reference		-	
Yes	1.02 (0.79, 1.32)	*0.89*	-	
Adjusted R-square*	**0.43**		**0.42**	

† Values are odds ratios (95% Cis) from a logistic regression analysis including all variables for which an effect estimate is presented, and reflect the risk of vitamin D deficiency (25(OH)D<50 nmol/L) per unit increase (continuous variables) or as compared to the reference (categorical variables).
‡ Values are odds ratios with 95% CIs from a logistic regression analysis with stepwise backward selection with $p<0.10$ as endpoint.
§$p>0.10$ in univariable analysis and therefore not included in the multivariable model.
* Nagelkerke pseudo R-square

Dietary determinants of vitamin D deficiency

Table 4.1.3 presents the associations between dietary characteristics in early childhood and vitamin D deficiency (<50 nmol/L) at the age of 6 years (*n*=1,915). After adjustment for sociodemographic and lifestyle factors, the risk of vitamin D deficiency was lower in children with a higher diet quality score (OR 0.87 (95% CI 0.78, 0.97) per point higher diet score) and in children with a high intake of margarines and cooking fats (OR 0.72 (95% CI 0.51, 1.00) for highest vs. lowest tertile). Intakes of dairy and cheese, fish and shellfish, infant formula, vitamin D, or vitamin D supplement use in early childhood were not associated with vitamin D status.

Table 4.1.3 Associations between dietary characteristics in early childhood and vitamin D deficiency (25 (OH)D <50nmol/L) in children aged 6 years (*n*=1,915)

	Odds ratio (95% CI)	***p*-value**
Diet score	0.87 (0.78, 0.97)	*0.01*
Dairy and cheese intake (highest vs. lowest tertile)	0.89 (0.64, 1.22)	*0.46*
Fish and shellfish intake (highest vs. lowest tertile)	1.02 (0.74, 1.40)	*0.92*
Margarines and cooking fats intake (highest vs. lowest tertile)	0.72 (0.51, 1.00)	*0.05*
Infant formula intake (highest vs. lowest tertile)§	-	
Adequate intake of vitamin D (≥10 µg/d)		
No	*Reference*	
Yes	0.81 (0.59, 1.12)	*0.20*
Vitamin D supplementation		
No	*Reference*	
Yes	1.08 (0.83, 1.40)	*0.58*

Values are odds ratios with 95% CIs from multivariable logistic regression analyses, and reflect the risk for vitamin D deficiency (25 (OH)D <50 nmol/L) per unit increase (continuous variables) or as compared to the reference group (categorical variables). Models are adjusted for child's sex, total energy intake, age at dietary assessment, age at vitamin D measurement, sunlight in the month before blood draw, season, ethnicity, weight status, and maternal age. Dietary factors are included in separate logistic regression models.
§ *p*>0.10 in univariable analysis and therefore not included in the multivariable model.

DISCUSSION

In this large population-based cohort, we observed a high prevalence of vitamin D deficiency in 6-year-old children, especially among non-Western children and in winter and spring. Other important determinants of vitamin D deficiency included a higher child's age, more television watching, less playing outside, less biking to school, lower maternal age, lower household income, multiparity, and higher maternal BMI. To the best of our knowledge, this is the largest study that assessed vitamin D status and its determinants in children in the Netherlands, and among the first large studies worldwide.[11-12]

Interpretation and comparison with previous studies

In our population, 30% of all children were vitamin D deficient (<50 nmol/L) and 66% had suboptimal vitamin D levels (<75 nmol/L). This prevalence is comparable to those observed in previous studies. In a large (*n*=6,275) survey among a multiethnic group of children from the U.S. (1-21 years), 70% had suboptimal 25 (OH)D levels (<75 nmol/L)[11] and in a study in the U.K. (*n*=1,102), 35% of the 4 to 8-year-old children was vitamin D deficient (<50 nmol/L).[33] In contrast,

two studies (*n*=380 and 781) in U.S. toddlers up to the age of 3 years, reported prevalences of vitamin D deficiency (<50 nmol/L) of only 12% and 15%.[10, 14] This may be explained by the younger age of these children, as previous studies reported that younger children generally have higher vitamin D levels.[14, 33-34] Within our study population, we also observed that older children had a higher risk of vitamin D deficiency. Potential explanations could be that older children less often receive vitamin D supplements or that they spend less time playing outdoors.[33]

As expected, we observed large differences in vitamin D concentrations between children of different ethnicities.[35-37] Previous small studies in the Netherlands also reported a higher prevalence of vitamin D deficiency in immigrant children,[35] in children from asylum seekers,[36] or in Turkish and Moroccan children,[37] compared to native Dutch children. Similarly, a large survey in 10,015 children in Germany reported a higher prevalence of deficiency in 3 to 17-year-old immigrant children (76% <50 nmol/L) compared to non-immigrant children (62%).[12] Differences in vitamin D levels between different ethnicities might be explained by higher levels of skin pigmentation, which limits cutaneous vitamin D synthesis.[3] or by other genetic differences.[38] Also cultural aspects, such as wearing concealing clothes or spending not much time outside, either by the mothers during pregnancy or by the children themselves, might explain the differences.[39] In line with this, season was another important determinant of vitamin D status in our population. Previous studies also reported lower vitamin D levels in winter than in summer,[14, 33, 40] and similarly, in subjects living at higher latitudes within Europe compared to those living at lower latitudes.[33, 40]

Important modifiable factors associated with vitamin D status are time spent outside and sedentary behavior. In our study, higher vitamin D levels were observed in children who played outside more often, who watched less television, and spent more time biking to school. Other studies in children observed similar associations for outdoor exercise,[15, 33] and for television watching or computer use.[11, 33]

Unfortunately, we had no data on vitamin D intake at the age of 6 years. Nevertheless, we examined whether dietary characteristics in early childhood were related to vitamin D status at the age of 6 years, showing that vitamin D intake and vitamin D supplement use were not associated with vitamin D status. Previous studies reported either no association,[12-13, 15, 40-41] or positive associations[11, 40, 42] between dietary intake and blood levels of vitamin D children. Dietary intake of vitamin D is known to have a smaller contribution to vitamin D status than cutaneous production in response to sun exposure.[3] In our study, also the time gap between dietary measurement and vitamin D status measurement might explain why we did not observe an association. However, a higher overall diet quality and higher intake of margarines and cooking fats in early childhood were associated with a lower risk of vitamin D deficiency. There is evidence that dietary habits track from early to later childhood,[43] and one might speculate that overall diet quality may better track into later childhood[44] than vitamin D intake specifically. In the Netherlands, dairy products are usually not fortified with vitamin D, hence other foods such as fish, meat, margarines, cooking fats, and infant formula might be more important sources for children. This may explain the lower risk of vitamin D deficiency in relation to the intake of margarines and cooking fats in our study. Some previous studies reported that children who received infant formula had higher vitamin D levels than children who received breastfeeding.[10, 14] However, in our study, neither formula intake at the

age of 1 year nor history of breastfeeding was associated with vitamin D status at the age of 6 years, which might be explained by the emphasis on vitamin D supplementation for infants in the Netherlands.[45]

In line with most previous studies in children,[10, 13, 15, 35] we did not observe differences in vitamin D status between boys and girls. In our population, child anthropometric factors were not strongly related to vitamin D status. Children with underweight had a higher risk of vitamin D deficiency than normal weight children, while there were no significant differences for overweight or obese children. This is in contrast to previous studies that reported higher prevalences of vitamin D deficiency in obese children.[11, 15, 33] The fact that we did not observe associations with obesity may be explained by the young age of our population or because the association observed in other studies may be explained by other sociodemographic and lifestyle variables for which we adjusted for in our analyses.

Several parental sociodemographic and lifestyle factors were associated with child vitamin D deficiency. In line with a few previous studies, a higher risk of vitamin D deficiency was associated with a lower socioeconomic status and less health-conscious behaviors.[12, 14]

The Dutch Health Council recommends vitamin D supplementation for all children younger than 4 years of age (5 μg/d until 2008 and 10 μg/d thereafter).[46] In older children, vitamin D supplementation is only recommended for those with a dark skin or limited sunlight exposure. This recommendation is supported by our results. However, it might be argued that all children, also above the age of 4 years, could benefit from vitamin D supplementation, given the high prevalence of vitamin D deficiency observed in our study and previous studies. Indeed, the American Academy of Pediatrics recommends a dietary vitamin D intake of 400 IU/d (10 μg/d) for all children and adolescents, and vitamin D supplementation up to the same amount in those who do not obtain this dietary intake.[23] Health care providers should be aware of the high prevalence of vitamin D deficiency in childhood and future studies are needed to assess whether interventions to increase vitamin D levels in childhood will improve health outcomes.

Methodological considerations

A strength of our study is that we measured vitamin D status in a large and ethnically diverse sample of 4,167 children, with a highly sensitive and accurate method.[19] We assessed circulating serum 25 (OH)D levels, the best and most widely used indicator of vitamin D status.[3, 21] A limitation of our study is the loss to follow-up for blood sampling, which was mainly because of non-consent for venipuncture. Non-response analysis showed that younger children, girls, and children of mothers with a lower educational level or lower household income were more likely to have no available blood samples. Although the differences were small, they might have affected the prevalence of vitamin D deficiency in our population. For example, we observed a higher prevalence of vitamin D deficiency in older children and in children from households with a lower income. The selective loss to follow up could therefore have resulted in an overestimation (age) or underestimation (income) of vitamin D deficiency prevalence in our sample. An important strength of this study is its population-based cohort design from early life onward, with detailed measurements of many sociodemographic and lifestyle determinants, which were not always considered in previous

studies. [4, 9, 12, 14] However, information on some potential determinants, including amount of physical activity and time spent outside, was limited. However, we did have information on participation in sports, television watching, and playing outside, as indicators for these determinants. We also lacked information on sun exposure habits (i.e., clothing, sun screen use) and on skin pigmentation. We used the birth countries of the parents to define and categorize ethnicity. Dietary data was available at the age of 1 year. Possibly, the associations between dietary vitamin D intake and vitamin D status would have been stronger if we would have had dietary data at the time of the vitamin D assessment. Nevertheless, it is well known that dietary vitamin D intake is a much weaker predictor of vitamin D status than sun exposure.[3]

Conclusions

In this large population-based cohort of 6-year-old children, vitamin D deficiency is highly prevalent, especially among non-Western children and in winter and spring. In addition to ethnicity and season, we identified other important modifiable (i.e., maternal BMI, folic acid use during pregnancy, child television watching, playing outside, biking to school) and non-modifiable (i.e., maternal age, parity, household income, child's age) factors associated with vitamin D status among children. Supplementation or lifestyle changes are important in order to prevent vitamin D deficiency in children. Future studies are needed to determine whether vitamin D deficiency during childhood may affect later health.

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SUPPLEMENT CHAPTER 4.1

Additional Supplemental Material for this chapter: http://jn.nutrition.org/content/early/2015/02/18/jn.114.208280/suppl/DCSupplemental

Supplement 4.1.1 Prevalence of vitamin D deficiency within specific ethnic groups

		Serum 25 (OH)D concentration, nmol/L			
		<25 *(<10 ng/mL)*	**25 to <50** *(10-20 ng/mL)*	**50 to <75** *(20-30 ng/mL)*	**≥75** *(≥ 30 ng/mL)*
	n	Severely deficient (%)	Deficient (%)	Sufficient (%)	Optimal (%)
Western					
Dutch	2,346	2.4	13.5	39.9	44.2
Other European	308	2.9	24.0	39.3	33.8
American	105	5.0	24.0	43.0	31.4
Oceanian	8	0.0	0.0	25.0	75.0
Turkish and Moroccan					
Turkish	289	16.3	36.3	33.6	13.8
Moroccan	247	19.8	36.8	30.4	13.0
African					
Cape Verdean	116	19.8	36.8	30.4	13.0
Surinamese, Creole	115	23.5	32.2	28.7	15.7
Dutch Antillean	132	18.9	38.6	20.5	22.0
African, other	94	18.1	37.2	25.5	19.1
Asian					
Indonesian	23	8.7	21.7	34.8	34.8
Surinamese, Hindustani	124	25.8	31.5	29.0	13.7
Surinamese, unspecified	47	21.3	27.7	25.5	25.5
Asian, other	94	15.2	24.2	41.4	19.2

Values are percentages for vitamin D status within different ethnic groups.

Supplement 4.1.2 Participant characteristics stratified by serum 25 (OH)D concentrations in children aged 6 years (*n*=4,167)

	Serum 25 (OH)D concentration, nmol/L				
	<25 *(<10 ng/mL)*	**25 to <50** *(10-20 ng/mL)*	**50 to <75** *(20-30 ng/mL)*	**≥75** *(≥ 30 ng/mL)*	
	Severely deficient	Deficient	Sufficient	Optimal	***p*-value***
	(*n*=259)	(*n*=983)	(*n*=1,522)	(*n*=1,403)	
Child characteristics					
25 (OH)D level (nmol/L)	19.0 (7.4-24.4)	39.0 (25.5-49.0)	62.6 (50.1-74.0)	89.0 (75.0-155.0)	<0.001
Age (y)	6.2 (5.7-8.5)	6.1 (5.7-8.3)	6.0 (5.7-7.9)	6.0 (5.7-7.5)	<0.001
Male (%)	50.2	53.5	52.0	49.8	0.32
Ethnicity (%)					<0.001
Western	20.9	47.7	73.9	85.9	
Moroccan and Turkish	33.5	23.4	11.6	5.2	
African	28.3	20.3	8.7	5.6	
Asian	17.4	8.6	5.8	3.3	
Birthweight Z-score	-0.4 (1.0)	-0.1 (1.0)	-0.0 (1.0)	0.0 (1.0)	<0.001
Breastfeeding (%)					0.02
Never	7.5	5.5	7.4	9.5	
Partial	77.4	68.4	60.2	61.2	
Exclusive ≥ 4 months	15.1	26.1	32.3	29.3	
Amount of sunlight in the month before blood draw (h/d)	2.5 (1.3, 7.4)	3.6 (1.3, 9.0)	5.3 (1.3, 9.5)	6.2 (1.6, 9.9)	<0.001
Season of blood sampling (%)					<0.001
Winter	49.0	33.9	19.5	10.0	
Spring	29.7	34.6	31.4	20.4	
Summer	3.9	10.1	24.4	41.2	
Fall	17.4	21.5	24.7	28.4	
Height at age 6 years (Z-score)	-0.4 (1.0)	-0.2 (1.0)	-0.2 (1.0)	-0.1 (1.0)	<0.001
Weight status at age 6 years					<0.001
Underweight	5.4	5.7	3.5	5.1	
Normal weight	67.6	72.6	80.3	83.2	
Overweight	17.0	14.9	13.4	9.4	
Obese	10.0	6.8	2.8	2.3	
Participation in sports (%)	24.4	40.2	48.2	48.6	<0.001
TV watching ≥2h/d (%)	48.2	29.9	17.1	13.0	<0.001
Computer use ≥1h/d (%)	16.1	10.2	7.0	5.1	<0.001
Playing outside during day ≥1h/d (%)	32.8	45.0	60.4	72.6	<0.001
Walking to school ≥10 min/d (%)	47.1	33.8	27.0	20.9	<0.001
Biking to school ≥ 10 min/d (%)	4.0	8.4	15.9	21.6	<0.001

Supplement 4.1.2 (continued) Participant characteristics stratified by serum 25 (OH)D concentrations in children aged 6 years (*n*=4,167)

	Serum 25 (OH)D concentration, nmol/L				
	<25 *(<10 ng/mL)*	**25 to <50** *(10-20 ng/mL)*	**50 to <75** *(20-30 ng/mL)*	**≥75** *(≥ 30 ng/mL)*	
	Severely deficient	Deficient	Sufficient	Optimal	***p*-value***
	(*n*=259)	(*n*=983)	(*n*=1,522)	(*n*=1,403)	
Parental characteristics					
Maternal age (y)	34.9 (5.6)	36.2 (5.4)	37.6 (4.8)	38.1 (4.3)	<0.001
Multiparous at enrollment (%)	59.0	48.9	42.9	40.4	<0.001
Maternal folic acid use during pregnancy (%)					<0.001
Start periconceptional	10.0	30.3	45.9	54.8	
Start in first ten weeks	22.0	31.4	33.4	31.7	
Never	68.0	38.3	20.7	13.5	
Maternal smoking during pregnancy (%)					<0.001
Never,	67.0	72.4	75.7	77.3	
In first trimester	9.0	8.2	10.2	8.4	
Continued	24.0	19.3	14.1	14.3	
Maternal alcohol consumption during pregnancy (%)					<0.001
Never	69.2	54.4	42.6	35.6	
In first trimester	9.7	12.1	14.2	15.6	
Continued	21.1	33.5	43.2	48.8	
Maternal educational level (%)					<0.001
Primary	16.8	8.6	2.7	1.1	
Secondary	59.9	46.3	36.3	34.0	
Higher	23.4	45.2	61.0	64.9	
Household income (%)					<0.001
<€2000/mo	67.8	38.5	19.8	14.7	
€2000 to <€3200/mo	19.7	24.5	26.6	24.1	
≥€3200/mo	12.5	37.0	53.6	61.2	
Marital status (%)					<0.001
Married/living together	76.2	78.2	88.3	89.9	
No partner/not living together	23.8	21.8	11.7	10.1	
Maternal employment, paid job (%)	52.7	67.4	77.6	81.7	<0.001
Paternal employment, paid job (%)	76.2	91.5	95.6	96.3	<0.001
Smoking in the household (%)	27.1	15.8	10.8	11.1	<0.001

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, and medians (95% range) for continuous variables with a skewed distribution.

* *p*-values for differences in means between groups of serum 25 (OH)D concentration, assessed with ANOVA for continuous variables with a normal distribution, Kruskal-Wallis test for continuous variables with a skewed distribution, and Chi-Square test for categorical variables.

Chapter 4.2

Vitamin D status in childhood & body composition

Manuscript based on this chapter:

Trudy Voortman, Ruchika Mehra, Rebecca C. Richmond, Fernando Rivadeneira, Janine F. Felix, Vincent W.V. Jaddoe, Edith H. van den Hooven, Oscar H. Franco. Vitamin D status and adiposity in a multiethnic cohort of school-aged children: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Vitamin D deficiency has been associated with adiposity in adults and adolescents. However, there is limited information about this association in young children. We examined the cross-sectional association between vitamin D status and measures of adiposity in 4,104 school-aged children participating in a large multiethnic cohort in the Netherlands.

Methods: At their median age of 6.0 years (95% range 5.7 to 8.0), we measured children's serum 25-hydroxyvitamin D (25(OH)D) concentrations and classified vitamin D status into optimal (25(OH)D ≥75 nmol/L), sufficient (50 to <75 nmol/L), and deficient (<50 nmol/L). We measured anthropometrics and body fat (using dual-energy X-ray absorptiometry) and we calculated age- and sex-specific standard deviation scores (SDS) for body mass index (BMI), fat mass index (FMI), body fat percentage (BF%), and android/gynoid (A/G) fat ratio. In a subsample of 2,714 children we used established genetic correlates of 25(OH)D levels and BMI as instrumental variables to evaluate causality.

Results: After adjustment for children's and parents' sociodemographic and lifestyle factors, 25(OH)D status was not associated with measures of adiposity. However, we observed interactions between 25(OH)D levels and sex on measures of adiposity. After adjustment for confounders, vitamin D deficient girls had a 0.17 SD (95% CI 0.02, 0.31) higher BF% than girls with optimal vitamin D levels. Similar results were observed for BMI and FMI in girls. Vitamin D status was not significantly associated with adiposity in boys. Furthermore, instrumental variable analyses provided no evidence for a causal relation between 25(OH)D levels and adiposity measures.

Conclusion: Our results suggest possible inverse associations between 25(OH)D levels and measures of body fat in girls, but not in boys. Future studies should further explore these sex differences and the causality of this association.

INTRODUCTION

Childhood obesity is an important public health problem, with major consequences not only for the child's health and well-being, but also for cardiometabolic health in later life. Vitamin D deficiency is also highly prevalent in both adults and children. Large population-based studies, including ours (Chapter 4.1), have reported a high prevalence (up to 70%) of suboptimal vitamin D levels in children who are otherwise healthy.[1-6] Studies in adults have reported an inverse association between obesity and 25-hydroxyvitamin D (25(OH)D) concentrations (reviewed by [7]) and several cross-sectional studies among school-age and adolescent children have also observed significantly lower 25(OH)D concentrations in children with excess body weight, body fat, or abdominal obesity.[2, 4, 8-14]

The causality and direction of effect of the described inverse relation between 25(OH)D concentrations and obesity remains unclear, i.e., do children with lower vitamin D levels have an increased risk of adiposity,[12, 15] or is obesity is associated with increased risk of vitamin D deficiency,[2, 9, 11] or is the association merely due to residual confounding. On the one hand, vitamin D is known to increase adipose tissue lipolysis and to decrease adipogenesis,[16] whereas on the other hand, vitamin D is stored in adipose tissue and obesity may therefore lead to decreased levels of circulating 25(OH)D.[17] The direction of the association could therefore be both ways.[18] One longitudinal study in 479 children aged 5 to 12 years, has shown greater increases in BMI and waist circumference over time in vitamin D deficient children compared to children with optimal vitamin D status at baseline, suggesting that vitamin D deficiency may lead to adiposity.[15]

Few studies have reported on the association between vitamin D status and adiposity in young children and most of these studies were small or did not adjust for lifestyle variables that might confound the association.[4, 9, 12, 14] We assessed the association between vitamin D status and detailed measures of body composition in a large multiethnic cohort of young children, in which we considered several potential confounders, and we examined whether these associations differ by age, sex, and ethnicity. In addition, we explored the causality of the association using SNPs for 25(OH)D concentrations and for BMI as instrumental variables.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward, in Rotterdam, the Netherlands.[19-20] The study was approved by the local medical ethics committee and written informed consent was obtained for all children. Around the age of 6 years, 6,690 children visited our research center, of whom 4,473 gave consent for a blood draw and 4,167 had data on 25(OH)D concentrations (Chapter 4.1, Figure 4.1.1).[6] The analyses in the present study include 4,104 children, with a median age of 6.0 years (95% range 5.7 to 8.0), who had data available on both 25(OH)D concentrations and body composition.

Vitamin D assessment

We assessed serum 25(OH)D concentrations, the most widely used indicator for vitamin D status.21 Non-fasting blood samples were drawn by antecubital venipuncture and blood samples were stored at -80 °C until analysis. Vitamin D measurements were conducted at the Endocrine Laboratory of the VU University Medical Center (VUMC, Amsterdam, the Netherlands). Serum 25(OH)D was measured using isotope dilution on-line solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS), a highly sensitive and specific method for 25(OH)D quantification.[22-23]

In short, a deuterated internal standard (IS: 25(OH)D3- d6) (Synthetica, Oslo, Norway) was added to the samples and 25(OH)D was released from its binding protein(s) with acetonitrile. Samples were extracted and analyzed by XLC-MS/MS (a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands)) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The limit of quantitation was 4.0 nmol/L; intra-assay coefficients of variation (CV) were <6% and inter-assay CVs were <8% for concentrations between 25 and 180 nmol/L. Following previous studies in pediatric populations, we categorized 25(OH)D into optimal (≥75 nmol/L), sufficient (50 to <75 nmol/L), deficient (25 to <50 nmol/L), and severely deficient (<25 nmol/L).[4, 11, 24-25]

Body composition assessment

Height (m) was determined in standing position to the nearest millimeter without shoes using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight (kg) was measured using a mechanical personal scale (SECA, Almere, the Netherlands) and BMI (kg/m^2) was calculated. Whole body dual-energy X-ray absorptiometry (DXA) scans were performed (iDXA, GE-Lunar, 2008, Madison, WI, USA), that analyzed fat, lean and bone mineral mass of the whole body and of specific regions using enCORE software (version13.6). Children were positioned on the DXA table without shoes, heavy clothing and metal objects in supine position and were asked not to move. Quality assurance tests were run every day using a standard calibration block of tissue-equivalent material, which showed CVs <0.5%. We calculated body fat percentage (BF%) by expressing total fat mass as percentage of total body weight. Body fat and fat-free mass were also standardized for height in analogy to BMI, by calculating fat mass index (kg/m^2) (FMI) and fat-free mass index (kg/m^2) (FFMI).[26] The ratio of android/gynoid fat mass (A/G ratio) was calculated as measure of body fat distribution.[27] Age- and sex-specific SD-scores were calculated for all body composition outcomes using data from all children in our study population with body composition data at the age of 6 years (n=6,491).[20]

Covariates

Information on folic acid supplement use in early pregnancy was obtained from a questionnaire at enrollment.[20] Child's sex and birthweight were available from medical records and hospital registries and sex- and gestational age-specific SD-scores for birthweight were calculated.[28] We defined ethnicity of the child on the basis of the countries of birth of the parents,[29] and categorized into: Western (Dutch, other European, American, Oceanian); Moroccan; Turkish; African

(Surinamese Creole, Antillean, Cape Verdean, other African); or Asian (Indonesian, other Asian, Surinamese Hindustani).[6] Information on breastfeeding was obtained from delivery reports and postnatal questionnaires.

In a subgroup of 2,714 children, cord blood samples were collected at birth and were genotyped using Illumina Infinium II HumanHap610 Quad Arrays following manufacturer's protocols and imputed to the combined HapMap Phase II CEU, CHB/JPT, YRI panel.[30] For instrumental variable analyses, we calculated genetic scores for vitamin D status and BMI. In line with previous studies,[18, 32] we calculated an unweighted vitamin D synthesis score as the sum of the 25(OH)D-increasing alleles of rs12785878 (near DHCR7) and rs10741657 (near CYP2R1). We calculated a genetic risk score for BMI using 29 of 32 independent variants that have previously been shown to be robustly associated with BMI.[33-34] Three of these 32 variants were not available in our dataset.[34] The score was weighted for the effect size of each variant in this previous meta-analysis.[33]

Around the child's age of 6 years we used a questionnaire to ask the parents about household income, maternal educational level, and maternal employment status. Information on child's participation in sports (yes/no) and watching television or using a computer (screen time) at 6 years of age were obtained via a questionnaire. The date of blood sampling was categorized into summer, fall, winter, and spring based on the Dutch standard seasons. Maternal anthropometrics were measured in the research center at the same day as the body composition measurements of their child and BMI was calculated.[20]

Statistical analyses

Vitamin D status was evaluated both as a continuous and categorical variable and measures of body composition were evaluated as continuous variables. Differences in subject characteristics between the different vitamin D status categories were assessed using ANOVA for normally distributed variables, Chi-square tests for categorical variables, and Mann-Whitney test for non-normally distributed variables. We used linear regression models to study the observational associations of serum 25(OH)D levels with BMI, BF%, FMI, and A/G ratio. We chose to use vitamin D levels as independent variable and adiposity measures as dependent variables, but the temporal direction of the association cannot be assessed in our study.

Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for ethnicity and season of blood collection, because we have previously shown that these variables are strongly associated with child vitamin D status.[6] Model 3 was further adjusted for maternal educational level, BMI, employment status, and folic acid supplement use during pregnancy; for household income; and for children's birthweight Z-score, breastfeeding, participation in sports, playing outdoors during the daytime, and screen time at the age of 6 years. These covariates were included because they changed the effect estimate of vitamin D status in model 2 with ≥10%. To handle missing data of covariates, multiple imputation was used to create 10 complete datasets with use of the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. Because we observed similar effect estimates in the original and imputed datasets, we present pooled estimates from the imputed datasets.

We tested potential nonlinear trends for the associations by adding the quadratic term of 25(OH)D levels to the models. To assess whether the associations were different by sex, age, or ethnicity we evaluated the statistical interaction by adding the product term of the covariate with 25(OH)D as a continuous variable to model 3 with unstandardized measures of body composition as dependent variables. As a sensitivity analysis, we repeated the analyses in participants with a Western ethnicity only and we repeated the analyses including a category of 25(OH)D <25 nmol/L (severe deficiency).

Bidirectional Mendelian randomization analyses were performed in a subgroup of the children with genetic data available (*n*=2,714). We used the vitamin D synthesis score as instrument for 25(OH)D concentrations and the BMI genetic risk score as an instrument for BMI. We performed two-stage least squares regression analyses using the vitamin D synthesis score as instrument for 25(OH)D levels; and using the BMI allelic score as instrument for obesity. These analyses were adjusted for age, sex, season of blood draw, and the first four principal components based on the genetic data to account for ethnicity. We used the Durbin-Wu-Hausman (DWH) test for endogeneity to examine the difference between the observational and instrumented effect estimates. Analyses were conducted using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and Stata 13 (StataCorp, College Station, TX, USA).

RESULTS

Subject characteristics

Descriptive characteristics of the children and their parents are presented in Chapter 4.1, Table 4.1.1. Most children had a Western ethnic background (69.0%) and had mothers with a higher educational level (57.3%). Mean ± SD 25(OH)D concentration was 65.1 ± 28.2 nmol/L. As described in detail in Chapter 4.1, 29.8% of the children in our study population were vitamin D deficient (25(OH)D <50 nmol/L) and only 33.7% had optimal vitamin D levels (25(OH)D ≥75 nmol/L).[6] Median BMI was 15.9 kg/m^2 (95% range 13.7 to 21.1) and median BF% was 23.5 (95% range 16.2 to 38.1).

Associations between 25(OH)D concentrations and body composition

In analyses adjusted for age and sex only (model 1), 25(OH)D concentrations were inversely associated with all measures of adiposity (Table 4.2.1). In models additionally adjusted for season and ethnicity (model 2), effect estimates strongly attenuated, but 25(OH)D levels remained inversely associated with BMI, BF%, and FMI, but no longer with distribution of body fat (A/G ratio). After further adjustment for child and parental sociodemographic and lifestyle factors (model 3), all effect estimates further attenuated and were no longer statistically significant (Table 4.2.1). Important confounders in this last model were maternal educational level, household income, and child participation in sports. Vitamin D status was not associated with child height, weight, or FFMI (Supplement 4.2.1).

Table 4.2.1 Associations between 25(OH)D concentrations and body composition at age 6 years in the whole group (*n*=4,104)

Serum 25(OH)D	BMI (SDS)	BF% (SDS)	FMI (SDS)	A/G ratio (SDS)
Model 1				
Continuous (per 10 nmol/L)	**-0.04 (-0.05, -0.03)**	**-0.04 (-0.05, -0.03)**	**-0.04 (-0.05, -0.03)**	**-0.02 (-0.03, -0.01)**
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	**0.10 (0.03, 0.17)**	**0.10 (0.04, 0.17)**	**0.11 (0.04, 0.17)**	0.05 (-0.02, 0.12)
Deficient (<50 nmol/L)	**0.26 (0.18, 0.33)**	**0.28 (0.21, 0.35)**	**0.29 (0.22, 0.36)**	**0.12 (0.05, 0.20)**
p for trend	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Model 2				
Continuous (per 10 nmol/L)	**-0.02 (-0.03, -0.01)**	**-0.03 (-0.04, -0.01)**	**-0.03 (-0.04, -0.01)**	-0.01 (-0.02, 0.00)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.07 (0.00, 0.14)	**0.08 (0.01, 0.15)**	**0.08 (0.01, 0.15)**	0.04 (-0.03, 0.11)
Deficient (<50 nmol/L)	**0.13 (0.04, 0.22)**	**0.18 (0.09, 0.27)**	**0.17 (0.08, 0.26)**	0.07 (-0.02, 0.16)
p for trend	***<0.01***	***<0.01***	***<0.01***	*0.10*
Model 3				
Continuous (per 10 nmol/L)	-0.01 (-0.02, 0.00)	-0.01 (-0.02, 0.00)	-0.01 (-0.02, 0.00)	0.00 (-0.01, 0.01)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.04 (-0.03, 0.11)	0.05 (-0.02, 0.12)	0.05 (-0.03, 0.12)	0.03 (-0.05, 0.10)
Deficient (<50 nmol/L)	0.07 (-0.02, 0.16)	0.08 (-0.01, 0.18)	0.08 (-0.01, 0.17)	0.02 (-0.07, 0.12)
p for trend	*0.15*	*0.07*	*0.07*	*0.57*

Values are based on multivariable linear regression models and reflect differences with 95% confidence intervals, in body composition outcomes (in age- and sex-specific SDS) for categories of sufficient and deficient vitamin D, with optimal as reference; or per 10 nmol/L higher 25(OH)D concentration. **Bold** values indicate statistically significant results ($p<0.05$).

Model 1 is adjusted for age and sex.

Model 2 is additionally adjusted season of blood draw and ethnicity

Model 3 is additionally adjusted for maternal education, maternal employment, maternal BMI, maternal use of folic acid supplements in early pregnancy, household income, child SDS birthweight, breast feeding exclusivity in the first four months of life, and child's participation in sports, playing outdoors during the daytime, and screen time.

Abbreviations: A/G ratio, android/gynoid fat mass ratio; BMI, body mass index; BF%, body fat percentage; FMI, fat mass index.

P for trend is obtained by using 25(OH)D levels in three categories as ordinal variable in linear regression models

Stratified analyses

We observed significant or borderline significant interactions between 25(OH)D concentration and sex for BMI (p<0.01), FMI (p<0.01), BF% (p<0.01), and A/G ratio (p=0.08). In regression analyses stratified for child sex, 25(OH)D levels were inversely associated with BMI, FMI, and BF% in girls, but not in boys (Table 4.2.2). After adjustment for confounders (model 3), vitamin D deficient girls had a 0.13 SDS higher BMI (95% CI 0.04, 0.22), a 0.17 SD higher BF% (95% CI 0.02, 0.31), and a 0.17 SD higher FMI (95% CI 0.08, 0.26) than girls with optimal vitamin D status. Levels of 25(OH)D were not associated with A/G ratio. Vitamin D status was not associated with body composition boys. Vitamin D status did not differ between boys and girls in our population.[6]

We also observed a significant negative interaction between 25(OH)D levels and child age for BMI, FMI, and BF% (all p<0.05), but not for A/G ratio (p=0.98). The inverse association between 25(OH)D and measures of adiposity became stronger with increasing age. After stratification for sex, the interaction with age was only present in girls, not in boys. In models stratified on age above or below the median of 6.0 years, effect estimates were larger in older than in younger girls (Supplement 4.2.2). Trends were significant for body fat percentage and fat mass index. There were no significant interactions of 25(OH)D with child ethnicity on measures of adiposity.

Table 4.2.2 Associations between vitamin D status and body composition at age 6 years, stratified by sex

Serum 25(OH)D	BMI (SDS)	BF% (SDS)	FMI (SDS)	A/G ratio (SDS)
Boys(n=2,072)				
Continuous (per 10 nmol/L)	0.01 (-0.02, 0.02)	0.00 (-0.02, 0.02)	0.00 (-0.02, 0.02)	-0.00 (-0.02, 0.02)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.03 (-0.07, 0.13)	0.05 (-0.05, 0.15)	0.05 (-0.05, 0.15)	0.02 (-0.09, 0.12)
Deficient (<50 nmol/L)	-0.08 (-0.22, 0.06)	-0.05 (-0.19, 0.10)	-0.06 (-0.19, 0.08)	-0.05 (-0.19, 0.10)
p for trend	*0.41*	*0.76*	*0.68*	*0.63*
Girls (n=1,959)				
Continuous (per 10 nmol/L)	**-0.02 (-0.04, -0.00)**	**-0.02 (-0.04, -0.00)**	**-0.02 (0.04, -0.00)**	-0.00 (-0.02, 0.02)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.07 (-0.03, 0.17)	0.04 (-0.07, 0.14)	0.05 (-0.05, 0.15)	0.01 (-0.10, 0.12)
Deficient (<50 nmol/L)	**0.16 (0.01, 0.30)**	**0.17 (0.02, 0.31)**	**0.16 (0.02, 0.30)**	-0.02 (-0.17, 0.14)
p for trend	***0.03***	***0.04***	***0.03***	*0.90*

Values are based on multivariable linear regression models and reflect differences with 95% confidence intervals, in body composition outcomes (in age- and sex-specific SDS) for categories of sufficient and deficient vitamin D, with optimal as reference; or per 10 nmol/L higher 25(OH)D concentration. **Bold** values indicate statistically significant results (p<0.05).

Model is adjusted for maternal education, maternal employment, maternal BMI, maternal use of folic acid supplements in early pregnancy, household income, child SDS birthweight, breast feeding exclusivity in the first four months of life, child's age and ethnicity, season of blood draw, and child's participation in sports, playing outdoors during the daytime, and screen time (model 3).

Abbreviations: A/G ratio, android/gynoid fat mass ratio; BMI, body mass index; BF%, body fat percentage; FMI, fat mass index.

P for trend is obtained by using 25(OH)D levels in three categories as ordinal variable in linear regression models

Additional analyses

There was no evidence for a nonlinear association. Sensitivity analyses restricted to children with a Western ethnicity showed similar results as observed in the whole group (data not shown). We examined the relation between the genetic instruments with their respective phenotypes and found that the vitamin D synthesis score was associated with a 3.2 nmol/L higher 25(OH)D concentration per additional average allele (p=5.8×10^{-10}) and explained 1.1% of the variance in 25(OH)D levels (F-statistic =39.2). Each average allele increase in the BMI genetic risk score was associated with a 0.05 higher BMI-SDS (p=2.7×10^{-21}, F-statistic =91.3) and the BMI score explained 3.1% of the variance in BMI-SDS. We performed bidirectional instrumental variable analyses in the whole group and stratified by sex. Results of these analyses did not support a causal relation between 25(OH)D levels and adiposity in either direction (Supplements 4.2.3 and 4.2.4)

DISCUSSION

In this population-based study of 6-year-old children, we observed an interaction between vitamin D levels and sex on adiposity parameters: vitamin D deficient girls have a higher BMI and body fat percentage than girls with an optimal vitamin D status; whereas such associations are not present in boys.

Interpretation and comparison with previous studies

In line with previous studies,[2, 4, 8-12, 14, 35-36] we observed a higher BMI, BF%, and FMI in children with lower 25(OH)D levels. However, associations were strongly attenuated and remained statistically significant after adjustment for sociodemographic and lifestyle confounders only among girls. Important confounding variables in our population were ethnicity, participation in sports, maternal educational level, household income, and maternal folic acid supplement use during pregnancy (a proxy for maternal healthy lifestyle). Playing outside and screen time were less important confounding variables. Many of the previously published studies on vitamin D status and obesity in children only adjusted their analyses for basic confounders such as age, sex, ethnicity, and season,[8, 10, 37] some for dietary factors or proxies of physical activity,[2, 4, 9, 11-12, 14, 36] whereas some did not adjust at all.[35, 38] Our findings suggest that parental sociodemographic variables may explain part of the association between vitamin D deficiency and adiposity and that results from these previous studies may have been hampered by residual confounding.

We observed significant inverse associations between vitamin D levels and measures of adiposity in girls, mainly in the older girls within our study population (>6.0 years). Contrary to our observation, Kouda *et al.* found a significant inverse association between 25(OH)D level and body fat in boys, but not in girls, in a cross-sectional analysis in 521 children aged 11 years.[13] These sex differences could be caused by differences in growth and fat deposition between boys and girls from early ages. In our study population, we previously observed stronger associations between dietary exposures and measures of adiposity in girls than in boys (e.g., for our analyses on protein intake described in Chapter 2.3).[39-40] This might be because of the age of the children at which we measured body composition measures. Around the age of 6 years, a rise in BMI takes place, the so-

called adiposity rebound, which has been shown to occur earlier in girls than in boys.[41] Possibly, we might only be able to observe an association with measures of body composition in children during or after this adiposity rebound has taken place. This might explain the contrasting results with the study from Kouda *et al.*, and may explain our findings in girls and older children, but not in boys and younger children.

In a cohort study with repeated anthropometric measurements in 5 to 12-year-old children (*n*=479), children with vitamin D deficiency at baseline had a greater increase in BMI and waist circumference after a median follow-up of 2.5 years than children with adequate vitamin D levels.[15] These results suggest that low vitamin D status could lead to adiposity. However, in this study no information was available on 25(OH)D levels at follow-up to also investigate the association between obesity and later vitamin D status. Interestingly, although the statistical interaction with sex was not significant, the authors of this study also observed an association of baseline 25(OH)D concentrations with an increase in height in girls – but not in boys.[15] However, associations with measures of obesity did not differ between boys and girls.

A potential inverse relation between vitamin D levels and body fat could be explained by various mechanisms. Low vitamin D status could predispose an individual to adiposity as suggested by the results from the abovementioned longitudinal study.[15] The vitamin D receptor is omnipresent in the human body and vitamin D has shown to be involved in several processes, for example in stimulating lipolysis and reducing adipogenesis in adipose tissue.[3, 7] In addition, obesity could contribute to lower circulating 25(OH)D by adipose sequestration of fat soluble vitamin D,[17] as suggested by results from a large bi-directional Mendelian randomization analysis in adults on BMI and 25(OH)D levels.[18] This latter study observed no significant association of higher 25(OH)D levels as predicted by genetic instruments with BMI, whereas a 10% higher genetically instrumented BMI was association with 4.2% lower 25(OH)D levels.[18] Finally, the relation might not be causal, but both vitamin D deficiency and obesity might share common causes, such as a lack of outdoor physical activity, low diet quality, or other lifestyle factors.

We performed exploratory Mendelian randomization analyses both in the whole group and stratified by sex, as the observational associations between vitamin D status and measures of adiposity were significant in girls only. We found no evidence for a causal relation between 25(OH)D concentrations and BMI or vice versa, however, we must note that our study did not have enough power to detect small differences, if any. Future studies observing an association between 25(OH)D levels and obesity in children, should further examine the causal direction of the association, for example by performing Mendelian randomization analyses across results of GWAS consortia constituting adequately powered settings, or by performing longitudinal analyses and measuring both vitamin D status and adiposity at multiple points in time.

Methodological considerations

Our study is among the first to report on the association between 25(OH)D levels and adiposity in a large number of young children from a multiethnic background. We used a highly sensitive method to measure vitamin D status[22] and we obtained detailed measures of body composition using DXA. Unlike many other studies, we could evaluate the role of a large number of potentially

confounding factors. Furthermore, we applied a Mendelian randomization approach to assess causality. However, if there are indeed small causal effects our study sample was too small to identify them.

A limitation of this study is that we did not have repeated measurements of vitamin D status and adiposity. Furthermore, we did not have detailed data on some potential determinants of vitamin D levels, such as sun exposure habits, parathyroid hormone levels, or skin pigmentation. We used birth countries of the parents to define ethnicity. We had no information on dietary intake of vitamin D intake or supplement use at the time of vitamin D and body composition measurements. However, as described in Chapter 4.1, vitamin D intake and supplement use in early childhood were not associated with 25(OH)D concentrations at the age of 6 years.[6]

Conclusions

In conclusion, in this population-based cohort in more than four thousand 6-year-old children, we observed associations between vitamin D deficiency and a higher body fat among girls, but not among boys. Further follow-up studies are needed to explore these sex differences and to evaluate the direction and causality of this association.

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SUPPLEMENT CHAPTER 4.2

Supplement 4.2.1 Associations of 25(OH)D status with height, weight and FFMI (*n*=4,104)

Serum 25(OH)D	Height (SDS)	Body weight (SDS)	FFMI (SDS)
Model 1			
Continuous (per 10 nmol/L)	0.01 (-0.00, 0.02)	**-0.02 (-0.03, -0.01)**	-0.00 (-0.01, 0.01)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	**0.07 (0.01, 0.13)**	0.06 (-0.01, 0.12)	0.06 (-0.00, 0.12)
Deficient (<50 nmol/L)	0.00 (-0.06, 0.07)	**0.11 (0.04, 0.17)**	0.05 (-0.01, 0.12)
p for trend	*0.11*	*<0.01*	*0.79*
Model 2			
Continuous (per 10 nmol/L)	0.01 (-0.01, 0.02)	-0.01 (-0.02, 0.01)	0.01 (-0.00, 0.02)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	**0.07 (0.01, 0.13)**	0.06 (-0.00, 0.12)	**0.07 (0.1, 0.13)**
Deficient (<50 nmol/L)	0.04 (-0.03, 0.10)	0.06 (-0.02, 0.13)	-0.01 (-0.07, 0.06)
p for trend	*0.37*	*<0.01*	***0.04***
Model 3			
Continuous (per 10 nmol/L)	-0.00 (-0.02, 0.01)	-0.00 (-0.02, 0.01)	0.01 (-0.00, 0.0)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.04 (-0.01, 0.10)	0.04 (-0.02, 0.10)	0.05 (-0.01, 0.11)
Deficient (<50 nmol/L)	0.05 (-0.03, 0.12)	0.04 (-0.03, 0.11)	0.00 (-0.07, 0.07)
p for trend	*0.45*	*0.67*	*0.07*

Values are based on multivariable linear regression models and reflect differences with 95% confidence intervals, in body composition outcomes (age- and sex-specific SDS) for categories of vitamin D status, with optimal as reference; or per 10 nmol/L higher 25(OH)D concentration. *P* for trend is obtained by using 25(OH)D levels in three categories as ordinal variable in linear regression models. **Bold** values indicate statistically significant results ($p<0.05$).

Model 1 is adjusted for age and sex; Model 2 is additionally adjusted season of blood draw and ethnicity;

Model 3 is additionally adjusted for maternal education, maternal employment, maternal BMI, maternal use of folic acid supplements in early pregnancy, household income, child SDS birthweight, breast feeding exclusivity in the first four months of life, and child's participation in sports, playing outdoors during the daytime, and screen time.

Abbreviations: A/G ratio, android/gynoid fat mass ratio; BMI, body mass index; BF%, body fat percentage; FMI, fat mass index.

Supplement 4.2.2 Covariate-adjusted associations between 25(OH)D status and body composition in girls stratified by median age

Serum 25(OH)D	BMI (SDS)	BF% (SDS)	FMI (SDS)	A/G ratio (SDS)
Girls ≤ 6.0 years (*n*=996)				
Continuous (per 10 nmol/L)	-0.00 (-0.02, 0.02)	-0.01 (-0.03, 0.00)	-0.01 (-0.03, 0.00)	-0.00 (-0.02, 0.02)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.03 (-0.09, 0.16)	0.03 (-0.09, 0.14)	0.03 (-0.09, 0.14)	-0.01 (-0.15, 0.13)
Deficient (<50 nmol/L)	0.07 (-0.09, 0.23)	0.10 (-0.05, 0.25)	0.09 (-0.06, 0.24)	0.01 (-0.16, 0.19)
p for trend	*0.37*	*0.19*	*0.26*	*0.93*
Girls > 6.0 years (*n*=998)				
Continuous (per 10 nmol/L)	**-0.04 (-0.07, -0.01)**	**-0.04 (-0.07, -0.01)**	**-0.04 (-0.07, -0.01)**	-0.01 (-0.04, 0.02)
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.08 (-0.0, 0.27)	0.10 (-0.06, 0.25)	0.08 (-0.09, 0.26)	0.04 (-0.14, 0.21)
Deficient (<50 nmol/L)	0.19 (-0.01, 0.40)	**0.24 (0.05, 0.43)**	**0.21 (0.01, 0.42)**	0.03 (-0.19, 0.24)
p for trend	*0.08*	***0.01***	***0.04***	*0.82*

Values are based on multivariable linear regression models and reflect differences with 95% confidence intervals, in body composition outcomes (in age- and sex-specific SDS) for categories of sufficient and deficient vitamin D, with optimal as reference; or per 10 nmol/L increase in 25(OH)D levels. *P* for trend is obtained by using 25(OH)D levels in three categories as ordinal variable in linear regression models **Bold** values indicate statistically significant results ($p<0.05$).

Models are adjusted for maternal education, maternal employment, maternal BMI, maternal use of folic acid supplements in early pregnancy, household income, child SDS birthweight, breast feeding exclusivity in the first four months of life, child ethnicity, season of blood draw, child age, child participation in sports, playing outdoors during the daytime, screen time (model 3).

Abbreviations: BMI, body mass index; BF%, body fat percentage; FMI, fat mass index; A/G ratio, android/gynoid fat mass ratio

Supplement 4.2.3 Association of 25(OH)D with measures of body composition using the vitamin D synthesis score as instrument to predict 25(OH)D levels (*n*=2,714)

	BMI (SDS)	BF% (SDS)	FMI (SDS)	A/G ratio (SDS)
All (*n*=2,714)				
Observed 25(OH)D	-0.013	-0.006	-0.009	-0.004
(per 10 nmol/L)	(-0.027, 0.002)	(-0.021, 0.008)	(-0.023, 0.006)	(0.019, 0.011)
Instrumented 25(OH)D	0.062	-0.017	0.010	-0.003
(per 10 nmol/L)	(-0.060, 0.185)	(-0.136, 0.102)	(-0.109, 0.130)	(-0.125, 0.118)
DWH p-value	*0.22*	*0.86*	*0.75*	*0.99*
Girls (*n*=1,338)				
Observed 25(OH)D	-0.017	-0.006	-0.012	-0.005
(per 10 nmol/L)	(-0.037, 0.004)	(-0.026, 0.014)	(-0.032, 0.009)	(-0.026, 0.016)
Instrumented 25(OH)D	-0.058	-0.076	-0.077	-0.108
(per 10 nmol/L)	(-0.183, 0.067)	(-0.205, 0.052)	(-0.204, 0.050)	(-0.244, 0.028)
DWH p-value	*0.51*	*0.27*	*0.30*	*0.12*
Boys (*n*=1,376)				
Observed 25(OH)D	-0.007	-0.005	-0.005	-0.002
(per 10 nmol/L)	(-0.028, 0.014)	(-0.026, 0.015)	(-0.026, 0.016)	(-0.022, 0.019)
Instrumented 25(OH)D	0.310	0.097	0.186	0.204
(per 10 nmol/L)	(-0.030, 0.650)	(-0.167, 0.360)	(-0.105, 0.476)	(-0.086, 0.495)
DWH p-value	*0.02*	*0.43*	*0.15*	*0.12*

Regression coefficients can be interpreted as the change in body composition outcomes (SDS) per 10 nmol/L increase in 25(OH)D levels, either observed or predicted using the vitamin D synthesis allele score, while keeping age, sex, and ethnicity (principal components) constant.

Supplement 4.2.4 Association of BMI with 25(OH)D levels using the BMI genetic risk score as instrument to predict BMI (*n*=2,714)

	25(OH)D (nmol/L) (95% CI)
All (*n*=2,714)	
Observed BMI (SDS)	-0.82 (-1.81, 0.17)
Instrumented BMI (SDS)	-1.70 (-7.17, 3.76)
DWH p-value	*0.75*
Girls (*n*=1,338)	
Observed BMI (SDS)	-1.15 (-2.59, 0.28)
Instrumented BMI (SDS)	-6.18 (-14.92, 2.57)
DWH p-value	0.25
Boys (*n*=1,376)	
Observed BMI (SDS)	-0.35 (-1.72, 1.02)
Instrumented BMI (SDS)	2.36 (-4.84, 9.57)
DWH p-value	0.45

Regression coefficients can be interpreted as the change in 25(OH)D (nmol/L) per SDS increase in body composition measures, either as measured in the research center or as predicted using the BMI genetic risk score, while keeping age, sex, and ethnicity (principal components) constant.

Chapter 4.3

Vitamin D status in childhood & cardiometabolic health

Manuscript based on this chapter:

Trudy Voortman, Edith H. van den Hooven, Anna Vitezova, Vincent W.V. Jaddoe, Oscar H. Franco Vitamin D status and cardiometabolic health in childhood: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Vitamin D levels have been associated with cardiovascular and metabolic health in adults. However, limited information is available on young children. We aimed to examine the association between vitamin D status and cardiometabolic health factors in 4,167 children participating in a multiethnic population-based cohort study.

Methods: At the children's age of 6 years, we measured serum 25-hydroxyvitamin D (25(OH)D) levels and classified vitamin D status into optimal (25(OH)D ≥75 nmol/L), sufficient (50 to <75 nmol/L), and deficient (<50nmol/L). At the same moment, we assessed children's body fat percentage, blood pressure, and blood concentrations of HDL cholesterol, triacylglycerol, and insulin. Age- and sex-specific SD-scores of these cardiometabolic measures were combined into a cardiometabolic risk factor score.

Results: After adjustment for age, sex, ethnicity, season, and several lifestyle and sociodemographic factors, vitamin D deficient children had 0.12 SD (95% CI 0.03, 0.22) higher insulin concentrations than children with optimal vitamin D levels. Vitamin D status was not associated with blood pressure, blood lipids, or the cardiometabolic risk factor score.

Conclusion: In our population-based cohort of young children, higher vitamin D levels are associated with lower insulin levels, but not with other measures of cardiometabolic health. Further studies are needed to examine whether vitamin D deficiency in childhood predicts insulin sensitivity in later life.

INTRODUCTION

Studies in adults suggest that vitamin D insufficiency is associated with several cardiovascular and metabolic risk factors. In observational studies, low serum 25-hydroxyvitamin D (25(OH)D) levels are associated with hypertension, insulin resistance, and cardiovascular disease.[1-2] Subclinical cardiometabolic risk factors, such as high blood pressure, high cholesterol levels, and insulin resistance, are already present in childhood[3] and are suggested to predict type 2 diabetes and cardiovascular disease in later life.[4-5] Furthermore, as has been described in Chapters 4.1 and 4.2, vitamin D deficiency in childhood is common[6-7] and has been linked to childhood obesity[6, 8-15] However, whether vitamin D levels are related to cardiometabolic health already in childhood has not been studied extensively.

To our knowledge, only one previous large population-based study examined this association in children. In a large sample (*n*=6,175) of 1 to 21-year-olds from the 2001-2004 U.S. National Health and Nutrition Examination Survey (NHANES), vitamin D deficiency (25(OH)D <37.5 nmol/L) was associated with a high blood pressure, elevated CRP levels, and low HDL cholesterol levels.[6] Another analysis in a subgroup of 3,577 12 to 19-year-olds from the same sample (NHANES 2001-2004) showed that low 25(OH)D levels were associated with hypertension, hypertension, hyperglycemia, and metabolic syndrome, but not with dyslipidemia.[16] This study was however limited by a lack of information on several important potential confounders, such as season of 25(OH)D measurement and time spent outdoors.[6, 16]

Other studies investigating the relation between vitamin D and cardiometabolic health in children were small and mainly focused on obese children: The results of these studies are inconsistent with regard to the associations between vitamin D and blood pressure,[17-18] blood lipids,[17, 19, 20] and markers of insulin sensitivity.[8, 18, 21-25]

Therefore we aimed to examine the associations between 25(OH)D status and several individual cardiometabolic factors and a combined cardiometabolic risk factor score in 4,167 6-year-old children from a large population-based cohort in the Netherlands.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[26] The study was conducted in accordance with the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus University Medical Center, Rotterdam. Written informed consent was obtained from parents for all children. A total of 7,893 children were available for postnatal follow-up,[26] of whom 6,690 visited the research center at the age of 6 years. For 4,167 of these children, information on 25(OH)D concentration and one or more cardiometabolic outcome measures was available (Chapter 4.1, Figure 4.1.1). The exact number of children in the current analyses slightly differs per outcome, ranging from 4,044 for blood pressure to 4,140 for cholesterol levels (Table 4.3.2).

Vitamin D assessment

We assessed 25(OH)D concentrations in serum, which is the most widely used indicator of vitamin D status.[27] Non-fasting blood samples were drawn by antecubital venipuncture at a median age of 5.9 years (95% range 5.6 to 6.6) in a dedicated research center in the Sophia Children's Hospital in Rotterdam. Blood samples were stored at -80 °C until analysis. Vitamin D measurements were conducted at the Endocrine Laboratory of the VU University Medical Center (VUMC, Amsterdam, the Netherlands). Serum 25(OH)D was measured using isotope dilution on-line solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS), a highly sensitive and specific method for 25(OH)D quantification.[28-29] In short, 25(OH)D was released from its binding protein(s) and a deuterated internal standard (IS: 25(OH)D3- d6) was added. Samples were extracted and analyzed using XLC-MS/MS (a Symbiosis online SPE system, Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The limit of quantitation was 4.0 nmol/L. Quality control samples demonstrated intra-assay and inter-assay coefficients of variation of <6% and <8% respectively for concentrations between 25 and 180 nmol/L. Following previous studies in pediatric populations, we categorized 25(OH)D into optimal (≥75 nmol/L), sufficient (50 to <75 nmol/L), deficient (50 to <25 nmol/L) and severely deficient (<25 nmol/L).[7, 11, 14, 30-31]

Cardiometabolic health assessment

Children's cardiometabolic health factors were measured in the research center at the same day as blood was drawn for vitamin D measurement.[32] Total body, android, and gynoid fat mass were measured using a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA), which analyzed fat, lean and bone mineral mass using enCORE software version 13.6. Children were placed on the DXA table without shoes, heavy clothing and metal objects, in supine position with their hands lying flat and pronated and were asked not to move. Quality assurance tests were run every day using a standard calibration block of tissue-equivalent material. Repeated measurements of this block had variation coefficients of <0.5%. Body fat percentage (BF%) was calculated by expressing total fat mass as percentage of total body weight, and android fat mass was divided by gynoid fat mass to obtain the ratio.

Insulin, C-peptide, C-reactive protein (CRP), total cholesterol, HDL cholesterol (HDL-C), and LDL cholesterol (LDL-C), and triacylglycerol (TAG) concentrations in blood samples were measured with enzymatic methods (using a Cobas 8000 analyzer, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay and inter-assay coefficients of variation ranging from 0.69 to 1.57%. Because of possible acute inflammation, children with CRP levels ≥27.9 mg/L were excluded and remaining values were dichotomized into <3 mg/L and ≥3mg/L.[33] While the children were lying, systolic and diastolic blood pressure (BP) were measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmomanometer Datascope Accutorr Plus™ (Paramus, NJ, USA). For our analyses we used mean SBP and mean DBP of the last three measurements. For all cardiometabolic outcomes we calculated age- and sex-specific SD-scores (SDS) on the basis of the total Generation R Study population with data on cardiometabolic health at age 6 years (n 4,414 to 6,491).[26]

In addition to the individual cardiometabolic outcomes, we applied a continuous score for overall cardiometabolic health. In line with previous studies that defined scores for a metabolic syndrome-like phenotype in children[33] and as described in Chapter 2.3, we used the sum of age- and sex-specific SD-scores of five components: BF%, blood pressure (including both SBP and DBP), HDL-C levels, TAG levels, and insulin levels. The SD-scores for HDL-C were multiplied by -1 because higher HDL-C levels reflect better cardiometabolic health. The SD-scores for SBP and DBP were multiplied by 0.5 so that each contributed half to the blood pressure component. Thus, the cardiometabolic risk factor score was calculated as: BF% SDS + 0.5 × SBP SDS + 0.5 × DBP SDS + TAG SDS + (-1× HDL-C SDS) + insulin SDS, with a higher score reflecting less optimal cardiometabolic health.[35]

Covariates

Information on maternal educational level, folic acid supplement use in early pregnancy, household income, and parity was obtained with a questionnaire at enrollment in the study.[26] Ethnicity of the child was defined on the basis of the countries of birth of the parents,[36] and was categorized into: Western (Dutch, other European, American, Oceanian); Moroccan; Turkish; African (Surinamese Creole, Antillean, Cape Verdean, other African); or Asian (Indonesian, other Asian, Surinamese Hindustani).[7] In a subgroup of 2,714 children, cord blood samples were collected at birth and were genotyped using Illumina Infinium II HumanHap610 Quad Arrays following manufacturer's protocols, and were imputed to the combined HapMap Phase II CEU, CHB/JPT, YRI panel.[37] In line with previous studies,[38,39] and as described in more detail in Chapter 4.2, we calculated a genetic score for vitamin D synthesis based on two markers.[40]

At the children's age of 6 years, their time spent watching television or using a computer (screen time) and time spent playing outside during the daytime were assessed using questionnaires. Height was determined at the research center in standing position to the nearest millimeter without shoes by a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands) and body mass index (BMI) (kg/m^2) was calculated. The date of blood sampling was categorized into summer, fall, winter, and spring based on the Dutch standard seasons.

Statistical analyses

Concentrations of 25(OH)D were analyzed both as continuous variable and categorized on the basis of clinical cut-offs, with optimal vitamin D levels (≥75 nmol/L) as reference category. Because of a low prevalence of severe vitamin D deficiency, we combined the deficient (50 to <25 nmol/L) and severely deficient groups (<25 nmol/L) for our main analyses. We used linear regression models to study the observational associations of serum 25(OH)D concentrations with cardiometabolic factors. Model 1 was adjusted for age and sex; model 2 was additionally adjusted for child ethnicity and season of blood draw; and model 3 was further adjusted for maternal educational level, parity, and folic acid supplement use during early pregnancy; household income; and child's screen time and playing outside during the daytime at 6 years of age. Covariates were included in the regression models on the basis of previous literature or a significant change (≥10%)

in effect estimate for at least one of the outcomes. We considered but did not include the following variables because they did not fulfill the 10%-criterion: maternal age, maternal BMI, smoking during pregnancy, alcohol use during pregnancy, birthweight, gestational age, breastfeeding in infancy, and child's participation in sports at the age of 6 years.

To assess whether the associations were different by sex, age, ethnicity, or body fat percentage of the child, we evaluated the statistical interaction by adding the product term of the covariate and 25(OH)D levels to model 3. Stratified analyses were conducted if the interaction term was significant (p<0.05). As sensitivity analyses, we repeated the analyses in participants with a Western ethnicity only and we repeated the analyses including the category of 25(OH)D <25 nmol/L (severe deficiency). For outcomes for which we observed significant associations, we additionally performed instrumental variable analysis in a subgroup of the children (n=2,714) to examine causality of the association.[39] We used a genetic vitamin D synthesis score as instrument for 25(OH)D levels and adjusted the models for age, sex, and the first four principal components of our genetic data to account for ethnicity (Chapter 4.2, Supplement 4.2.1).

To handle missing data of covariates (up to 34%), a multiple imputation procedure (n=10 imputations) was performed with use of the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. The multiple imputation procedure was based on the correlation between each variable that had missing values and the other subject characteristics.[41] Because we observed similar effect estimates in the original and imputed datasets, we report pooled estimates from the imputed dataset. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and Stata 13 (StataCorp, College Station, TX, USA).

RESULTS

Subject characteristics

Characteristics of the children and their mothers are presented in Table 4.3.1. Mean (±SD) 25(OH)D level was 65.1 ± 28.2 nmol/L. As described in Chapter 4.1, 29.8% of the children in our study population were vitamin D deficient (25(OH)D <50 nmol/L) and only 33.7% had optimal vitamin D levels (25(OH)D ≥75 nmol/L).[7]

Associations between 25(OH)D concentrations and cardiometabolic health

The associations between 25(OH)D levels and cardiometabolic factors are presented in Table 4.3.2. In models adjusted for age and sex only (model 1), a higher 25(OH)D concentrations was associated with a lower body fat percentage, lower insulin levels, lower SBP and DBP, but also lower HDL cholesterol levels and higher TAG levels. Further adjustment for season and ethnicity (model 2) attenuated all the effect estimates into non-significant for all cardiometabolic outcomes except body fat percentage and insulin levels. In the final multivariable model (model 3), higher 25(OH)D concentrations remained associated with lower insulin levels (-0.02 SD per 10 nmol/L (95% CI -0.04, -0.01)), but were no longer associated with body fat percentage (Table 4.3.2). Vitamin D status was not associated with concentrations of total cholesterol, LDL cholesterol, C-peptide, or CRP (Supplement 4.3.1).

Table 4.3.1 Subject characteristics (*n*=4,167)

	Mean ± SD, median (95% range), or percentage
Parental characteristics	
Folic acid supplement use during pregnancy (%)	
Never	31.7
Start ≤10 weeks	31.0
Start periconceptional	37.3
Maternal education level (%)	
Primary	8.1
Secondary	44.1
Higher	47.7
Household income (%)	
Low	34.2
Mid	9.4
High	56.4
Child characteristics	
Child's sex, girls (%)	48.5
Child's ethnicity (%)	
Western	69.0
Moroccan	6.2
Turkish	7.2
African	11.4
Asian	6.3
Child age (y)	6.0 (5.7-8.0)
Season (%)	
Winter	21.5
Spring	28.3
Summer	25.4
Fall	24.7
Playing outside daytime (h/d)	1.3 (0.1-4.0)
Screen time (h/d)	1.5 (0.2-4.8)
BMI (kg/m^2)	15.8 (13.7-21.3)
Body fat percentage (%)	23.8 (16.2-38.1)
Systolic blood pressure (mmHg)	60.4 ± 6.7
Diastolic blood pressure (mmHg)	102.4 ± 8.0
HDL cholesterol (mmol/L)	1.35 ± 0.31
Triacylglycerol (mmol/L)	0.95 (0.39-2.34)
Insulin (pmol/L)	112 (17-398)

Values are percentages, means ± SD for normally distributed variables, or medians (95% range) for continuous variables with a skewed distribution.

Additional analyses

No significant interactions were observed for 25(OH)D levels with age, sex, ethnicity, or body fat percentage of the child, except for one significant interaction between BF% and 25(OH)D levels on TAG levels (p=0.03). Stratification for tertiles of BF% revealed that the association of 25(OH)D levels with TAG was less strong in children with a higher BF%, although not significant in any of the groups. Sensitivity analyses restricted to children with a Western ethnicity showed similar results as observed in the whole group. We performed instrumental variable analyses for the observed association with insulin concentrations using the vitamin D synthesis score composed of two markers as instrument for 25(OH)D. Results of these analyses did not support a causal relation of 25(OH)D levels on insulin concentrations (Supplement 4.3.2).

DISCUSSION

The results of this large population-based study in 6-year-old children suggest no consistent associations between vitamin D status and cardiometabolic health. We observed an inverse association between 25(OH)D and insulin concentrations, however, this association was not observed for C-peptide concentrations and was not supported by findings from instrumental variable analyses. After adjustment for season, ethnicity, and other sociodemographic and lifestyle factors, we observed no associations between 25(OH)D concentrations and children's blood pressure, blood lipids, or a combined cardiometabolic risk factor score.

Interpretation and comparison with previous studies

Several studies have examined the association between vitamin D status and cardiometabolic health and disease in adult populations. In many observational studies, low serum 25(OH)D concentrations have been shown to be associated with hypertension, insulin resistance, and cardiovascular disease.[1-2] However, data from randomized controlled trials on vitamin D supplements are not conclusive.[42-43]

Only a few studies have examined these associations in children, including one other large population-based study. Using data from this cross-sectional survey in the U.S. (NHANES 2001-2004), two separate studies reported associations between vitamin D deficiency and several cardiometabolic risk factors. In the first study, among 6,175 children and adolescents aged 1 to 21 years, vitamin D deficiency (defined as 25(OH)D <37.5 nmol/L) was associated with a higher blood pressure, elevated CRP levels, and lower HDL cholesterol levels.[6] Similarly, in another cross-sectional analysis in a subsample of 3,577 adolescents aged 12 to 19 years from the same survey, low 25(OH)D levels were associated with hypertension, hypertension, hyperglycemia, and metabolic syndrome, but not with dyslipidemia.[16] However, both analyses in this study were limited by a lack of information on several potentially important confounding factors, such as season of 25(OH)D measurement and time spent outdoors.[6]

Other previous studies investigating the relation between vitamin D and cardiometabolic health in children were small and mainly focused on obese children. Overall, these studies reported inconsistent results on the presence or absence of associations with blood pressure, blood lipids, and CRP.[8, 17, 19-20] Nevertheless, in line with our findings, several of these studies reported associations between higher vitamin D concentrations and improved measures of insulin sensitivity in children.[8, 18, 22, 44] However, most of these studies reported only simple correlations with no or minimal adjustment for potential confounding factors.

In our analyses, we observed that vitamin D status was associated with several of the examined cardiometabolic markers in crude analyses, but that these associations were mostly explained by ethnicity of the child and season of examination, and further explained by other sociodemographic and lifestyle variables. This suggests that previous studies may have been hampered by residual confounding. In our adjusted analyses, only the association between 25(OH)D and insulin concentrations remained statistically significant.

Table 4.3.2 Associations of vitamin D status and cardiometabolic health factors at the age of 6 years

	BF% (SDS) *n*=4,104	Insulin (SDS) *n*=4,100	SBP (SDS) *n*=4, 044	DBP (SDS) *n*=4,044	HDL cholesterol (SDS) *n*=4,140	Triacylglycerol (SDS) *n*=4,125	Cardiometabolic risk factor score *n*=3,892
Model 1							
Per 10 nmol/L	***0.04 (-0.05, -0.03)***	***-0.02 (-0.03, -0.01)***	***-0.01 (-0.02, 0.00)***	***-0.03 (-0.04, -0.01)***	***-0.01 (-0.02, -0.00)***	***0.02 (0.01, 0.03)***	**-0.04 (-0.06, -0.01)**
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	**0.12 (0.05, 0.19)**	0.02 (-0.05, 0.10)	0.03 (-0.05, 0.10)	0.04 (-0.03, 0.11)	0.06 (-0.01, 0.13)	**0.08 (-0.15, -0.01)**	0.04 (-0.15, 0.23)
Deficient (<50 nmol/L)	**0.32 (0.24, 0.39)**	**0.09 (0.01, 0.17)**	**0.10 (0.03, 0.18)**	**0.19 (0.12, 0.27)**	**0.11 (0.04, 0.19)**	**0.15 (-0.23, -0.08)**	0.16 (-0.04, 0.37)
p for trend#	**<0.01**	**0.03**	**0.01**	**<0.01**	**<0.01**	**<0.01**	0.06
Model 2							
Per 10 nmol/L	***0.03 (-0.04, -0.01)***	***-0.02 (-0.04, -0.01)***	*0.01 (-0.01, 0.02)*	*0.00 (-0.02, 0.01)*	*0.00 (-0.02, 0.01)*	*0.01 (-0.00, 0.02)*	*-0.03 (-0.07, 0.00)*
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	**0.08 (0.01, 0.16)**	0.03 (-0.05, 0.10)	-0.02 (-0.09, 0.06)	-0.01 (-0.09, 0.06)	0.04 (-0.03, 0.12)	-0.05 (-0.13, 0.03)	0.03 (-0.17, 0.23)
Deficient (<50 nmol/L)	**0.18 (0.09, 0.27)**	**0.11 (0.02, 0.21)**	-0.02 (-0.11, 0.07)	0.05 (-0.04, 0.14)	0.06 (-0.03, 0.15)	-0.07 (-0.16, 0.02)	0.18 (-0.06, 0.42)
p for trend#	***<0.01***	***0.02***	*0.61*	*0.32*	*0.18*	*0.06*	*0.16*
Model 3							
Per 10 nmol/L	*-0.01 (-0.02, 0.00)*	***-0.02 (-0.04, -0.01)***	*0.01 (-0.00, 0.02)*	*0.00 (-0.01, 0.02)*	*0.00 (-0.02, 0.01)*	*0.01 (-0.01, 0.02)*	*-0.01 (-0.05, 0.02)*
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.03 (-0.04, 0.10)	0.03 (-0.05, 0.11)	-0.02 (-0.10, 0.05)	-0.02 (-0.09, 0.06)	0.04 (-0.03, 0.12)	-0.05 (-0.13, 0.02)	-0.01 (-0.21, 0.19)
Deficient (<50 nmol/L)	0.04 (-0.04, 0.12)	**0.12 (0.03, 0.22)**	-0.04 (-0.13, 0.05)	0.03 (-0.07, 0.12)	0.05 (-0.04, 0.15)	-0.08 (-0.17, 0.02)	0.08 (-0.17, 0.33)
p for trend#	*0.07*	***0.01***	*0.34*	*0.65*	*0.25*	*0.08*	*0.56*

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age- and sex-specific SD-scores) and in cardiometabolic risk score per 10 nmol/L increase in 25(OH)D levels, and categories of vitamin D status, compared to optimal levels. **Bold** values indicate statistically significant results.

Model 1: Adjusted for age and sex

Model 2: Same as model 1 and additionally adjusted for season and ethnicity

Model3: Same as model 2 and additionally adjusted for maternal education, parity, and folic acid supplement use in early pregnancy; household income; and child's screen time and playing outside during the daytime at 6 years.

Tests for trend were conducted using the categories of 25(OH)D levels as an ordinal variable in linear regression analyses.

However, these findings for insulin levels were not supported by exploratory instrumental variable analyses and were not supported by associations of vitamin D status with C-peptide concentrations. C-peptide is released from pancreatic beta-cells during cleavage of insulin from proinsulin; and because of its longer half-life, C-peptide blood concentrations are a more stable marker of insulin secretion than actual insulin concentrations.[45] This suggests that the observed associations for insulin concentrations in our study may also have been explained by residual confounding. Further large-scale studies are needed to examine the association between vitamin D status and cardiometabolic health in children, preferably using repeated measurements to identify the direction of the associations, if any, and adjusting for sociodemographic and lifestyle factors that may confound the associations.

Methodological considerations

Important strengths of this study are its prospective population-based design and the large number of subjects being studied. Although the current analyses are cross-sectional, prospectively collected information on many potential confounders was available. Unlike several previous studies examining these associations, we could evaluate the role of a large number of potentially confounding factors and we were able to perform a meticulous stepwise adjustment for confounders. Other strengths of our study are the detailed measurements of childhood cardiometabolic health.

A limitation of this study is that we did not have repeated measurements of vitamin D status and cardiometabolic health. Therefore we were not able to assess the direction of the potential association between vitamin D status and insulin levels. Another limitation is that blood samples were non-fasting, which may have introduced errors in the measurement of blood lipids and insulin concentrations. Furthermore, although we had information on several potential confounding factors, residual confounding in the observed association between 25(OH)D and insulin concentrations may still have occurred.

Conclusions

In conclusion, results of this large cohort study among 6-year-old children suggest that serum vitamin D levels are inversely associated with insulin concentrations, but not with blood lipids, blood pressure, or combined cardiometabolic health. Further research is needed to explore the potential link between vitamin D and insulin and to examine potential long-term cardiometabolic effects of vitamin D deficiency in childhood.

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SUPPLEMENT CHAPTER 4.3

Supplement 4.3.1 Associations of vitamin D status and C-peptide, total and LDL cholesterol, CRP levels at the age of 6 years

	C-peptide (SDS) *n*=4,100	Total cholesterol (SDS) *n*=4,140	LDL cholesterol (SDS) *n*=4,125	CRP (OR) *n=4,342*
Model 1				
Per 10 nmol/L	*-0.01 (-0.01, 0.01)*	*0.01 (-0.01, 0.02)*	*0.01 (-0.01, 0.02)*	*1.06 (0.95, 1.19)*
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.01 (-0.08, 0.10)	0.00 (-0.09, 0.09)	-0.01 (-0.09, 0.08)	1.08 (0.90, 1.28)
Deficient (<50 nmol/L)	-0.03 (-0.13, 0.06)	-0.05 (-0.14, 0.04)	-0.03 (-0.13, 0.06)	**1.34 (1.11, 1.61)**
p for trend#	0.67	0.19	0.41	**<0.01**
Model 2				
Per 10 nmol/L	*-0.02 (-0.02, 0.01)*	*0.01 (-0.05, 0.08)*	*0.01 (-0.01, 0.03)*	*1.06 (0.95, 1.19)*
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.02 (-0.07, 0.10)	-0.01 (-0.07, 0.07)	-0.01 (-0.10, 0.08)	1.01 (0.84, 1.21)
Deficient (<50 nmol/L)	0.00 (-0.11, 0.10)	-0.08 (-0.18, 0.03)	-0.07 (-0.17, 0.04)	1.03 (0.83, 1.26)
p for trend#	*0.88*	*0.10*	*0.17*	*0.95*
Model 3				
Per 10 nmol/L	*-0.01 (-0.03, 0.01)*	*0.01 (-0.00, 0.03)*	*0.01 (-0.01, 0.03)*	*1.06 (0.95, 1.19)*
Optimal (≥ 75 nmol/L)	Reference	Reference	Reference	Reference
Sufficient (50 to <75 nmol/L)	0.02 (-0.07, 0.11)	-0.01 (-0.09, 0.08)	-0.01 (-0.10, 0.08)	0.99 (0.82, 1.18)
Deficient (<50 nmol/L)	0.02 (-0.07, 0.13)	-0.08 (-0.19, 0.03)	-0.07 (-0.18, 0.04)	0.94 (0.76, 1.16)
p for trend#	*0.57*	*0.11*	*0.17*	*0.31*

Values are based on multivariable linear regression models and reflect differences (95% CI) in individual cardiometabolic outcomes (age and sex adjusted SD-scores) and in cardiometabolic risk score per 10 nmol/L increase in 25(OH)D levels, and categories of vitamin D status, compared to optimal levels. **Bold** values indicate statistically significant results.
Model 1: Adjusted for age and sex
Model 2: Same as model 1 and additionally adjusted for season and ethnicity
Model 3: Same as model 2 and additionally adjusted for maternal education, parity, and folic acid supplement use in early pregnancy; household income; and child's screen time and playing outside during the daytime at 6 years.
Tests for trend were conducted using the categories of 25(OH)D levels as an ordinal variable in linear regression analyses.

Supplement 4.3.2 Association of 25(OH)D with insulin concentrations using the vitamin D synthesis score as instrument to predict 25(OH)D levels (*n*=2,714)

	Insulin (SDS)
Observed 25(OH)D (per 10 nmol/L)	-0.013 (-0.027, 0.002)
Instrumented 25(OH)D (per 10 nmol/L)	0.005 (-0.124, 0.134)
DWH p-value	*0.66*

Regression coefficients can be interpreted as the change in insulin (SDS) per 10 nmol/L higher 25(OH)D levels, either observed or predicted using the vitamin D synthesis allele score, while keeping age, sex, and ethnicity (first 4 principal components) constant.

Chapter 5

Dietary patterns

Chapter 5.1

The development, validation & determinants of a diet quality score for preschool children

Manuscript based on this chapter:
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ABSTRACT

Background: Although many studies have examined health effects of infant feeding, studies on diet quality shortly after the weaning and lactation period are scarce. Our aims were to develop and evaluate a diet score that measures overall diet quality in preschool children, and to examine the sociodemographic and lifestyle determinants of this score.

Methods: On the basis of national and international dietary guidelines for young children, we constructed a diet score containing ten components: intake of vegetables; fruit; bread and cereals; rice, pasta, potatoes, and legumes; dairy; meat and eggs; fish; oils and fats; candy and snacks; and sugar-sweetened beverages. The total score ranged from 0 to 10 on a continuous scale, and was standardized to an energy intake of 1200 kcal/d with the residual method. The score was evaluated in 3,629 children participating in the Generation R Study, a population-based prospective cohort. Food consumption of the children was assessed with a food-frequency questionnaire at their age of 1 year.

Results: Mean (± SD) diet score was 4.1 ± 1.3. The food-based diet score was positively associated with intakes of many nutrients, including *n*-3 fatty acids (0.25 SD increase (95% CI 0.22, 0.27) per 1 point increase in the diet score), dietary fiber (0.32 (95% CI 0.30, 0.34)), and calcium (0.13 (95% CI 0.11, 0.16)); and inversely associated with intakes of sugars (-0.28 (95% CI -0.31, -0.26)) and saturated fat (-0.03 (95% CI -0.05, -0.01)). A higher diet score was associated with several health-conscious behaviors, such as maternal folic acid supplement use during pregnancy, no smoking during pregnancy, and less television watching of the child.

Conclusion: We developed a novel food-based diet score for preschool children that could be applied in future studies to compare diet quality in early childhood and to investigate associations between diet in early childhood and growth, health, and development.

INTRODUCTION

Dietary behaviors and food preferences develop early in life,[1] and there is evidence that they remain stable during childhood,[2] and from childhood to adulthood.[3] Furthermore, an inadequate diet in childhood affects child health[4] and may also affect health later in life.[5] Therefore, it is important to study diet already in early childhood. Although many studies have examined infant feeding, studies on diet quality shortly after the weaning and lactation period are scarce.

One way to examine diet is to focus on intakes of individual foods or nutrients. A complementary approach is to use holistic measures of overall diet, such as dietary patterns or diet scores.[6-7] Diet scores or patterns can either be data-driven, on the basis of dietary intakes in the population; or they can be predefined, for example on the basis of dietary guidelines.[7-8] An advantage of the data-driven approach is that it reflects actual dietary patterns in the population. A disadvantage, however, is that a pattern that is considered to be healthier does not necessarily reflect a diet that is actually healthy.[7, 9] Dietary guidelines, on the other hand, are developed on the basis of known relations between diet and health. Hence, guideline-driven approaches may better reflect a desirable dietary pattern. Another advantage of a guideline-driven approach are that different populations can be more easily compared. However, disadvantages of a predefined dietary pattern or index are that the variation between subjects might be small and that there is often scientific debate on what constitutes the healthiest diet.[7, 9]

There are a few previously published diet indices for children,[4, 10] but: these were not developed for preschool children,[11-13] they focused on dietary variety or behaviors rather than quality,[14-15] they were constructed for specific health outcomes,[16] or they included intake of specific micronutrients for which data is not commonly available in observational studies.[17] Therefore we aimed to develop a new food-based diet quality index for evaluating overall diet quality in preschool children. Secondly, we aimed to evaluate the construct validity of this score in preschool children participating in the Generation R Study, a population-based cohort in the Netherlands. Our third aim was to study the associations of various sociodemographic and lifestyle factors with the diet score in participants from the Generation R Study.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort in Rotterdam, the Netherlands.[18] The study was approved by the local medical ethics committee and written consent was given by parents. Pregnant women were enrolled between 2001 and 2005, and 7,893 live born children were available for postnatal follow-up. A food-frequency questionnaire (FFQ) to assess diet of the children around the age of 1 year was sent to 5,088 mothers who provided consent for follow-up and had sufficient mastery of the Dutch language. In total, 3,650 (72%) of these mothers returned the questionnaire.[19] After exclusion of subjects with invalid dietary data (n=18) and withdrawn consent (n=3), information on diet was available from 3,629 children (Figure 5.1.1). Mothers of a subgroup of the cohort, consisting of Dutch children only (n=1,106), received an additional FFQ around their child's age of 2 years.[18] This FFQ was completed for 844 children (94%) (Supplement 5.1.1).

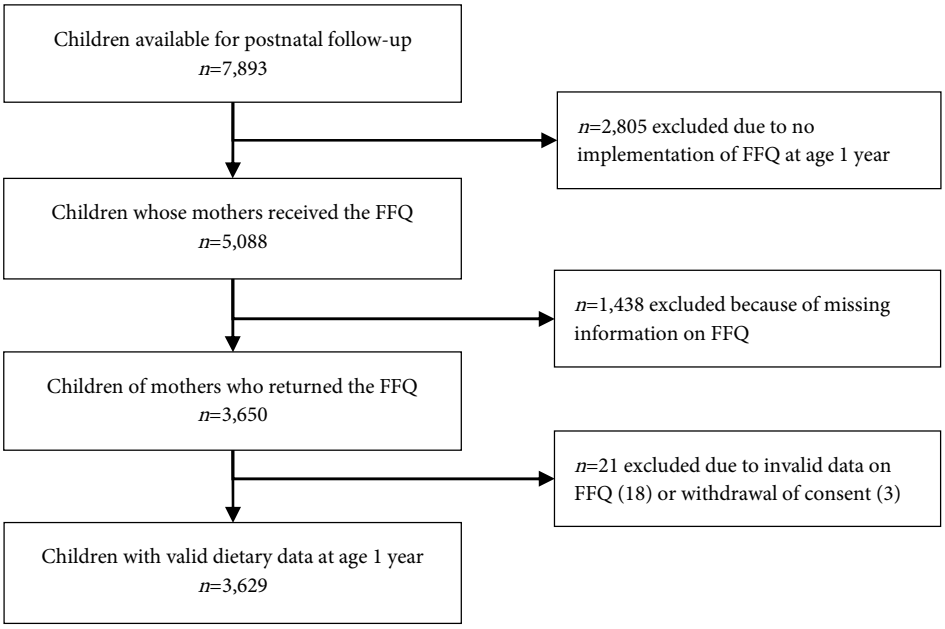

Figure 5.1.1 Flowchart of the study participants with dietary data at the age of 1 year

Dietary assessment

Dietary intake was assessed when the children were 1 year old (median age 12.9 months (95% range 12.2 to 19.0), with a semi-quantitative FFQ.[19] This FFQ was developed in cooperation with the division of Human Nutrition of Wageningen University, the Netherlands. An existing FFQ for Dutch adults was modified to only include foods frequently consumed during the second year of life according to a Dutch National Food Consumption Survey in 941 Dutch children aged 9-18 months.[20] 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 consisted of 211 food items and included questions on the frequency of consumption, serving sizes, type of food items, and preparation methods over the last month. Information on frequencies and servings was converted into grams per day on the basis of standardized portion sizes and nutrient intakes were calculated with the use of the Dutch Food Composition Table 2006.[21]

The FFQ was evaluated against three 24-h recalls in a representative sample of 32 Dutch children with a median age of 14 months (95% range 6 to 20) living in Rotterdam.[19] The three 24-h recalls were carried out by phone by trained nutritionists. Interclass correlation coefficients for nutrient intakes ranged from 0.36 to 0.74 (Supplement 5.1.2).

An almost identical FFQ was completed for a subgroup of 844 Dutch children at a median age of 2 years (median age 24.9 months, 95% range 24.3 to 27.6). This FFQ consisted of 230 food items, and compared to the FFQ at the age of 1 year, included more items on specific dairy products, nuts and seeds, and toddler foods; and fewer items on specific types of infant formula. For 777 children, dietary data was available at both 1 year and 2 years of age, with a median time difference between the two measurements of 12 months (95% range 5 to 14).

Construction of the diet score

We used national and international dietary guidelines as a basis for the development of the diet score. These guidelines were the few available food-based guidelines for preschool children with quantitative recommendations: from the Netherlands (1-3 years),[22] Germany (1 year),[23] Switzerland (1 year),[24] Flanders (1.5-3 years),[25] and Northern Ireland (1-5 years).[26] We also considered U.S. food-based recommendations for the general population from the age of 2 years[27] and other scientific literature on foods that were not consistently included in these dietary guidelines (e.g., sugar-sweetened beverages, fish, and whole milk).[28-30] Summaries of these guidelines are available upon request from the corresponding author.

Based on the guidelines and literatures we decided to include the following ten food groups in the diet score: vegetables; fruit; bread and cereals; rice, pasta, potatoes, and legumes; dairy; meat, poultry, eggs and meat substitutes; fish; oils and fats; candy and snacks; and sugar-sweetened beverages (Table 5.1.1). To better distinguish between more and less healthy diets, and in line with several of the guidelines,[22-24, 27] we took account of the healthier and less healthy options within the food groups: we only included recommended food items from the food groups (e.g., whole-wheat bread, but not white bread for the bread and cereal component), except for the snacks and the sugar-sweetened beverages components (Table 5.1.1).

Table 5.1.1 Food groups, cut-off levels, and food items included in the diet score

Food group	Cut-off level	Summary of included items	Summary of excluded items
Vegetables	≥ 100 g/d	Fresh vegetables, frozen or canned vegetables	Pickles
Fruit	≥ 150 g/d	Fresh fruit, canned fruit without added sugar	Canned fruit with added sugar, fruit juice
Bread and cereals	≥ 70 g/d	Whole-wheat bread or crackers, oatmeal, muesli without added sugar	White bread or crackers, breakfast cereals with added sugar
Rice, pasta, potatoes, and legumes	≥ 70 g/d	Boiled or steamed potatoes, whole-wheat pasta, couscous, whole-grain rice, legumes	Fried potatoes, French fries, white pasta, white rice
Dairy	≥ 350 g/d*	Semi-skimmed and skimmed milk and yogurt without added sugars, soy milk without added sugars, low-fat and reduced-fat cheeses (≤30% fat in dry matter)	Milk products with added sugars, full-fat cheeses, whole milk and yogurt
Meat, eggs and meat substitutes	≥ 35 g/d	Low-fat meat, eggs, tofu, tempeh	Fat and processed meat
Fish	≥ 15 g/d†	Fresh or canned fish	Fish fingers
Oils and fats	≥ 25 g/d	Low-fat margarine products (≤16g saturated fat and ≤1g trans-fat per 100g), vegetable oils, liquid cooking or frying fat	Butter, solid cooking or frying fats
Candy and snacks	≤ 20 g/d‡	Ice cream, potato chips, cookies, candy bars, fried snacks, cakes	-
Sugar-sweetened beverages	≤ 100 g/d‡	Soft drinks, lemonade	-

*Milk equivalents, on the basis of recommendations for 300 mL milk and 10 g cheese
†On the basis of recommendations for adults: 30% of the recommended weight of fish, meat and eggs combined (50 g)
‡ On the basis of portion sizes in several guidelines and combined within a maximum of 120 kcal/d (10 E%)

Cut-off values for the intakes of foods were derived from recommendations in existing guidelines.[22-27] For each food group, we calculated the ratio of the reported intake and the recommended intake, an approach that has previously been used for other diet indices.[13, 31] For instance, a child with a fruit intake of 120 g/d is assigned a score of 0.8 (120 divided by 150 g/d) for the fruit component. For each component the score was truncated at 1, meaning that if a child exceeds the recommended intake for a food group the score for this food group is 1. For the candy and snacks and the sugar-sweetened beverages components, children were assigned a score of 0 for intakes at or above the maximum cut-off (Table 5.1.1), and were assigned a score proportional between 0 and 1 if they consumed less than this cut-off, with a higher score for lower intakes. The scores for the single components were added together, resulting in an overall score that ranged from 0 to 10 on a continuous scale, with a higher score representing a healthier diet.

The score was adjusted for energy intake to control for overconsumption or underconsumption and to reduce the potential measurement error in the dietary assessment.[32] The diet score was standardized to an energy intake of 1200 kcal/d, which is the recommended energy intake for 1 to 4-year-old children in the Netherlands,[33] and in line with energy recommendations in other guidelines.[23, 25]

Parental characteristics

Information on maternal ethnicity, educational level, disease history, marital status, parity, and folic acid supplement use in early pregnancy; on household income; and on paternal age, educational level, and smoking was obtained from questionnaires at enrollment.[18] Parental educational level was dichotomized into higher and primary/secondary education.[34] Net household income was categorized into <€2,200 and ≥€2,200 per month.[35] Ethnicity of the mother 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 a country other than the Netherlands, that country determined ethnicity; if both parents were born in countries other than the Netherlands, the mother's country applied.[36] Diet of the mother during pregnancy was assessed at enrollment (median 13.5 weeks of gestation) with a semi-quantitative FFQ, and total energy and fiber intake were calculated.[37] Maternal smoking and alcohol consumption during pregnancy were assessed with questionnaires in each trimester.[38] At enrollment, maternal and paternal anthropometrics were measured without shoes or heavy clothing at the research center, and body mass index (BMI) was calculated.[18]

Child characteristics

Information on child's sex, birthweight, and gestational age at birth was available from medical records and hospital registries.[18] Information on vitamin and mineral supplement use (yes/no) at the age of 1 year was obtained from the previously mentioned FFQ. Ethnicity of the child was defined in the same way as for the mothers.[36] Child's height and weight were measured at the Community Child Health Centers at a median age of 14 months (95% range 13 to 16) and weight-for-age and height-for-age Z-scores were calculated on the basis of Dutch reference curves.[39] Information about breastfeeding was obtained from delivery reports and postnatal questionnaires at 2, 6 and 12 months after delivery. Breastfeeding was categorized into: never; partial; or exclusive

for at least 4 months.[19] Timing of introduction of solids in the first year of life was obtained from the FFQ administered at 1 year and was categorized into: <3 months, 3-6 months, or ≥6 months.[40] Information on doctor-diagnosed food allergies and hospital admission was obtained through questionnaires at the child's ages of 6 and 12 months.[18] Day care attendance in the first year of life was assessed in a questionnaire at the age of 12 months and was categorized into ≤24 or >24 h/wk.[41] Child's picky eating behavior was assessed with a questionnaire at the age of 1.5 years and categorized into picky or non-picky eating.[42] Information about sleep duration and television watching was collected with questionnaires at the age of 2 years. Nighttime sleep duration was dichotomized into <11.5 or ≥11.5 h/night.[43] Average duration of television watching was categorized into <1 or ≥1 h/d.[44]

Statistical analyses

The nutrient residual method was used to standardize the diet score and the component scores to a total energy intake of 1200 kcal/d.[45] Briefly, we used linear regression analysis to calculate energy-adjusted diet score residuals for each subject, with energy intake as independent variable and the diet score as dependent variable. Subsequently, the predicted score for a daily energy intake of 1200 kcal was added as a constant. We compared the diet score with previously defined data-driven dietary patterns for the 1 year-old children with Pearson correlations.[19] We also compared the diet score at the age of 1 year with the score at the age of 2 years with Pearson correlations.

The construct validity of the diet score was assessed by evaluating the associations of the food-based diet score with intakes of nutrients considered to be healthy (e.g., vitamins, dietary fiber, and *n*-3 fatty acids) or unhealthy (e.g., saturated fat, sodium, and sugars). For this we used linear regression models with energy-adjusted nutrient intake as dependent variable and the diet score as independent variable, adjusted for child's age and sex, and in separate models additionally adjusted for current breastfeeding and intake of infant formula (energy-adjusted). To adjust for multiple testing, a Bonferroni correction was applied and a p-value of 0.0017 (0.05/29 tests) was used as threshold of statistical significance. In addition to these models with nutrient intakes as continuous variables, we assessed the association between the diet score and adequate nutrient intakes as specified by European guidelines for young children.[46]

Linear regression models were used to analyze the associations of various sociodemographic and lifestyle factors with the diet score. Variables with $p<0.10$ in univariable models were included in one multivariable model. To retain only the strongest determinants, we performed a stepwise backward elimination procedure on the full multivariable model, with $p<0.10$ as endpoint.

To diminish potential bias associated with attrition, missing values of sociodemographic and lifestyle variables were multiple imputed by generating ten independent datasets with use of the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. The multiple imputation procedure was based on the correlation between each variable with missing values and other subject characteristics.[47-48] Because we found similar effect estimates, we present the pooled regression coefficients after the multiple imputation procedure.

We performed several sensitivity analyses. Firstly, since the FFQ was developed and validated for Dutch children but different ethnicities are included in our cohort, analyses were repeated in

children with a Dutch ethnic background only. Secondly, we performed sensitivity analyses in which children who still received breastfeeding (*n*=319) or a substantial amount of infant formula (i.e., more than 500 kcal/d, *n*=477) were excluded. Finally, we repeated our analyses after we excluded one random child per twin pair to account for clustering. All statistical analyses were performed with SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Subject and diet score characteristics

Most of the mothers were highly educated (66%), were nulliparous at enrollment (59%), and did not smoke during pregnancy (78%). Most of the children had a Dutch ethnicity (68%) (Table 5.1.2). After standardization to an energy intake of 1200 kcal/d, the diet score ranged from 0.6 to 8.8 with a mean (± SD) of 4.1 (± 1.3) in the 1-year-old children in our study population (Supplement 5.1.3). Most children had relatively high component scores for the intakes of fruit (median standardized score 0.80) and bread and cereal (0.81), but relatively low scores for the intakes of meat, eggs and meat substitutes (0.23); fish (0.15); and candy and snacks (0.18) (Supplement 5.1.4). Among Dutch children (*n*=2,413) for whom we previously derived data-driven dietary patterns with principal component analysis.[19] the diet score had a strong positive correlation with adherence to the 'health-conscious' dietary pattern (r=0.47, $p<0.001$) and a slight inverse correlation with adherence to the 'Western-like' dietary pattern (r=-0.12, $p<0.01$) (further details in Chapter 5.2).

The standardized diet score in the 844 Dutch children at the age of 2 years ranged from 0.7 to 8.4, with a mean of 4.6 ± 1.1. For 777 children, dietary data was available at both 1 and 2 years of age. In this subgroup, mean diet score was 4.2 ± 1.4 at the age of 1 year, increasing to 4.8 ± 1.2 at the age of 2 years ($p<0.001$). This increase was driven by an increase in all individual component scores, except for the candy and snacks component (Supplement 5.1.4). The correlation between the diet score at the two ages was 0.39 ($p<0.001$).

Table 5.1.2 Characteristics of the study participants (*n*=3,629)

	Mean, median, or percentage
Parental characteristics	
Maternal age (y)	31.8 (21.2-39.8)
Maternal ethnicity (%)	
Dutch	65.7
Other Western	7.5
Moroccan	3.2
Turkish	4.8
Surinamese and Antillean	10.4
Other non-Western	8.4
Maternal BMI at enrollment (kg/m^2)	23.5 (18.8-35.3)
Parity at enrollment (%)	
0	59.2
1	29.3
2 or more	11.5

Table 5.1.2 (continued) Characteristics of the study participants (*n*=3,629)

	Mean, median, or percentage
Married/living with partner (%)	90.9
Education level (%)	
No higher education	38.5
Higher	61.4
Net household income (%)	
<2,200/mo	34.4
≥€2,200/mo	65.6
Smoking during pregnancy (%)	
Never	77.9
Until pregnancy was known	9.7
Continued	12.4
Folic acid supplement use in early pregnancy (%)	
Never	16.6
Started in the first 10 weeks	31.1
Started periconceptionally	52.3
Child characteristics	
Girls (%)	51.0
Ethnicity (%)	
Dutch	68.0
Other Western	7.8
Moroccan	3.7
Turkish	4.4
Surinamese and Antillean	9.7
Other non-Western	6.4
Breastfeeding (%)	
Never	9.1
Partial	62.8
Exclusive for at least 4 months	28.1
Child characteristics at dietary measurement	
Age at FFQ (mo)	12.9 (12.2-19.0)
Total energy intake (kcal/d)	1270 (663-2279)
Still receiving breast milk (%)	8.8
Still receiving infant formula (%)	69.8
Intake of breast milk and infant formula combined (E%)	19.8 (0.0-57.2)
Supplement use (%)	
Multivitamin supplements (%)	2.6
Vitamin D supplements (%)	46.5
Vitamin A supplements (%)	8.1
Fluor supplements (%)	1.1
Iron supplements (%)	0.6
Other supplements (%)	1.6
Diet score (standardized to 1200 kcal/d)	4.2 ± 1.3

Values are percentages for categorical variables, means ± SD for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

Associations between the diet score and intakes of nutrients

We evaluated the construct validity of the diet quality score by assessing the associations between the diet score and intakes of nutrients (Table 5.1.3). With regard to macronutrients; a one point higher diet score was associated with a 0.40 SD higher protein intake (95% CI 0.38, 0.42), with a 0.07 SD higher fat intake (95% CI 0.05, 0.09), and with a 0.21 SD lower carbohydrate intake (95% CI -0.23, -0.18). More specifically, the diet score was positively related to intakes of polysaccharides and dietary fiber (0.32 (95% CI 0.30, 0.34) SD for fiber), but inversely to the intake of monosaccharides and disaccharides (-0.28 (95% CI -0.31, -0.26)). Furthermore, the diet score was inversely related to the intake of saturated fatty acids (-0.03 (95% CI -0.05, -0.01)), and positively associated with the intake of polyunsaturated fatty acids, mainly with *n*-3 fatty acids (0.25 (95% CI 0.22, 0.27)).

The diet score was positively associated with intakes of several vitamins and minerals (Table 5.1.3). However, in models adjusted for age and sex only, the diet score was inversely associated with vitamins C, D and E, iron, and zinc. After further adjustment for intake of infant formula and current breastfeeding, these associations became significantly positive, except for iron intake. The diet score was also positively associated with sodium intake. In the subgroup of 25-month-old children, intake of formula and breast milk was very low, and at this age associations between the diet score and nutrient intakes were all in the expected direction, except for a positive association between the diet score and sodium intake (Table 5.1.3).

Similar results were obtained in sensitivity analyses in which one child per twin pair was excluded (results not shown) or in which non-Dutch children were excluded (Online Supplement). In analyses from which children who still received breast milk or a substantial amount of infant formula were excluded, results were similar to those of the whole group after adjustment for intake of formula and breastfeeding (results not shown). In line with results from the models with nutrient intakes as continuous variable, the diet score was also associated with achieving recommended micronutrient intakes (Supplement 5.1.6).

Table 5.1.3 Associations between the diet score and intakes of nutrients in children at the ages of 1 year and 2 years

	1 year *n*=3,629 β (95% CI)†	1 year *n*=3,629 β (95% CI) †#	2 years *n*=844 β (95% CI)†
Macronutrients			
Protein	0.43 (0.41, 0.45)*	0.40 (0.38, 0.42)*	0.45 (0.40, 0.50)*
Vegetable protein	0.34 (0.31, 0.36)*	0.20 (0.18, 0.22)*	0.32 (0.26, 0.38)*
Animal protein	0.23 (0.21, 0.26)*	0.28 (0.25, 0.30)*	0.25 (0.20, 0.30)*
Fat	-0.02 (-0.05, 0.01)	0.07 (0.05, 0.09)*	0.08 (0.02, 0.14)*
Saturated fat	-0.04 (-0.06, -0.02)*	-0.03 (-0.05, -0.01)*	-0.06 (-0.10, -0.01)
Monounsaturated fat	-0.06 (-0.09, -0.04)*	0.06 (0.04, 0.08)*	0.11 (0.05, 0.17)*
Polyunsaturated fat	0.13 (0.11, 0.16)*	0.14 (0.12, 0.17)*	0.22 (0.16, 0.27)*
n-3 fatty acids	0.33 (0.31, 0.36)*	0.25 (0.22, 0.27)*	0.30 (0.24, 0.35)*
n-6 fatty acids	0.25 (0.22, 0.27)*	0.14 (0.12, 0.16)*	0.21 (0.15, 0.27)*
Carbohydrates	-0.13 (-0.16, -0.11)*	-0.21 (-0.23, -0.18)*	-0.20 (-0.26, -0.14)*
Monosaccharides and disaccharides	-0.26 (-0.28, -0.24)*	-0.28 (-0.31, -0.26)*	-0.32 (-0.37, -0.27)*
Polysaccharides	0.19 (0.17, 0.22)*	0.13 (0.10, 0.15)*	0.23 (0.17, 0.29)*
Dietary fiber	0.34 (0.31, 0.36)*	0.32 (0.30, 0.34)*	0.42 (0.37, 0.47)*
Micronutrients			
Beta-carotene	0.36 (0.34, 0.38)*	0.35 (0.33, 0.38)*	0.40 (0.34, 0.45)*
Vitamin B6	0.31 (0.29, 0.33)*	0.26 (0.24, 0.28)*	0.44 (0.38, 0.49)*
Vitamin B12	0.22 (0.19, 0.47)*	0.22 (0.20, 0.25)*	0.31 (0.25, 0.36)*
Vitamin C	-0.07 (-0.10, -0.05)*	0.05 (0.02, 0.07)*	0.04 (-0.02, 0.10)
Vitamin D	-0.21 (-0.23, -0.19)*	0.05 (0.04, 0.05)*	0.05 (-0.01, 0.11)
Vitamin E	-0.14 (-0.16, -0.11)*	0.08 (0.07, 0.10)*	0.19 (0.13, 0.25)*
Calcium	-0.02 (-0.05, 0.01)	0.13 (0.11, 0.16)*	0.21 (0.15, 0.27)*
Food folate (naturally present)	0.47 (0.45, 0.49)*	0.36 (0.34, 0.38)*	0.46 (0.41, 0.51)*
Total folate (incl. added folic acid)	0.01 (-0.02, 0.03)	0.03 (0.00, 0.05)	0.09 (0.03, 0.15)*
Iron	-0.25 (-0.27, -0.22)*	-0.02 (-0.03, -0.01)	0.13 (0.07, 0.18)*
Magnesium	0.38 (0.36, 0.40)*	0.32 (0.29, 0.34)*	0.41 (0.36, 0.47)*
Phosphorus	0.34 (0.31, 0.37)*	0.34 (0.32, 0.36)*	0.52 (0.46, 0.59)*
Potassium	0.34 (0.31, 0.36)*	0.32 (0.30, 0.34)*	0.46 (0.40, 0.53)*
Selenium	0.42 (0.39, 0.44)*	0.30 (0.28, 0.32)*	0.37 (0.32, 0.43)*
Sodium	0.24 (0.21, 0.26)*	0.10 (0.08, 0.13)*	0.28 (0.23, 0.34)*
Zinc	-0.10 (-0.13, -0.08)*	0.12 (0.11, 0.14)*	0.20 (0.14, 0.25)*

Values are regression coefficients from linear regression models with the diet score as independent variable and energy-adjusted standardized nutrient intakes as dependent variables. Regression coefficients can be interpreted as the difference in nutrient intake (in SD) with a 1 point higher diet score with, while keeping energy intake constant.

† Models are adjusted for child's age and sex.

#Additionally adjusted for current intake of infant formula (energy-adjusted) and current breastfeeding (yes/no)

* $p<0.0017$ (0.05/29 because of Bonferroni correction)

Sociodemographic and lifestyle determinants of the diet score

Associations between the diet score and various parental and child sociodemographic and lifestyle factors are presented in Table 5.1.4. In the multivariable model that included all variables kept after the backward selection procedure, the diet score was higher in children of mothers with health-conscious behaviors during pregnancy, such as a higher fiber intake, folic acid supplement use, no smoking, and having quit alcohol use. Furthermore, children with two or more older siblings had a 0.24 lower diet score than children who were their mother's firstborns (95% CI -0.33, -0.15). The diet score was not associated with maternal age, education, ethnicity, BMI, or marital status; household income; or paternal age, education, BMI or smoking.

The diet score was slightly higher in boys (0.11 (95% CI 0.19, 0.04) and in children with a higher weight-for-age. Turkish children had a 0.28 lower diet score (95% CI -0.47, -0.09) and Surinamese/Antillean children a 0.26 lower score (95% CI -0.42, -0.17) compared to children with a Western ethnicity. A lower diet score was associated with current intake of breast milk or infant formula, and with picky eating. Children who were breastfed in the first four months of life, who were introduced to complementary foods after 6 months of age, who currently receive food supplements, and who watch television less than 1 h/d had a higher diet score. The child's daycare attendance, sleep duration, or having a food allergy were not significantly associated with the diet score.

The full multivariable model without backward selection showed similar results (Table 5.1.4). In this model, older children had a slightly lower diet score, whereas in univariable models, higher age was associated with a higher diet score (Supplement 5.1.5). For the multivariable model presented in Table 5.1.4, similar results were obtained after exclusion of one child per twin pair, or children who still received breast milk or a substantial amount of infant formula (results not shown). In analyses in Dutch children only, maternal smoking and alcohol use during pregnancy were no longer associated with the diet score; the effect estimates for the other determinants were similar to those in the whole group (Online Supplement).

DISCUSSION

We developed a novel food-based diet score that can be used to evaluate overall diet quality of preschool children. Subsequently, the diet quality score was applied in a large cohort of 12 to 19-month-old children in the Netherlands. Evaluation of the diet score in this cohort showed that the diet quality score had adequate construct validity since it was positively associated with intake of nutrients considered to be healthy and inversely with intake of nutrients considered to be unhealthy. Furthermore, a higher diet quality score was associated with achieving recommended nutrient intakes. A higher diet score of the children was also linked to other health-conscious behaviors of mothers and children, such as less television watching and not smoking during pregnancy, but was not associated with sociodemographic or lifestyle factors of the fathers.

Development and construct validity of the diet score

The components and cut-off values of the diet score were defined on the basis of several food-based dietary guidelines for preschool children. Besides the decisions on what food groups to include, no additional weights were applied. It is not plausible that all food groups have the same health impact, however, there is also not enough evidence to assign relative contributions for the different food groups to overall health.[8] To control for different levels of food intakes, we adjusted the score for energy intake. This removes part of the variation due to differences in age and body size, partly controls for underconsumption and overconsumption, and might remove part of the measurement error in the dietary assessment.[32]

As a first step in validating the diet score, we assessed its construct validity, i.e., whether the score actually measures the construct being investigated: a healthy diet. Because our diet score was based on food groups, we used nutrient intakes and achieving recommended micronutrient intakes as alternative measures of healthy diet to assess construct validity. The diet score was positively associated with the intake of healthy nutrients, such as dietary fiber, polyunsaturated fatty acids, and vitamins. Exceptions were iron and total folate, which were not significantly associated with the diet score at the age of 1 year. This might be explained by the fact that many products for young children (e.g., biscuits, infant cereals) are fortified with folic acid and iron. Most of these products are not included in the diet score because of their high sugar content and/or because they are low in dietary fiber. Indeed, the diet score was positively associated with naturally present folate, but inversely with added folic acid. The lack of an association between the score and iron intake might also be caused by the contribution of fat meat products to iron intake in the children's diet.

In the diets of the 1-year-old children, the diet score was also inversely related to intakes of nutrients that are generally available in high amounts in infant formula or breast milk, such as vitamin D, vitamin E, and zinc. After adjusting for intake of formula, or after exclusion children who still received breastfeeding or large amounts of formula, associations between the diet score and these nutrients became positive, both for continuous nutrient intakes and for achieving recommended intakes. This indicates that our diet score is a good indicator of a healthy overall diet when one does not consider intake of breast milk or infant formula, which is no longer specifically recommended after the age of 1 year.

As expected, the score was inversely associated with the intake of monosaccharides and disaccharides, and with saturated fat. However, the diet score was positively associated with sodium intake. A potential explanation could be that many healthy food items are still rich in sodium (e.g., whole bread, canned vegetables or fish). Also, FFQs are limited in estimating sodium intake and our FFQ did not include items on added salt (i.e., during cooking or during consumption of a meal). Therefore, our estimates of sodium intake should be interpreted with caution. Overall, of the 29 examined foods and nutrients, 26 showed associations in the expected direction, indicating that the diet score showed adequate construct validity. We aim to evaluate the predictive validity of the score for later health, growth and development in future studies.

Table 5.1.4 Associations between parental and child sociodemographic and lifestyle factors and the diet score at the age of 1 year (*n*=3,629)

	Multivariable model[†]		Multivariable model after stepwise backward selection[‡]	
	β (95% CI)	*p*-value	β (95% CI)	*p*-value
Parental characteristics[§]				
Maternal age (y)	0.01 (-0.01, 0.02)	0.16	-	
Maternal ethnicity				
Western	*reference*			
Moroccan	0.34 (-0.09, 0.77)	0.12	-	
Turkish	-0.15 (-0.54, 0.24)	0.44	-	
Surinamese and Antillean	-0.02 (-0.21, 0.17)	0.92	-	
Other non-Western	-0.07 (-0.22, 0.08)	0.93	-	
Parity at enrollment				
0	*reference*		*reference*	
1	-0.01 (-0.10, 0.08)	0.84	0.01 (-0.09, 0.09)	0.98
2 or more	-0.26 (-0.40, -0.11)	<0.01	-0.24 (-0.33, -0.15)	<0.01
Maternal BMI at enrollment (kg/m^2)	-0.01 (-0.02, 0.00)	0.23	-	
Maternal education level				
No higher education	*reference*			
Higher	0.08 (-0.07, 0.23)	0.10	-	
Paternal education level				
No higher education	*reference*			
Higher	-0.02 (-0.16, 0.12)	0.74	-	
Marital stage				
Married or living together	*reference*			
No partner	0.05 (-0.09, 0.20)	0.47	-	
Net household income				
< €2200/mo	*reference*			
≥ €2200/mo	0.09 (-0.07, 0.11)	0.87	-	
Maternal energy intake during pregnancy (per 100 kcal/d)	0.00 (-0.01, 0.01)	0.99	-	
Maternal fiber intake during pregnancy (per 10 g/d)	0.43 (0.34, 0.52)	<0.01	0.44 (0.35, 0.53)	<0.01
Maternal smoking during pregnancy				
Never	*reference*		*reference*	
Until pregnancy was known	0.01 (-0.12, 0.14)	0.89	-0.01 (-0.16, 0.13)	0.86
Continued during pregnancy	-0.13 (-0.25, -0.01)	0.03	-0.17 (-0.29, -0.04)	0.01
Maternal alcohol use during pregnancy				
Never	*reference*		*reference*	
Until pregnancy was known	0.12 (0.00, 0.24)	0.06	0.15 (0.02, 0.27)	0.02
Continued during pregnancy	0.00 (-0.09, 0.10)	0.96	0.02 (-0.09, 0.12)	0.75
Maternal folic acid supplement use in early pregnancy				
Never	*reference*		*reference*	
Started in the first 10 weeks	0.29 (0.13, 0.44)	<0.01	0.29 (0.14, 0.44)	<0.01
Started periconceptionally	0.23 (0.06, 0.40)	<0.01	0.23 (0.08, 0.38)	<0.01

Table 5.1.4 (continued) Associations between parental and child sociodemographic and lifestyle factors and the diet score at the age of 1 year (*n*=3,629)

	Multivariable model†		Multivariable model after stepwise backward selection‡	
	β (95% CI)	*p*-value	β (95% CI)	*p*-value
Child characteristics§				
Sex				
Male	*reference*		*reference*	
Female	-0.11 (-0.19, -0.04)	<0.01	-0.10 (-0.18, -0.02)	<0.01
Birthweight (per 100 g)	0.00 (-0.01, 0.01)	0.50	-	
Age (mo)	-0.03 (-0.05, 0.00)	0.02	-0.03 (-0.05, 0.00)	0.08
Ethnicity				
Western	*reference*		*reference*	
Moroccan	0.10 (-0.30, 0.50)	0.62	0.17 (-0.03, 0.38)	0.10
Turkish	-0.04 (-0.35, 0.27)	0.83	-0.28 (-0.47, -0.09)	<0.01
Surinamese and Antillean	-0.24 (-0.45, -0.03)	0.03	-0.26 (-0.42, -0.17)	<0.01
Other non-Western	0.16 (-0.03, 0.36)	0.10	0.17 (-0.11, 0.28)	0.64
Breastfeeding in the first 4 months				
Exclusive	*reference*		*reference*	
Partial	-0.19 (-0.29, -0.09)	<0.01	-0.20 (-0.30, -0.10)	<0.01
Never	-0.14 (-0.29, 0.00)	0.05	-0.16 (-0.32, -0.01)	0.05
Currently receiving breast milk				
No	*reference*		*reference*	
Yes	-0.62 (-0.77, -0.47)	<0.01	-0.60 (-0.75, -0.46)	<0.01
Intake of infant formula at 1 year (energy-adjusted) (per 100 mL)	-0.14 (-0.16, -0.13)	<0.01	-0.14 (-0.16, -0.13)	<0.01
Timing of introduction of solids				
≥ 6 mo	*reference*		*reference*	
3-6 mo	-0.09 (-0.17, 0.00)	0.04	-0.09 (-0.17, -0.01)	0.03
< 3 mo	-0.19 (-0.37, -0.01)	0.03	-0.19 (-0.36, -0.02)	0.03
Currently receiving food supplements*				
No	*reference*		*reference*	
Yes	0.22 (0.14, 0.31)	<0.001	0.22 (0.14, 0.31)	<0.01
Picky eating at age 1.5 year				
No	*reference*		*reference*	
Yes	-0.24 (-0.33, -0.14)	<0.01	-0.24 (-0.37, -0.11)	<0.01
History of any food allergy at age 1 year				
No	*reference*			
Yes	-0.13 (-0.34, 0.09)	0.24	-	
Television watching at age 2 years				
< 1 h/d	*reference*		*reference*	
≥ 1 h/d	-0.11 (-0.20, -0.03)	<0.01	-0.13 (-0.21, -0.05)	<0.01
Sleep duration at age 2 years				
<11.5 h/night	*reference*			
≥11.5 h/night	0.07 (-0.02, 0.16)	0.14	-	
Weight-for-age at 14 mo (Z-score)	0.09 (0.02, 0.16)	0.02	0.06 (0.01, 0.10)	0.02
Height-for-age at 14 mo (Z-score)	-0.05 (-0.09, 0.01)	0.11	-	

†Values are regression coefficients with 95% confidence intervals from a linear regression analysis that included all variables described in the table.
‡Values are regression coefficients with 95% confidence intervals from a linear regression analysis with stepwise backward selection up to $p<0.10$
§Variables with $p>0.10$ in univariable analyses were not included in the multivariable model (Supplement 5.1.5).

Sociodemographic and lifestyle determinants

The mean diet score in our study population at the age of 1 year was 4.1 ± 1.3, on a theoretical scale from 0 to 10, indicating that diet quality could be improved. We investigated the associations with several sociodemographic and lifestyle factors to identify potential determinants of diet quality. In our sample boys had a slightly higher diet score than girls, whereas previous studies observed better diet quality in girls (reviewed by [49]). Turkish and Surinamese/Antillean, but not Moroccan children, had a lower diet score than children with a Western ethnicity. However, our FFQ was constructed and validated for Dutch children, which means that dietary intakes might be less accurately assessed in non-Dutch ethnicities.

Previous studies have shown that as children get older, diet quality tends to decrease,[13, 17, 50-51] which might be explained by parents' lower levels of control over the diets of their school-going children.[17] In our group of young children, age was not strongly associated with the diet score; and within the subgroup of children of whom dietary data was available at two points in time, the diet score increased between the age of 1 year and 2 years. This increase in diet quality can be explained by the increase in consumption of normal table foods and decrease in formula and toddler foods: in a subgroup of children for whom we had dietary data at both 1 year and 2 years of age and who did not receive infant formula or breast milk at either time point (n=259), mean diet score was the same at both ages (4.8 ± 1.3).

As expected, a higher diet score was associated with health-conscious behaviors, including no maternal smoking during pregnancy, folic acid supplement use in early pregnancy, maternal fiber intake, exclusive breastfeeding, food supplement use, and less time spent watching television of the child. These associations were significant after adjustment for sociodemographic variables. As expected, children who were picky eaters had a lower diet quality score than children who were not. Interestingly, children with two or more older siblings had a lower diet score than children who were their mother's firstborns. This is in line with results from previous studies in young children in which a poorer diet quality was observed in children with more siblings.[52-53] Parents who have more children might be less strict with controlling the diets of their children. Also, children with older siblings might be exposed to more unhealthy food products through their older brothers or sisters.

Methodological considerations

As individuals do not consume single nutrients or foods, but combinations of food items; intakes of nutrients are correlated and their function and metabolism may interact.[6-7] An advantage of a diet score is that it takes these interrelations into account.[54] Also, studying this diet score in relation to health outcomes could facilitate future development of food-based dietary guidelines for this age group. Moreover, the score provides an overall measure of a healthy diet which can be useful as potential covariate in epidemiological studies on other nutrition and lifestyle factors.[6] A limitation of diet scores is that there is no general consensus on what constitutes a healthy diet, therefore there are differences in the components and cut-offs among diet scores.[7] We based our score on current guidelines and scientific literature, but we were limited by the scarcity of quantitative food-based dietary guidelines for young children, a problem that has been acknowledged before.[4]

We evaluated the diet score in a large multiethnic group of children from the Netherlands with an age range between 12 and 19 months and in a smaller subgroup of Dutch children between 24 and 28 months of age. In this population, the score had good construct validity for intake of nutrients, especially for children who no longer receive large amounts of infant formula or breast milk. Because breastfeeding and infant formula intake are no longer recommended in the guidelines and therefore not included in the diet score, the score will be lower in children who consume a substantial amount of breast milk of formula. This should be taken into account when applying the diet score in subsequent studies. Future studies should examine whether our diet score can also be used for slightly older preschool children and in other population and countries.

Of all mothers who received the FFQ, 72% returned the questionnaire. Mothers who returned the questionnaire were generally more highly educated and had a healthier lifestyle. This selection toward a more healthy population may have underestimated true associations of sociodemographic and unhealthy lifestyle factors with the diet score. The FFQ was sent to Dutch speaking mothers only, but with different ethnic backgrounds. A limitation is that this FFQ was developed and validated for Dutch children,[19] whereas our study population was multiethnic. However, sensitivity analyses restricted to Dutch children revealed similar results, indicating that in our analyses no large bias due to ethnicity was present. Another limitation of FFQs in general, is that it relies on memory and reported food intakes are subject to substantial measurement error.[32] By adjusting for total energy intake, we aimed to reduce the measurement error.[32, 55] Evaluation of our FFQ against three 24-h recalls showed intraclass correlation coefficients for nutrient intakes from 0.36 to 0.74. These are not optimal but in line with coefficients obtained in other validation studies of dietary measurements.[56] Unfortunately, we were not able to validate our FFQ against energy or protein intake assessed with doubly labeled water or 24-h urine nitrogen.[32]

An advantage of predefined diet scores is that they better reflect an actual healthy diet and may better predict health outcomes than data-driven dietary patterns. In our population, the diet score was positively associated with adherence to a previously identified 'health-conscious' data-driven dietary pattern and inversely with a 'Western-like' dietary pattern.[19] Future studies are needed to explore whether the predefined diet score can better predict growth, health and disease outcomes in children than *a posteriori*-derived dietary patterns.

Conclusions

Our diet score for preschool children is positively associated with intakes of healthy nutrients and inversely associated with intakes of unhealthy nutrients in a population of 1- and 2-year-old children. The diet score was also associated with several other maternal and child lifestyle factors. The diet score can be a useful instrument for evaluating overall diet quality in preschool children. Furthermore, this diet score can be used in future studies that investigate the associations between childhood diet and health outcomes.

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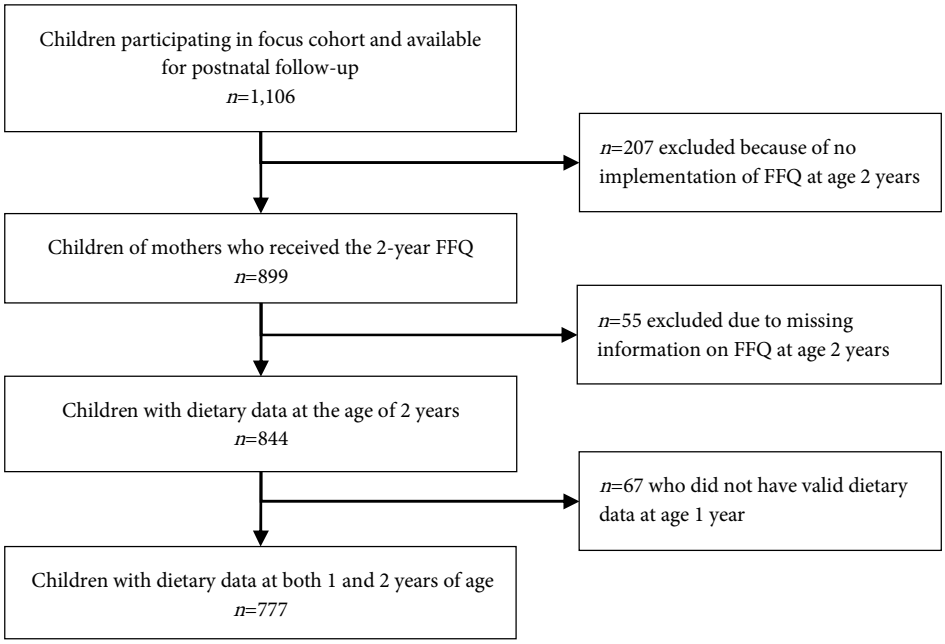
SUPPLEMENT CHAPTER 5.1

Additional Supplemental Material for this chapter can be found online at http://jn.nutrition.org/content/145/2/306/suppl/DCSupplemental

Supplement 5.1.1 Flowchart of the study participants with dietary data at the age of 2 years

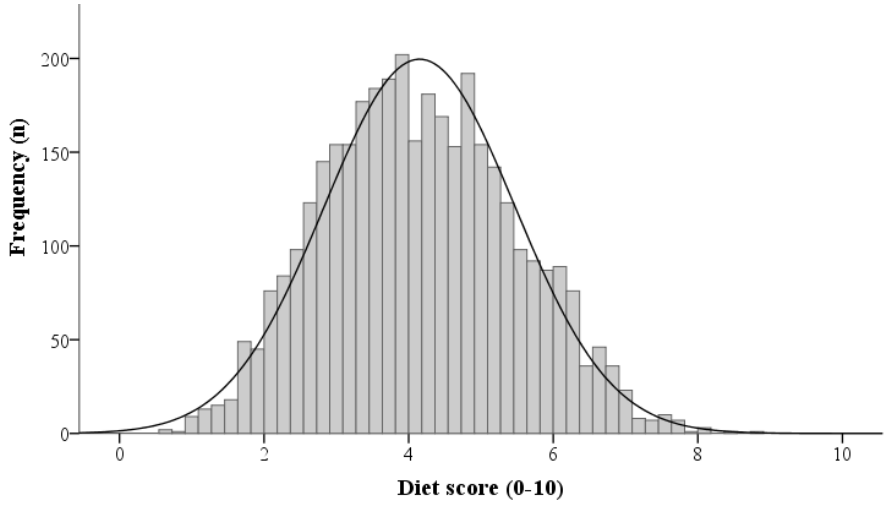


Supplement 5.1.2 Evaluation of the FFQ against three 24-h recalls in 32 Dutch children

	Intraclass correlation coefficient	*p*-value
Protein (g/d)	0.74	<0.01
Fat (g/d)	0.36	0.11
Carbohydrates (g/d)	0.48	0.02
Dietary fiber (g/d)	0.68	<0.01
Calcium (mg/d)	0.53	0.02
Iron (mg/d)	0.48	<0.01
Vitamin C (mg/d)	0.63	<0.01
Vitamin D (μg/d)	0.74	<0.01
Vitamin E (mg/d)	0.42	0.07

Values are unadjusted intraclass correlation coefficients

Supplement 5.1.3 Histogram of the diet score, standardized to 1200 kcal/d, in the study participants at the age of 1 year (*n*=3,629)



Supplement 5.1.4 Median score per diet score component, standardized to 1200 kcal/d, in the study participants at the ages of 1 and 2 years

	All children with dietary data		Children with dietary data at both 1 and 2 years	
	1 year (*n*=3,629)	2 years (*n*=844)	1 year (*n*=777)	2 years (*n*=777)
Component	Median score	Median score	Median score	Median score
Vegetables	0.41	0.46	0.40	0.46
Fruit	0.80	0.86	0.82	0.85
Bread and cereal	0.81	0.94	0.90	0.93
Rice, pasta, potatoes, and legumes	0.30	0.43	0.31	0.44
Dairy	0.24	0.68	0.28	0.68
Meat, eggs, and meat substitutes	0.23	0.43	0.22	0.43
Fish	0.15	0.22	0.11	0.22
Oils and fats	0.23	0.55	0.28	0.55
Candy and snacks	0.18	0.13	0.20	0.13
Sugar-sweetened beverages	0.11	0.68	0.06	0.68

Supplement 5.1.5 Crude associations between sociodemographic and lifestyle factors and the diet score at 1 year of age (*n*=3,629)

	Univariable model	
	β (95% CI)	***p*-value**
Parental characteristics		
Maternal age (y)	0.01 (0.00, 0.02)	0.007
Maternal ethnicity		
Western	*reference*	
Moroccan	-0.02 (-0.26, 0.23)	0.879
Turkish	-0.56 (-0.77, -0.36)	<0.001
Surinamese and Antillean	-0.53 (-0.67, -0.39)	<0.001
Other non-Western	-0.14 (-0.30, 0.02)	0.082
Parity at enrollment		
0	*reference*	
1	0.05 (-0.05, 0.14)	0.328
2 or more	-0.31 (-0.46, -0.17)	<0.001
Maternal BMI (kg/m^2)	-0.02 (-0.03, -0.01)	0.003
Maternal education level		
No higher education	*reference*	
Higher	0.27 (0.11, 0.43)	0.001
Marital stage		
Married or living together	*Reference*	
No partner	-0.22 (-0.37, -0.06)	0.007
Net household income		
< €2200/mo	*reference*	
≥ €2200/mo	0.23 (0.14, 0.32)	<0.001
Maternal history of diabetes, hypertension or hypercholesterolemia		
No	*reference*	
Yes	-0.05 (-0.30, 0.20)	0.701
Maternal energy intake during pregnancy (per 100 kcal/d)	0.01 (0.00, 0.02)	0.069
Maternal fiber intake during pregnancy (per 10 g/d)	0.05 (0.04, 0.06)	<0.001
Maternal smoking during pregnancy		
Never	*reference*	
Until pregnancy was known	-0.03 (-0.18, 0.12)	0.703
Continued during pregnancy	-0.32 (-0.46, -0.18)	0.001
Maternal alcohol use during pregnancy		
Never	*reference*	
Until pregnancy was known	0.28 (0.14, 0.42)	<0.001
Continued during pregnancy	0.15 (0.05, 0.25)	0.005
Maternal folic acid supplement use in early pregnancy		
Never	*reference*	
Started in the first 10 weeks	0.56 (0.41, 0.71)	<0.001
Started periconceptionally	0.55 (0.41, 0.69)	<0.001
Paternal age (y)	0.01 (-0.01, 0.01)	0.632
Paternal BMI (kg/m^2)	-0.01 (-0.02, 0.01)	0.224
Paternal education level		
No higher education	*reference*	
Higher	0.14 (0.02, 0.26)	0.022
Paternal smoking		
No	*reference*	
Yes	-0.03 (-0.13, 0.07)	0.555

Supplement 5.1.5 (continued) Crude associations between sociodemographic and lifestyle factors and the diet score at 1 year of age (*n*=3,629)

	Univariable model	
	β (95% CI)	***p*-value**
Child characteristics		
Sex		
Male	*reference*	
Female	-0.11 (-0.20, -0.02)	0.012
Birthweight (per 100 g)	0.01 (0.01, 0.02)	0.001
Gestational age at birth (mo)	0.01 (-0.02, 0.03)	0.424
Age (mo)	0.04 (0.02, 0.06)	0.001
Ethnicity		
Western	*reference*	
Moroccan	-0.01 (-0.24, 0.22)	0.940
Turkish	-0.53 (-0.74, -0.32)	<0.001
Surinamese and Antillean	-0.61 (-0.76, -0.46)	<0.001
Other non-Western	-0.08 (-0.26, 0.11)	0.404
Breastfeeding		
Full in first 4 mo	*reference*	
Partially in first 4 mo	-0.28 (-0.39, -0.18)	<0.001
Never	-0.22 (-0.40, 0.04)	0.014
Currently receiving breast milk		
No	*reference*	
Yes	-0.12 (-0.27, 0.02)	0.092
Intake of infant formula at age 1 year (energy-adjusted) (per 100 mL)	-0.14 (-0.15, -0.13)	<0.001
Timing of introduction of solids		
≥ 6 mo	*reference*	
3-6 mo	-0.17 (-0.26, -0.08)	<0.001
< 3 mo	-0.37 (-0.57, -0.18)	<0.001
Currently receiving food supplements		
No	*reference*	
Yes	0.49 (0.41, 0.58)	<0.001
Picky eating at age 1.5 year		
No	*reference*	
Yes	-0.32 (-0.42, -0.22)	<0.001
History of any food allergy at age 1 year		
No	*reference*	
Yes	-0.26 (-0.49, -0.04)	0.031
Hospital admission in the first year of life		
No	*reference*	
Yes	-0.03 (-0.17, 0.10)	0.587
Day care attendance at age 1 year		
≤ 24 h/wk	*reference*	
> 24 h/wk	-0.01 (-0.14, 0.12)	0.868
Television watching at age 2 years		
< 1 h/d	*reference*	
≥ 1 h/d	-0.22 (-0.30, -0.13)	<0.001
Sleep duration at age 2 years		
<11.5 h/night	*reference*	
≥11.5 h/night	0.16 (0.05, 0.26)	<0.001
Weight-for-age at 14 mo (Z-score)	0.07 (0.03, 0.12)	0.004
Height-for-age at 14 mo (Z-score)	0.02 (-0.03, 0.07)	0.048

Values are regression coefficients with 95% confidence intervals from univariable linear regression analyses.

Supplement 5.1.6 Association between the diet score and the odds of meeting recommended intakes of micronutrients (*n*=3,629)

	Adequate intakes 1 to 3-year-old children*	Odds ratio (95% CI) †#	Odds ratio (95% CI) †$
n-3 fatty acids (mg/d)	≥250 mg/d	2.53 (2.27, 2.82)	2.07 (1.83, 2.34)
Dietary fiber (g/d)	≥10 g/d	2.30 (2.04, 2.60)	2.34 (2.04, 2.68)
Vitamin C (mg/d)	≥20 mg/d	-**	-**
Vitamin D (µg/d)	≥10 µg/d	0.67 (0.63, 0.71)	1.39 (1.22, 1.57)
Vitamin E (mg/d)	≥6 mg TE/d	0.91 (0.84, 0.97)	1.39 (1.27, 1.53)
Calcium (mg/d)	≥600 mg/d	1.09 (1.00, 1.19)	1.62 (1.46, 1.80)
Total folate (µg/d)	≥100 µg/d	1.24 (1.10, 1.40)	2.19 (1.85, 2.59)
Iron (mg/d)	≥8 mg/d	0.68 (0.63, 0.74)	0.91 (0.81, 1.02)
Magnesium (mg/d)	≥85 mg/d	-**	-**
Phosphorus (mg/d)	≥460 mg/d	1.89 (1.46, 2.45)	2.24 (1.63, 3.09)
Potassium (mg/d)	≥800 mg/d	1.93 (1.76, 2.11)	2.59 (2.29, 2.92)
Selenium (µg/d)	≥20 µg/d	2.33 (2.18, 2.49)	2.14 (1.99, 2.29)
Sodium (mg/d)	170-370 mg/d	-**	-**
Zinc (mg/d)	≥4 mg/d	1.23 (1.09, 1.38)	2.52 (2.10, 3.03)

* According to the EFSA Panel on Dietetic Products Nutrition and Allergies (NDA) (2013). Scientific Opinion on nutrient requirements and dietary intakes of infants and young children in the European Union. Parma, Italy, European Food Safety Authority (EFSA).

† Values are odds ratios from logistic regression models with the diet score as independent variable and reaching adequate intake (yes/no) as dependent variables.

Models are adjusted for child's age and sex.

$ Additionally adjusted for current intake of infant formula (energy-adjusted) and current breastfeeding (yes/no)

** ≤10 children in one group (6 children did not reach adequate intake for magnesium, 10 children did not reach adequate vitamin C intake, and only 4 children had adequate sodium intake)

Chapter 5.2

Dietary patterns in early childhood & body composition

Manuscript based on this chapter:

Trudy Voortman*, Elisabeth T. M. Leermakers*, Vincent W.V. Jaddoe, Albert Hofman, Oscar H. Franco, Edith H. van den Hooven, Jessica C. Kiefte-de Jong. *A priori* and *a posteriori* dietary patterns at the age of 1 year and body composition at the age of 6 years: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Dietary patterns have been linked to obesity in adults, however, not much is known about this association in early childhood. We examined associations of different types of *a posteriori* and *a priori*-defined dietary patterns in toddlers with body composition at school age.

Methods: This study was performed in 2,026 Dutch children participating in a population-based cohort study. Dietary intake at the age of 1 year was assessed with a validated food-frequency questionnaire. At the children's age of 6 years, we measured their anthropometrics and body fat mass (with dual-energy X-ray-absorptiometry) and calculated age- and sex-specific SD-scores for body mass index (BMI), fat mass index (FMI), and fat-free mass index (FFMI). Three dietary pattern approaches were used: 1) *A priori*-defined diet quality was assessed with a guideline-based diet score for preschool children. 2) With principal component analysis (PCA), two patterns based on variation in food group intake were extracted which we labeled 'health-conscious' and 'Western-like'. 3) Reduced-rank regression (RRR) was used to identify two dietary patterns on the basis of on variations in FMI and FFMI.

Results: After adjustment for confounders, children in the highest quartile of the *a priori*-defined diet score or the 'health-conscious' PCA-pattern at 1 year of age had a higher FFMI at age 6 years than children in the respective lowest quartile (0.19 SD (95% CI 0.08, 0.30) per SD higher diet score). These patterns were not associated with FMI. Adherence to the 'Western-like' PCA-pattern was not associated with body composition. The first RRR-derived pattern was positively associated with both FMI and FFMI and was characterized by high intake of meat, fish, sauces, and sugar-containing beverages. The second RRR-pattern was positively associated with FFMI, and inversely with FMI and was characterized by intake of whole grains, pasta and rice, and vegetable oils.

Conclusion: Our results from both *a priori* and *a posteriori*-derived dietary patterns suggest that patterns characterized by high intake of vegetables, grains, and vegetable oils in early childhood are beneficial for later body composition; whereas patterns characterized by high intake of refined grains, meat, and sugar-containing beverages may be associated with unfavorable body composition.

INTRODUCTION

Childhood adiposity is of great concern because of its adverse consequences for both short and long term health.[1] Diet in early childhood may be an important target for prevention of childhood obesity, but nevertheless, there are not many studies that examined overall diet of preschool children in relation to later body composition.[2-3] Analysis of dietary patterns can be used to characterize overall food intake. This method has emerged as a complementary approach for studying diet in addition to examining intake of individual nutrients or foods.[4-5] Three main approaches have been used to identify dietary patterns. Dietary patterns can be defined *a priori,* for instance on the basis of existing dietary guidelines; they can be defined *a posteriori,* using information on dietary intake of the population; or they can be derived *a posteriori* based on the variation in specific markers related to health.[6]

Previous studies in adults have shown that high adherence to *a posteriori*-derived 'Western-like' or 'Empty calorie' dietary patterns is associated with higher risk of obesity, whereas high adherence to so-called 'Prudent' or 'Heart Healthy' dietary patterns is linked with lower obesity risk.[7-9] Likewise, higher scores on *a priori*-defined diet quality indices, such as the Mediterranean diet score, the Healthy Eating Index (HEI), or the Diet Quality Index (DQI), have been associated with a lower prevalence of obesity and less weight gain in adult populations.[10-13] A few studies in children also reported associations between higher scores on *a posteriori*-derived 'Snacking' or high-fat and low-fiber dietary patterns and a higher risk for obesity,[14-15] and similarly, between higher *a priori*-defined diet quality scores and a lower risk for obesity.[16-17]

However, studies on overall diet in young children shortly beyond the weaning period, in relation to later obesity are scarce.[2] Therefore, we explored the associations between dietary patterns in children at the age of 1 year and body mass index (BMI), fat mass index (FMI), and fat-free mass index (FFMI) at the age of 6 years. We applied three different methods to characterize dietary patterns: 1) an *a priori*-defined diet quality score[18] based on dietary guidelines for preschool children as described in Chapter 5.1; 2) *a posteriori*-derived dietary patterns based on variations in food intake,[19] extracted using principal component analysis; and 3) *a posteriori*-defined patterns based on variations in body composition outcomes, identified using reduced-rank regression.

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[20] The study was conducted in accordance with the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam. Written informed consent was provided by the parents of all children. To avoid the influence of cultural differences in dietary patterns, our analyses were restricted to children with a Dutch ethnicity.[21] A total of 4,215 Dutch children participated in the preschool follow-up.[20] Data on dietary patterns were available for 2,413 of these children. At the age of 6 years, 2,026 (84%) of these children visited the research center and had anthropometrics measured, and body fat measures were available for 1,980 of them (Figure 5.2.1).

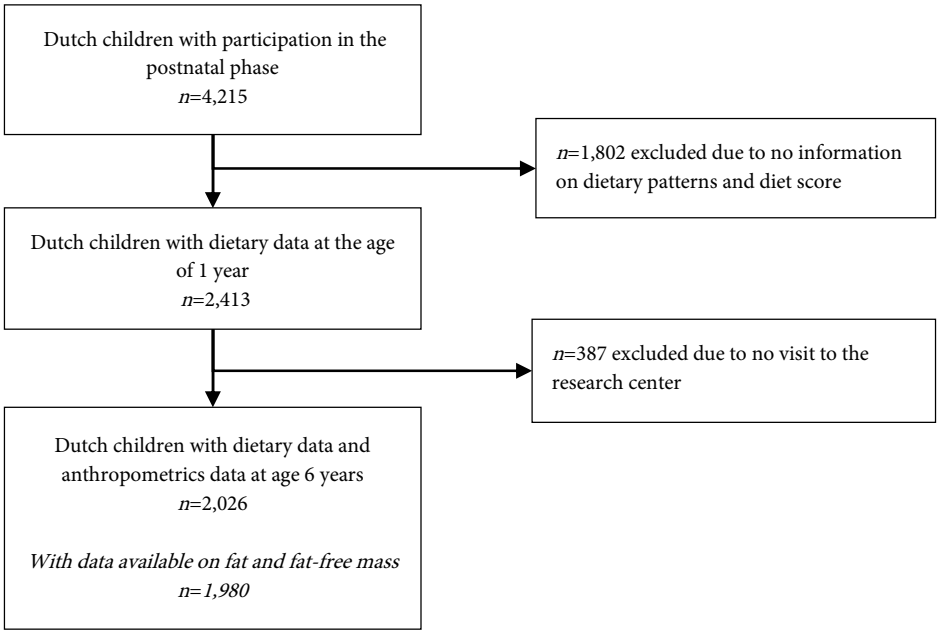


Figure 5.2.1 Flowchart of study participants included for the main analysis.

Dietary patterns

Dietary intake was assessed at a median age of 12.9 months (95% range 12.2 to 19.2) using a 211-item semi-quantitative food-frequency questionnaire (FFQ), which included foods that are frequently consumed by Dutch children between 9 and 18 months of age.[19] The FFQ was evaluated against three 24-h recalls in a representative sample of 32 Dutch children and intraclass correlation coefficients for nutrient intakes ranged from 0.4 to 0.7.[18-19]

An *a priori* dietary pattern was defined using a previously developed diet quality score for preschool children (described in Chapter 5.1).[18] This score was developed using dietary guidelines as a basis and includes intake of the following ten food groups: high intake of vegetables; fruit; bread and cereals; rice, pasta, potatoes, and legumes; dairy; meat, poultry, eggs and meat substitutes; fish; and fats and oils; and low intake of candy and snacks; and sugar-sweetened beverages (Table 5.2.1 and Chapter 5.1). The score ranges from 0 to 10 on a continuous scale, with a higher score representing a healthier diet. The diet score was standardized to an energy intake of 1200 kcal/d.[18]

A posteriori-derived dietary patterns on the basis of variation in food group intake were extracted using principal component analysis (PCA). With this method, patterns were identified that explained the maximum variation in the intake of 21 food groups.[19] Two major dietary patterns were extracted in our population: a 'health-conscious' dietary pattern, characterized by high intake of fruits, vegetables, oils, legumes, pasta, and fish; and a 'Western-like' dietary pattern characterized by high intake of snacks, animal fats, refined grains, confectionery and sugar-containing beverages. Together, these two PCA-derived patterns explained 24.5% of the total variance in food intake (Table 5.2.1).

A posteriori-derived dietary patterns on the basis of variation in FMI and FFMI were identified using reduced-rank regression (RRR).[22] We used this approach to identify dietary patterns that best predict childhood body composition.[22] For this method, we used age- and sex-adjusted FMI and FFMI as response variables and we used the same 21 food groups as used in the PCA as predictor variables. With this approach, two dietary patterns were extracted which explained the maximal variance in FMI and FFMI. The first RRR-pattern was positively correlated with both FMI and FFMI. Because the correlation was strongest for FMI we labeled this pattern 'high FMI' dietary pattern. The second RRR-pattern was positively correlated with FFMI, but inversely with FMI. We refer to this second pattern a 'high FFMI' dietary pattern. The two RRR-derived patterns together explained 2.6% of the variation in FMI and FFMI (Table 5.2.1).

Table 5.2.1. Food groups included in the dietary patterns

	Positive (+) or negative (-) score	Factor loadings from PCA†§ (after varimax rotation)		Factor loadings from RRR§	
	Diet quality score*	'Health-conscious' pattern	'Western-like' pattern	'High FMI' pattern	'High FFMI' pattern
Refined cereals	Not included		0.57	0.22	
Whole cereals	+				0.41
Pasta and rice	+	0.62			0.46
Dairy	+				0.27
Fruit	+	0.32			0.28
Soya substitutes	Not included				
Vegetables	+	0.74		0.40	0.38
Potatoes	+	0.61		0.34	
Soups and sauces	Not included		0.23	0.44	
Savory snacks	-		0.59		
Confections	-		0.72		
Vegetable oils	+	0.50			0.37
Other fats	+		0.58		0.20
Fish	+	0.22		0.42	
Shellfish	+				
Meat	+	0.21	0.27	0.30	
Eggs	+				
Legumes	+	0.59			
Sugar-containing beverages	-		0.59	0.30	
Non-sugar-containing beverages	Not included				0.28
Composite dishes	Not included				
Explained variation (%) in food group intake		16.3	8.2	5.7	12.5
Explained variation (%) in FMI and FFMI		0.4	0.1	1.8	0.8

*Further details in Chapter 5.1
†Reprinted from Kiefte-de Jong et al., 2013[19]
§Only factor loadings ≥|0.2| are reported

Body composition assessment

Children's anthropometrics and body composition were measured at a median age of 5.9 years (95% range 5.7 to 6.5) in a dedicated research center in the Sophia Children's Hospital in Rotterdam. Height was determined in standing position to the nearest millimeter without shoes with a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands) and body mass index (BMI) was calculated (body weight (kg)/height $(m)^2$).

Total body, android, and gynoid fat mass were measured using a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA). Fat, lean and bone mass of the total body and specific regions were analyzed using enCORE software version 13.6.[23] We calculated fat mass index (FMI) (fat mass (kg)/height $(m)^2$) and fat-free mass index (FFMI) (fat-free mass (kg)/height $(m)^2$).[24] Secondary outcome measures included android/gynoid ratio (android fat mass divided by gynoid fat mass); and body fat percentage (BF%) (fat mass as percentage of total body weight). We calculated age- and sex-specific SD-scores for all outcomes based on the total Generation R study population.

Covariates

We selected covariates as potential confounders based on a previously described association with one of the dietary patterns.[18-19] Information on maternal age, parity, folic acid supplement use, paternal education, paternal smoking and household income was obtained using questionnaires at enrollment in the study.[20] Educational level and household income were categorized into three groups following Dutch standard classifications.[25-26] Maternal smoking and alcohol use during pregnancy were assessed using questionnaires in each trimester and was categorized into never; until pregnancy was known; or continued during pregnancy.[27] Maternal anthropometrics were measured at enrollment at the research center, without shoes and heavy clothing.[20]

Information on breastfeeding was obtained from delivery reports and postnatal questionnaires and was categorized as never breastfeeding; any partial breastfeeding in the first 4 months of life and; exclusive breastfeeding in the first 4 months of life.[19] Timing of introduction of complementary feeding in the first year of life was assessed using the FFQ at age 1 year and categorized into three groups: <3 months, 3-6 months, or ≥6 months.[28] Child's height and weight around the age of 1 year were measured at the Community Child Health Centers, and BMI SD-scores were calculated using Dutch reference curves.[29] Television watching at the age of 2 years (h/d), as an indicator of sedentary behavior; and about participation in sports (yes, no) at the age of 6 years, as an indicator of physical activity, were assessed using questionnaires.

Statistical analyses

The dietary pattern scores were analyzed as continuous variables and categorized into quartiles with the first quartile as reference. All dietary patterns were expressed in SD-scores to facilitate comparability of the results. A higher SD-score represents a higher adherence to the dietary pattern. All body composition outcomes were expressed as age- and sex-specific SD-scores and analyzed as continuous variables.

We used multivariable linear regression models to assess the associations between the five dietary patterns and each of the body composition measures in separate models. Crude models were adjusted for child sex, age at dietary assessment, and total energy intake. The multivariable models were further adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, and smoking and alcohol use during pregnancy; paternal smoking and education; household income; child breastfeeding in the first four months of life, timing of introduction of complementary feeding, and television watching at the age of 2 years.

To assess whether the associations were different by sex, BMI-SDS at the age of 1 year, sedentary behavior at the age of 2 years, or participation in sports at the age of 6 years we evaluated the statistical interaction by adding the product term of the covariate and the dietary patterns to the multivariable models on BMI, FMI, and FFMI. To reduce potential bias associated with missing data, missing values of covariates were multiple imputed (n=10 imputations) using the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern.[30-31] Analyses were performed in each of 10 imputed datasets separately and results were pooled. We performed sensitivity analyses in which we excluded children who still received breast milk or a substantial amount of infant formula (i.e., more than 500 kcal/d) (n=386) at the time of dietary assessment. Also, we additionally adjusted for playing sports at the age of 6 years. Finally, we additionally adjusted the multivariable models for BMI-for-age at 1 year to assess whether dietary patterns at 1 year of age were associated with body composition at the age of 6 years independent of BMI at baseline. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Subject characteristics

Characteristics of the children and their parents are presented in Table 5.2.2. Most of the mothers were nulliparous (63.0%) and did not smoke during pregnancy (79.5%); and most of the children received breastfeeding exclusively (30.2%) or partially (60.1%) in their first four months of life. Mean (± SD) diet quality score at the age of 1 year was 4.2 (± 1.3) on a theoretical range of 0 to 10. The diet quality score, the 'health-conscious' PCA-derived pattern and the 'high FFMI' RRR-pattern were strongly positively correlated with each other (Supplement 5.2.1). At the age of 6 years, median (95% range) body fat percentage was 23.1% (16.2 to 34.3) and median BMI was 15.7 kg/m^2 (13.6 to 19.1).

Associations between dietary patterns and body composition

In multivariable adjusted models, a higher adherence to the PCA-derived 'health-conscious' dietary pattern or a higher diet quality score at the age of 1 year was associated with a higher BMI and FFMI at the age of 6 years (Table 5.2.3). Children in the highest quartile of the diet score had a 0.19 SD higher FFMI (95% CI 0.08, 0.30) than children in the lowest quartile. These patterns were not associated with FMI (Table 5.2.3), or with BF% or with android/gynoid ratio (Supplement 5.2.2). Adherence to the PCA-derived 'Western-like' dietary pattern at the age of 1 year was not consistently associated with any of the body composition measures the age of 6 years.

Table 5.2.2 Characteristics of the children and their parents (n=2,026)

	n	Median or percentage*
Parental characteristics		
Maternal age (y)	2,026	32.3 (22.9 – 39.9)
Maternal BMI at enrollment (kg/m^2)	1,849	23.3 (18.9 – 34.8)
Parity		
0	1,242	1,242 (63.0%)
1	557	557 (28.2%)
≥ 2	173	173 (8.8%)
Folic acid use (%)		
Start periconceptional	970	970 (63.5%)
Start in first 10 weeks	448	448 (29.3%)
Never	110	110 (7.2%)
Alcohol use during pregnancy (%)		
Never	517	517 (30.8%)
Until pregnancy was known	278	278 (16.6%)
Continued	881	881 (52.6%)
Smoking during pregnancy (%)		
Never	1,466	1,466 (79.5%)
Until pregnancy was known	186	186 (10.1%)
Continued	193	193 (10.5%)
Paternal smoking (%)		
No	947	947 (61.7%)
Yes	589	589 (38.3%)
Paternal education level (%)		
Primary or secondary school	542	542 (29.0%)
Higher education	1,328	1,328 (71.0%)
Net household income (%)		
< €2200/mo	359	359 (20.3%)
≥ €2200/mo	1,413	1,413 (79.7%)
Child characteristics		
Gender (%)		
Boys	1,002	1,002 (49.5%)
Girls	1,024	1,024 (50.5%)
Breastfeeding (%)		
Exclusive ≥ 4 months	552	552 (30.2%)
Partial	1,101	1,101 (60.2%)
Never	176	176 (9.6%)
Introduction complementary feeding (%)		
After 6 months	800	800 (39.6%)
3-6 months	1,136	1,136 (56.3%)
0-3 months	83	83 (4.1%)
Television watching at age 2 years (h/day)	1915	0.9 (0 – 2)
Child age at FFQ (mo)	2,026	12.9 (12.2 – 19.2)
Total energy intake (kcal/d)	2,026	1267 (737 – 2080)
Child age at 6-year visit (y)	2,026	5.9 (5.7 – 6.5)

* Values are valid percentages for categorical variables or medians (95% range) for continuous variables.

The 'high FMI' RRR-derived pattern, which was positively correlated with FMI and FFMI, was characterized by high intake of refined grains, meat, potatoes, fish, soups and sauces, and sugar-containing beverages (Table 5.2.1). After adjustment for confounders, a higher adherence to this pattern remained positively associated with both FMI and FFMI (Table 5.2.3) and was also associated with a higher BF% and a higher android/gynoid ratio (difference in BF% 0.14 (95% CI 0.04, 0.24) SD for highest vs. lowest quartile) (Supplement 5.2.2).

Table 5.2.3. Multivariable associations of dietary patterns at the age of 1 year with body composition at 6 years of age

	BMI (SDS) ***n*=2,026** β (95% CI)	**FMI (SDS)** ***n*=1,980** β (95% CI)	**FFMI (SDS)** ***n*=1,980** β (95% CI)
Diet quality score			
Per SD	**0.06 (0.02, 0.09)**	0.02 (-0.01, 0.05)	**0.06 (0.02, 0.10)**
Q1 (low adherence)	*Reference*	*Reference*	*Reference*
Q2	0.07 (-0.02, 0.16)	0.03 (-0.06, 0.11)	0.09 (-0.02, 0.20)
Q3	0.07 (-0.02, 0.16)	-0.01 (-0.09, 0.08)	**0.14 (0.02, 0.25)**
Q4 (high adherence)	**0.18 (0.08, 0.27)**	0.07 (-0.01, 0.16)	**0.19 (0.08, 0.30)**
'Health-conscious' pattern (PCA)			
Per SD	**0.04 (0.01, 0.08)**	0.01 (-0.03, 0.04)	**0.05 (0.01, 0.09)**
Q1 (low adherence)	*Reference*	*Reference*	*Reference*
Q2	0.03 (-0.07, 0.12)	0.02 (-0.07, 0.10)	0.02 (-0.09, 0.13)
Q3	**0.10 (0.01, 0.19)**	0.03 (-0.05, 0.12)	**0.13 (0.02, 0.24)**
Q4 (high adherence)	**0.14 (0.05, 0.24)**	0.04 (-0.05, 0.13)	**0.17 (0.06, 0.29)**
'Western-like' pattern (PCA)			
Per SD	0.00 (-0.04, 0.05)	-0.01 (-0.05, 0.03)	0.02 (-0.04, 0.07)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*
Q2	0.02 (-0.07, 0.11)	0.02 (-0.06, 0.11)	-0.01 (-0.12, 0.10)
Q3	**0.12 (0.02, 0.21)**	0.06 (-0.03, 0.15)	**0.15 (0.04, 0.27)**
Q4 (high adherence)	0.04 (-0.07, 0.14)	-0.01 (-0.11, 0.09)	0.09 (-0.04, 0.22)
'High FMI' dietary pattern (RRR)			
Per SD	**0.11 (0.07, 0.15)**	**0.10 (0.06, 0.13)**	**0.09 (0.04, 0.14)**
Q1 (low adherence)	*Reference*	*Reference*	*Reference*
Q2	**0.12 (0.03, 0.21)**	**0.10 (0.01, 0.18)**	0.08 (-0.03, 0.19)
Q3	**0.12 (0.03, 0.21)**	**0.09 (0.01, 0.18)**	0.07 (-0.04, 0.18)
Q4 (high adherence)	**0.25 (0.16, 0.35)**	**0.18 (0.10, 0.27)**	**0.23 (0.11, 0.35)**
'High FFMI' dietary pattern (RRR)			
Per SD	0.02 (-0.02, 0.06)	-0.03 (-0.07, 0.00)	**0.07 (0.02, 0.11)**
Q1 (low adherence)	*Reference*	*Reference*	*Reference*
Q2	-0.06 (-0.15, 0.03)	-0.06 (-0.15, 0.02)	-0.02 (-0.13, 0.10)
Q3	0.07 (-0.03, 0.16)	-0.01 (-0.10, 0.08)	**0.18 (0.06, 0.29)**
Q4 (high adherence)	0.06 (-0.04, 0.17)	-0.07 (-0.17, 0.03)	**0.19 (0.06, 0.32)**

Values are regression coefficients (95% confidence interval) that reflect the difference in outcome (age- and sex-specific SD-scores) per 1 SD higher dietary pattern score and for quartiles of exposure compared to the lowest quartile, based on imputed data. **Bold** values indicate statistically significant associations.

Models are adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child sex, breastfeeding in the first four months of life, timing of introduction of complementary feeding, age at dietary measurement, total energy intake at 1 year, and television watching at age 2 years.

Abbreviations: BMI, body mass index; FFMI, fat-free mass index; FMI, fat mass index; PCA, principal component analysis; RRR, reduced rank regression

The 'high FFMI' RRR-derived pattern, which was positively correlated with FFMI and inversely correlated with FMI, was characterized by high intake of whole grains, pasta and rice, dairy, fruit, vegetable oils and fats, and non-sugar-containing beverages (Table 5.2.1). After adjustment for confounders, a higher score on this pattern remained positively associated with FFMI (0.19 (95% CI 0.06, 0.32) SD for highest vs. lowest quartile), but was no longer significantly associated with FMI (Table 5.2.3). However, the 'high FFMI' RRR-pattern remained associated with a lower BF% and a lower android/gynoid ratio in the multivariable model (-0.12 (95% CI -0.23, -0.02) SD in BF% for highest vs. lowest quartile) (Supplement 5.2.2).

Additional analyses

Additional adjustment for BMI-for-age at the age of 1 year only slightly attenuated the effect estimates and all associations with FFMI remained significant (Supplement 5.2.3). The inverse association between the 'high FFMI' RRR-derived dietary pattern and FMI became statistically significant again.

We observed no significant interactions of the dietary patterns with child sex, BMI-SDS at the age of 1 year, or participation in sports at the age of 6 years on body composition. We observed a significant interaction (p=0.03) of the RRR-derived 'high FFMI' dietary pattern with television watching at the age of 2 years on FMI. Stratification using a cut-off for television watching of 1 h/d[32] revealed that a higher score for the 'high FFMI' dietary pattern was associated with a lower FMI in children who watched <1 h/d television (-0.07 SD (95% CI -0.12, -0.03) per 1 SD higher dietary pattern score), but not in those who watch ≥1 h/d (0.00 (95% CI -0.05, 0.06)).

We performed a sensitivity analysis in which we excluded children who still received breastfeeding or a substantial amount of infant formula at the time of dietary measurement (n=386). In this subgroup, the associations of the PCA-derived 'health-conscious' dietary pattern with BMI and FFMI slightly attenuated and were no longer statistically significant, whereas the association of the diet score and the RRR-derived dietary patterns with body composition remained similar (Supplement 5.2.4)

DISCUSSION

In a large population-based cohort of young children, we observed that higher adherence to an *a priori*-defined diet quality score or to an *a posteriori*-defined 'health-conscious' dietary pattern at the age of 1 year was associated with a higher fat-free mass index, but not with fat mass index at the age of 6 years. Using reduced-rank regression, we additionally identified dietary patterns that predict child body composition at age 6 years. A pattern that was associated with a higher FFMI, but not with FMI, was characterized by high intake of whole grains, pasta and rice, dairy, fruit, vegetable oils and fats, and non-sugar-containing beverages. Additionally, a pattern positively associated with both FMI and FFMI was identified, which was characterized by high intake of refined grains, meat, potatoes, fish, soups and sauces, and sugar-containing beverages. These associations were all independent of total energy intake and parental and child sociodemographic and lifestyle factors.

Interpretation and comparison with previous studies

We observed small but statistically significant positive associations between higher scores on PCA- or RRR-derived health-conscious dietary patterns or *a priori*-defined diet quality in early childhood and subsequent FFMI, but not with FMI. These three patterns were characterized by high intake of foods generally considered to be healthy (vegetables, fruit, whole grains, and vegetable oils). The associations with a higher FFMI suggest that these dietary patterns can be beneficial for health in later life, as higher lean mass is associated with improved cardiovascular and metabolic health.[33-35]

Three previous prospective studies examined the association of dietary patterns or indices in early childhood with body composition later in life,[36-38] of which only one separately assessed fat and fat-free mass.[38] In line with our results, this latter study in 536 children in the U.K. observed that a higher adherence to a PCA-derived 'infant guidelines' dietary pattern at the age of 12 months was associated with higher lean mass index but not with FMI at the age of 4 years.[38] This dietary pattern was characterized by a high intake of fruit, vegetables, cooked meat and fish, and rice and pasta; and low intake of commercial baby foods.[39] Other dietary patterns were not examined. The other two studies both assessed predefined diet quality on the basis of dietary guidelines. Adherence to the 'Raine Eating Assessment in Toddlers' index at 1 to 3 years of age in 2,562 Australian children was not consistently associated with BMI during childhood and adolescence.[37] In a large cohort of U.K. children (n=4,798), a higher score on a 'Complementary Feeding Utility Index', with measures adherence to complementary feeding guidelines, at the age of 6 months was also not associated with BMI at the age of 7 years after adjustment for sociodemographic variables, but was associated with a lower waist circumference.[36]

We observed less consistent associations between dietary patterns and later body fat or fat distribution. Although in our population adherence to a 'Western-like' dietary pattern was associated with a higher FMI in crude models, this association was explained by sociodemographic and lifestyle factors. The only patterns associated with later body fat after adjustment for confounders were the RRR-derived patterns, constructed on the basis of variation in body composition. The 'high FMI' dietary pattern – which was characterized by high intake of refined grains, meat, potatoes, fish, soups and sauces, and sugar-containing beverages – was associated with a higher FMI, a higher body fat percentage, and a higher android/gynoid fat ratio. The 'high FFMI' dietary pattern, characterized by intake of whole grains, pasta and rice, and vegetable oils, was associated with a lower body fat percentage, but was associated with a lower FMI only after additional adjustment for BMI-SDS at the age of 1 year.

We used reduced-rank regression as exploratory approach to identify which dietary patterns in early childhood explain most variation in body composition. In contrast to several previous studies,[15, 40] but in line with one other study,[41] we chose to use body composition measures as response variables, rather than nutrient intake or biomarkers. We used this approach because we were interested in exploring which patterns best predict body composition. Consequently, the primary results of this method are which food groups characterize the patterns, rather than the association of the patterns with body composition. Additionally, patterns based on variation in FMI and FFMI can be used in future studies to evaluate the relation between diet and other health outcomes, as body composition is a possible intermediate factor in many diet-disease associations.

Methodological considerations

An important strength of our study is that we had a prospective study design with detailed information available on a large number of potential confounders. Previously, several family sociodemographic and lifestyle characteristics have been related to child dietary patterns,[19] and to child body composition.[42] It is therefore essential to take these factors into consideration when studying the relation between diet and body composition. Previous studies were not always able to adjust for important factors such as parental BMI and lifestyle. Furthermore, while loss to follow up is usually an important limitation of prospective studies, more than 80% of all children in our study population with information on food intake at the age of 1 year participated in the body composition measurements at the age of 6 years.

A limitation of our study is that we measured food intake with an FFQ, which is known to be prone to measurement error.[43] However, an FFQ measures habitual diet rather than diet on just one or a few days, and is considered appropriate to use for dietary pattern analysis.[43] A major strength of our study is that we performed detailed measurements of child body composition using DXA. Many previous studies assessed body composition based on total body weight, whereas in our study we were able to distinguish between fat mass and fat-free mass. We observed that higher adherence to healthier dietary patterns was associated with a higher BMI but that this reflected a higher fat-free mass rather than fat mass, supporting the notion that BMI alone is not a good proxy for childhood obesity.[44]

Conclusions

Dietary patterns characterized by high intake of fruit, vegetables, grains, and vegetable oils at the age of 1 year, were associated with a higher fat-free mass index at the age of 6 years. Using reduced-rank regression we additionally identified a pattern that predicted a higher fat and fat-free mass index and a higher body fat percentage, which was characterized by high intake of refined grains, meat, potatoes, fish, soups and sauces, and sugar-containing beverages. Future studies should explore whether these differences in body composition track into later life and whether these differences are independent of later dietary patterns.

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SUPPLEMENT CHAPTER 5.2

Supplement 5.2.1 Correlations between the different dietary patterns

	Diet quality score	'Health-conscious' pattern (PCA)	'Western-like' pattern (PCA)	'High FMI' dietary pattern (RRR)	'High FFMI' dietary pattern (RRR)
Diet quality score	-	-	-	-	-
'Health-conscious' (PCA)	0.476	-	-	-	-
'Western- (PCA)	-0.107	-0.026	-	-	-
'High FMI' (RRR)	0.183	0.231	0.296	-	-
'High FFMI' (RRR)	0.328	0.491	0.038	0.003	-

Values are Pearson's correlation coefficients.

Supplement 5.2.2 Multivariable-adjusted associations of dietary patterns at 1 year of age with childhood height, weight, body fat percentage, and android/gynoid ratio at 6 years of age

	Weight (SDS) β (95% CI)	**Height (SDS)** β (95% CI)	**BF% (SDS)** β (95% CI)	**A/G ratio (SDS)** β (95% CI)
Diet score				
Per SD	**0.06 (0.03, 0.10)**	**0.05 (0.01, 0.09)**	0.00 (-0.03, 0.03)	-0.00 (-0.04, 0.04)
Q1	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.04 (-0.05, 0.13)	-0.01 (-0.13, 0.10)	0.01 (-0.08, 0.10)	-0.04 (-0.14, 0.06)
Q3	0.04(-0.05, 0.14)	-0.02 (-0.14, 0.09)	-0.05 (-0.14, 0.04)	-0.06 (-0.16, 0.04)
Q4	**0.19 (0.10, 0.29)**	**0.15 (0.03, 0.27)**	0.02 (-0.08, 0.11)	0.01 (-0.10, 0.11)
'Health-conscious' pattern (PCA)				
Per SD	0.03 (-0.01, 0.07)	0.01 (-0.04, 0.05)	-0.01 (-0.04, 0.03)	-0.01 (-0.05, 0.03)
Q1	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.04 (-0.06, 0.13)	0.05 (-0.07, 0.16)	0.01 (-0.09, 0.10)	0.02 (-0.08, 0.12)
Q3	**0.12 (0.02, 0.21)**	0.11 (-0.01, 0.22)	-0.01 (-0.11, 0.08)	0.02 (-0.08, 0.12)
Q4	**0.13 (0.03, 0.23)**	0.08 (-0.04, 0.20)	-0.01 (-0.11, 0.08)	-0.02 (-0.12, 0.09)
'Western-like' pattern (PCA)				
Per SD	0.00 (-0.04, 0.04)	0.00 (-0.05, 0.05)	-0.01 (-0.05, 0.04)	0.01 (-0.04, 0.06)
Q1	*Reference*	*Reference*	*Reference*	*Reference*
Q2	-0.01 (-0.11, 0.08)	-0.05 (-0.16, 0.07)	0.05 (-0.05, 0.14)	0.08 (-0.03, 0.18)
Q3	**0.11 (0.01, 0.20)**	0.04 (-0.07, 0.16)	0.03 (-0.07, 0.13)	0.08 (-0.02, 0.19)
Q4	0.03 (-0.08, 0.14)	0.02 (-0.11, 0.15)	-0.02 (-0.13, 0.09)	0.05 (-0.07, 0.17)
'High FMI' dietary pattern (RRR)				
Per SD	**0.10 (0.06, 0.14)**	**0.05 (0.00, 0.10)**	**0.08 (0.04, 0.12)**	**0.07 (0.02, 0.11)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*
Q2	**0.09 (0.00, 0.19)**	0.04 (-0.08, 0.15)	**0.10 (0.01, 0.19)**	0.08 (-0.02, 0.18)
Q3	**0.14 (0.05, 0.24)**	**0.12 (0.00, 0.24)**	0.08 (-0.01, 0.18)	0.06 (-0.04, 0.16)
Q4	**0.23 (0.13, 0.33)**	**0.13 (0.01, 0.25)**	**0.14 (0.04, 0.24)**	**0.14 (0.03, 0.25)**
'High FFMI' dietary pattern (RRR)				
Per SD	0.02 (-0.02, 0.06)	0.02 (-0.03, 0.07)	**-0.05 (-0.09,-0.01)**	**-0.05 (-0.09,-0.00)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*
Q2	-0.06 (-0.15, 0.04)	-0.03 (-0.15, 0.09)	-0.07 (-0.16, 0.03)	-0.07 (-0.17, 0.03)
Q3	**0.11 (0.01, 0.21)**	**0.13 (0.01, 0.25)**	-0.06 (-0.16, 0.04)	-0.03 (-0.14, 0.08)
Q4	0.04 (-0.07, 0.15)	0.01 (-0.12, 0.15)	**-0.12 (-0.23, -0.02)**	-0.12 (-0.23, 0.00)

Values are regression coefficients that reflect the difference in outcome (age- and sex-specific SD-scores) per 1 SD higher exposure and for quartiles of exposure compared to the lowest quartile, based on imputed data. Statistically significant values ($p<0.05$) are indicated in **bold**.
Models are adjusted for maternal age, BMI, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child sex, breastfeeding, timing of introduction of complementary feeding, age at dietary measurement, total energy intake at 1 year, and television watching at 2 years. BF% and A/G ratio are additionally adjusted for child height. Abbreviations: A/G ratio, android/gynoid ratio; BF%, body fat percentage; PCA, principal component analyses; RRR, reduced rank regression

Supplement 5.2.3 Associations of dietary patterns at 1 year of age with childhood body composition at 6 years of age: crude, confounder and baseline BMI adjusted models

	BMI (SDS) ***n*=2,026** β (95% CI)	**FMI (SDS)** ***n*=1,980** β (95% CI)	**FFMI (SDS)** ***n*=1,980** β (95% CI)
Diet quality score (per SD)			
Crude	**0.05 (0.02, 0.08)**	0.01 (-0.02, 0.04)	**0.07 (0.03, 0.11)**
Covariate adjusted (main model)	**0.06 (0.02, 0.09)**	0.02 (-0.01, 0.05)	**0.06 (0.02, 0.10)**
Additionally adjusted for BMI at 1 year	**0.04 (0.01, 0.07)**	0.01 (-0.02, 0.04)	**0.05 (0.01, 0.08)**
'Health-conscious' pattern (PCA) (per SD)			
Crude	**0.04 (0.00, 0.07)**	0.00 (-0.03, 0.04)	**0.05 (0.01, 0.09)**
Covariate adjusted (main model)	**0.04 (0.01, 0.08)**	0.01 (-0.03, 0.04)	**0.05 (0.01, 0.09)**
Additionally adjusted for BMI at 1 year	0.03 (-0.00, 0.07)	0.00 (-0.03, 0.03)	**0.04 (0.00, 0.08)**
'Western-like' pattern (PCA) (per SD)			
Crude	0.04 (-0.01, 0.08)	**0.05 (0.01, 0.09)**	0.00 (-0.04, 0.05)
Covariate adjusted (main model)	0.00 (-0.04, 0.05)	-0.01 (-0.05, 0.03)	0.02 (-0.04, 0.07)
Additionally adjusted for BMI at 1 year	0.01 (-0.03, 0.05)	-0.01 (-0.04, 0.03)	0.02 (-0.03, 0.07)
'High FMI' dietary pattern (RRR) (per SD)			
Crude	**0.14 (0.10, 0.18)**	**0.15 (0.11, 0.19)**	**0.08 (0.03, 0.13)**
Covariate adjusted (main model)	**0.11 (0.07, 0.15)**	**0.10 (0.06, 0.13)**	**0.09 (0.04, 0.14)**
Additionally adjusted for BMI at 1 year	**0.10 (0.06, 0.14)**	**0.09 (0.06, 0.13)**	**0.08 (0.03, 0.12)**
'High FFMI' dietary pattern (RRR) (per SD)			
Crude	0.00 (-0.04, 0.04)	**-0.06 (-0.10, -0.03)**	**0.08 (0.03, 0.12)**
Covariate adjusted (main model)	0.02 (-0.02, 0.06)	-0.03 (-0.07, 0.00)	**0.07 (0.02, 0.11)**
Additionally adjusted for BMI at 1 year	0.01 (-0.03, 0.04)	**-0.04 (-0.07, -0.01)**	**0.06 (0.01, 0.10)**

Values are regression coefficients that reflect the difference in outcome (age- and sex- specific SD-scores) per 1 SD higher exposure, based on imputed data.

Crude models are adjusted for child sex, age at dietary measurement and total energy intake at 1 year.

Covariate models are additionally adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child breastfeeding in the first four months of life, timing of introduction of complementary feeding, and television watching at age 2 years.

Baseline BMI adjusted models are additionally adjusted for BMI-SDS at the age of 1 year

Abbreviations: BMI, body mass index; FFMI, fat-free mass index; FMI, fat mass index; PCA, principal component analyses; RRR, reduced rank regression

Supplement 5.2.4 Associations of dietary patterns at 1 year of age with childhood body composition at 6 years of age in total population vs. in weaned children only

	BMI (SDS) ***n*=2,026 or 1,649** β (95% CI)	**FMI (SDS)** ***n*=1,980 or 1,609** β (95% CI)	**FFMI (SDS)** ***n*=1,980 or 1,609** β (95% CI)
Diet quality score (per SD)			
Total population	**0.06 (0.02, 0.09)**	0.02 (-0.01, 0.05)	**0.06 (0.02, 0.10)**
Weaned children only	**0.06 (0.02, 0.09)**	0.03 (-0.01, 0.06)	**0.05 (0.00, 0.09)**
'Health-conscious' pattern (PCA) (per SD)			
Total population	**0.04 (0.01, 0.08)**	0.01 (-0.03, 0.04)	**0.05 (0.01, 0.09)**
Weaned children only	0.03 (-0.01, 0.07)	-0.00 (-0.04, 0.04)	0.03 (-0.02, 0.08)
'Western-like' pattern (PCA) (per SD)			
Total population	0.00 (-0.04, 0.05)	-0.01 (-0.05, 0.03)	0.02 (-0.04, 0.07)
Weaned children only	-0.03 (-0.09, 0.02)	-0.04 (-0.09, 0.01)	0.00 (-0.06, 0.06)
'High FMI' dietary pattern (RRR) (per SD)			
Total population	**0.11 (0.07, 0.15)**	**0.10 (0.06, 0.13)**	**0.09 (0.04, 0.14)**
Weaned children only	**0.09 (0.04, 0.13)**	**0.08 (0.04, 0.12)**	**0.07 (0.01, 0.12)**
'High FMI' dietary pattern (RRR) (per SD)			
Total population	0.02 (-0.02, 0.06)	-0.03 (-0.07, 0.00)	**0.07 (0.02, 0.11)**
Weaned children only	0.02 (-0.03, 0.06)	-0.03 (-0.07, 0.01)	0.05 (-0.00, 0.10)

Values are regression coefficients that reflect the difference in outcome (age- and sex- specific SD-scores) per 1 SD higher exposure, based on imputed data.

Weaned children were defined as children who no longer receive breast milk or a substantial amount of infant formula (i.e., more than 500 kcal/d). Models are adjusted for child sex, age at dietary measurement and total energy intake at 1 year and maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child breastfeeding in the first four months of life, timing of introduction of complementary feeding, and television watching at age 2 years.

Abbreviations: BMI, body mass index; FFMI, fat-free mass index; FMI, fat mass index; PCA, principal component analyses; RRR, reduced rank regression.

Chapter 5.3

Dietary patterns in early childhood & cardiometabolic health

Manuscript based on this chapter:

Trudy Voortman*, Elisabeth T. M. Leermakers*, Vincent W.V. Jaddoe, Oscar H. Franco, Jessica C. Kiefte-de Jong, Edith H. van den Hooven. *A priori* and *a posteriori* dietary patterns at the age of 1 year and cardiometabolic health at age 6 years: the Generation R Study. *Submitted for publication.*

ABSTRACT

Background: Cardiometabolic risk may be set early in life. However, at the moment it is unclear whether overall diet during early childhood is associated with cardiometabolic health. Therefore, we aimed to study the associations of different dietary patterns of toddlers with cardiometabolic health at school age, and to assess whether these associations were explained by differences in body composition.

Methods: In 2,026 Dutch children participating in a population-based cohort study, food intake at the age of 1 year was assessed with a food-frequency questionnaire. Dietary patterns were derived using three approaches: 1) *A priori*-defined diet quality based on dietary guidelines. 2) 'health-conscious' and 'Western-like' patterns derived from principal component analysis (PCA) based on variation in food intake. 3) Two dietary patterns derived with reduced-rank regression (RRR) based on variation in body composition (i.e., fat mass index and fat-free mass index). At the age of 6 years, we measured the children's systolic and diastolic blood pressure and serum levels of insulin, HDL cholesterol, and triacylglycerol. We used age- and sex-specific SD-scores (SDS) for all outcomes and calculated a combined cardiometabolic risk factor score.

Results: After adjustment for confounders, children with high adherence to the 'health-conscious' PCA-derived pattern at 1 year of age had a lower cardiometabolic risk factor score at age 6 years (-0.07 SD (95% CI-0.12;-0.02) lower in risk score per SD increase in dietary pattern). This association did not change after adjustment for body composition. The pattern was not clearly associated with one of the individual risk factors. The 'Western-like' PCA-pattern tended to be associated with a higher cardiometabolic risk score, and the diet quality score with a lower cardiometabolic risk score, but not consistently. The RRR-patterns based on variations in body composition were not associated with cardiometabolic health.

Conclusion: Our results suggest that a 'health-conscious' dietary pattern at 1 year of age, is associated with a lower cardiometabolic risk factor score at 6 years of age. This association was not explained by differences in body composition.

INTRODUCTION

Cardiometabolic health in childhood tracks to later life and predicts the risks of adult cardiovascular disease, type 2 diabetes and premature death.[1-2] Thus, management of cardiometabolic risk factors in childhood can be essential to prevent these diseases in later life. Diet in early life is an important modifiable determinant of later cardiometabolic health. For example, we have previously shown that intake of sugar-containing beverages and protein (Chapter 2.3) during early childhood are associated with cardiometabolic health in later childhood.[3-5] Associations of dietary factors with cardiometabolic health may occur via changes in body composition, but nutrition may also affect cardiometabolic health directly.

In addition to evaluating the intake of food groups or nutrients, dietary patterns can be used to characterize overall food intake.[6-7] Dietary patterns can be defined using several approaches: either *a priori*, for instance on the basis of dietary guidelines; or *a posteriori* with the use of data of the study population. Furthermore, they can be defined on the basis of food intake, or on the basis of variation in specific health-related markers.[8] Studies in adults have shown that dietary patterns that are considered to be unhealthy or low diet quality scores are associated with higher cardiometabolic disease risk.[9-14] A few studies examined the associations of dietary patterns in childhood with cardiovascular or metabolic outcomes and reported inconsistent results.[15-20] Most of these studies examined diet in school-age children or adolescents and one cohort study has focused on dietary patterns in preschool children.[17, 19] Results from this British cohort study showed that a higher *a priori*-defined diet score in infancy was associated with a lower blood pressure at age 7-8 years, but not with blood lipids,[17] while a 'less-healthy' *a posteriori*-derived dietary pattern was associated with a higher blood pressure.[19] Nevertheless, it is unclear whether dietary patterns in young children are related to overall cardiometabolic health. Furthermore, a few studies, including previous analyses in our study (Chapter 5.2), linked dietary patterns in early childhood to later body composition,[4, 21-22] but it is not known if a possible effect of overall diet on cardiometabolic health occurs directly or via changes in body composition.

Therefore, we examined the associations of dietary patterns in children at the age of 1 year with cardiometabolic health at the age of 6 years. As dietary patterns we examined: 1) an *a priori*-defined diet quality score,[23] which was previously constructed on the basis of dietary guidelines for preschool children as described in Chapter 5.1; 2) *a posteriori*-derived dietary patterns, which were extracted using principal component analysis using variations in food intake,[24] and 3) *a posteriori*-derived patterns identified using reduced-rank regression on the basis of variations in body composition measures as described in Chapter 5.2.[4] Cardiometabolic outcomes included systolic and diastolic blood pressure, serum levels of insulin, HDL cholesterol, and triacylglycerol, and an overall cardiometabolic risk factor score. In addition, we assessed whether associations of dietary patterns with cardiometabolic health were explained by differences in body composition (fat and fat-free mass index).

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.[25] The study was conducted in accordance with the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam. Written informed consent was provided by caregivers for all children. To avoid the influence of cultural differences in dietary patterns, our analyses were restricted to children with a Dutch ethnicity.[24]

A total of 4,215 Dutch children participated in the preschool follow-up.[25] Data on dietary patterns were available for 2,413 of these children. At the age of 6 years, 2,026 (84%) of these children visited the research center and had one or more cardiometabolic outcomes available (Chapter 5.2, Figure 5.2.1). Because we did not have blood samples for all these children, the population for analysis for the different cardiometabolic outcomes ranged from 1,364 to 1,931 (Table 5.3.2). For 1,297 children we had information available on all cardiometabolic outcomes and therefore on the cardiometabolic risk factor score.

Dietary pattern assessment

When the children had a median age of 12.9 months (95% range 12.2 to 19.2), their mothers completed a 211-item semi-quantitative food-frequency questionnaire (FFQ). This FFQ was developed for this age group and included foods that are frequently consumed by Dutch children between 9 and 18 months old.[24] The FFQ was evaluated against three 24-h recalls in a representative sample of 32 Dutch children.[24] Intraclass correlation coefficients for nutrient intakes ranged from 0.4 to 0.7 (Chapter 5.1).[23-24]

We applied three approaches to identify dietary patterns, the same as described in Chapter 5.2.[4] As *a priori* dietary pattern we used a previously constructed diet quality score for preschool children,[23] which was developed on the basis of dietary guidelines and includes the following ten food groups: vegetables; fruit; bread and cereals; rice, pasta, potatoes, and legumes; dairy; meat, poultry, eggs and meat substitutes; fish; and fats and oils; candy and snacks; and sugar-sweetened beverages (Chapter 5.1). The diet score was standardized to a recommended energy intake of 1200 kcal/d and a higher diet quality score represents a healthier diet.[4]

Additionally, we used *a posteriori* dietary patterns explaining the maximum variation in food group intake, which were previously extracted using principal component analysis (PCA).[24] A 'health-conscious' dietary pattern was extracted, characterized by high intakes of fruits, vegetables, oils, legumes, pasta, and fish; and a 'Western-like' dietary pattern was extracted, characterized by high intakes of snacks, animal fats, refined grains, confectionery and sugar-containing beverages (Chapter 5.2, Table 5.2.1).

Furthermore, we examined *a posteriori*-derived dietary patterns explaining the maximum variation in fat mass index (FMI) and fat-free mass index (FFMI), which were identified using reduced-rank regression (RRR).[26] Age- and sex-specific FMI and FFMI were used as response variable and two dietary patterns were extracted. The first RRR-derived pattern was positively

associated with both FMI and FFMI and was labeled a 'high FMI' dietary pattern. This pattern was characterized by high intake of refined grains, meat, potatoes, fish, soups and sauces, and sugar-containing beverages. The second RRR-pattern was positively associated with FFMI and inversely with FMI and was named a 'high FFMI' dietary pattern. This 'high FFMI' dietary pattern was characterized by high intake of whole grains, pasta and rice, dairy, fruit, vegetable oils and fats, and non-sugar-containing beverages (Chapter 5.2, Table 5.2.1).[4]

Cardiometabolic health assessment

At a median age of 5.9 years (95% range 5.6 to 6.6), children visited our dedicated research center in the Sophia Children's Hospital in Rotterdam, where cardiometabolic outcomes were measured by well-trained staff.

Non-fasting blood samples were obtained, transported and stored as described previously.[27] Concentrations of insulin, HDL cholesterol (HDL-C), and triacylglycerol (TAG) were measured with enzymatic methods (using a Cobas 8000 analyzer, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay and inter-assay coefficients of variation ranging from 0.77 to 1.69%. While the children were lying, systolic (SBP) and diastolic blood pressure (DBP) were measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmomanometer Datascope Accutorr Plus™ (Paramus, NJ, USA). For our analyses we excluded the first measurement and used mean SBP and mean DBP of the remaining measurements.

Height (m) was determined in standing position to the nearest millimeter without shoes using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Body fat mass and fat-free mass were measured using a dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA) using enCORE software (version13.6). Body fat percentage (BF%) was calculated by expressing total fat mass as percentage of total body weight, and fat mass index (FMI) (fat mass (kg)/height $(m)^2$) and fat-free mass index (FFMI) (fat-free mass (kg)/height $(m)^2$) were calculated.[28] For all cardiometabolic outcomes we calculated age- and sex-specific SD-scores using data of the entire Generation R study population with information on cardiometabolic outcomes available (*n* ranging from 4,414 to 6,491).[25]

In addition to the individual cardiometabolic outcomes, we calculated a continuous score for overall cardiometabolic risk. In line with previous scores for a metabolic syndrome-like phenotype in children[29] and as described in Chapter 2.3, five components were included: BF%, blood pressure (including both SBP and DBP), HDL-C levels, TAG levels, and insulin levels. The cardiometabolic risk factor score was calculated as the sum of age- and sex-specific SD-scores of these five variables, as proposed previously for pediatric populations.[29] The SD-scores for HDL-C were multiplied by -1 since higher HDL-C levels reflect a lower cardiometabolic risk. The SD-scores for SBP and DBP were multiplied by 0.5 so that each contributed half to the blood pressure component. The cardiometabolic risk factor score was thus calculated as: BF% SDS + 0.5 × SBP SDS + 0.5 × DBP SDS + TAG SDS + (-1 × HDL-C SDS) + insulin SDS,[5] with a higher score reflecting less optimal cardiometabolic health.

Covariates

We selected covariates as potential confounders based on previously described associations with one of the dietary patterns.[4, 23-24] Information on maternal age, parity, folic acid supplement use, paternal education, paternal smoking and household income was obtained from questionnaires at enrollment in the study.[25] Educational level and household income were categorized into three groups according to Dutch standard classifications.[30-31] Parity was categorized into 0, 1, or ≥2. Start of folic acid supplement use was categorized as periconceptionally, within the first 10 weeks of pregnancy, or not before 10 weeks of pregnancy.

Information on maternal smoking and alcohol use during pregnancy was obtained from questionnaires in each trimester of pregnancy and both variables were categorized into never during pregnancy, until pregnancy was known, or continued during pregnancy.[32] Maternal anthropometrics were measured at enrollment at the research center and BMI was calculated (kg/m^2).[25]

Information about breastfeeding duration was obtained from a combination of delivery reports and postnatal questionnaires and was categorized as never breastfeeding, any breastfeeding in the first 4 months of life and, or exclusive breastfeeding in the first 4 months of life.[24] The timing of introduction of complementary feeding was assessed in the FFQ administered at the age of 1 year and was categorized into three groups: before 3 months, between 3 and 6 months, or after 6 months.[33] Child's anthropometrics around the age of 1 year were measured at the Community Child Health Centers and BMI SD-scores were calculated in accordance with Dutch reference curves.[34] Television watching at the age of 2 years (h/d) was used as an indicator of sedentary behavior and was assessed with a questionnaire filled out by the parents. Child height and weight at the age of 6 years were measured in our research center and were expressed in age- and sex-specific SDS.

Statistical analyses

All dietary patterns were expressed in SD-scores to facilitate comparability of the results and were analyzed as continuous variables. A higher SD-score represents a higher adherence to the dietary pattern. In addition, dietary patterns were categorized as quartiles and analyzed using the first quartile as reference. Correlations between the dietary patterns were examined with Pearson's correlations. All individual cardiometabolic outcomes were expressed as age- and sex-specific SDS and analyzed as continuous variables. The cardiometabolic risk factor score was analyzed as continuous variable as the sum of five SD-scores.

We used multivariable linear regression models to assess the associations between the five dietary patterns and each of the cardiometabolic measures in separate models. Crude models were adjusted for child sex, age at dietary assessment, and total energy intake. The multivariable models were further adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, and smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child breastfeeding in the first four months of life, timing of introduction of complementary feeding, television watching at the age of 2 years; and height SDS at the age of 6 years. In separate

analyses, we additionally adjusted these models for child FMI and FFMI at the age of 6 years, to assess whether potential associations of the dietary patterns with cardiometabolic health outcomes at the age of 6 years were explained by differences in body composition at age 6 years, as described in Chapter 5.2.

We checked for a potential interaction of the dietary patterns with child sex by adding the product term of sex and each dietary pattern to the multivariable models. Stratified analyses were performed in case of significant interaction (p<0.05). To assess if potential associations between the dietary patterns with the cardiometabolic risk factor score were driven by any of the individual cardiometabolic components, we performed sensitivity analyses in which we excluded each component from the score one by one.

To reduce potential bias associated with missing data, missing values of covariates were multiple imputed (n=10 imputations) using the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern.[35-36] Analyses were performed in each of ten imputed datasets separately and results were pooled. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Subject characteristics

Characteristics of the children and their parents are presented in Chapter 5.2, Table 5.2.1. Most of the mothers did not smoke during pregnancy (79.5%) and used folic acid supplements in the periconceptional period (63.5%). Most of the children had received partial (60.1%) or exclusive (30.2%) breastfeeding in the first four months. Mean (± SD) diet quality score at the age of 1 year was 4.2 (± 1.3) on a theoretical range of 0 to 10. Correlations between the dietary patterns are described in Chapter 5.2, in Supplement 5.2.1. At the age of 6 years, median BMI was 15.7 kg/m^2 (95% range 13.6-19.1).

Associations between dietary patterns and cardiometabolic risk factor score

In multivariable models, higher adherence to the 'health-conscious' PCA-derived dietary pattern in early childhood was associated with a lower cardiometabolic risk factor score at the age of 6 years (-0.069 SD lower cardiometabolic risk factor score (95% CI -0.0122, -0.015) per SD in adherence to the 'health-conscious' pattern) (Table 5.3.1). Additional adjustment for child FMI and FFMI at the age of 6 years did not change this association (-0.068 SD (95% CI -0.118, -0.018). Furthermore, the third quartile of the 'Western-like' PCA-derived pattern was associated with a higher cardiometabolic risk factor score and the third quartile of the diet quality score at 1 year was associated with a lower cardiometabolic risk factor score at age 6 years, when compared to the lowest quartile. However, for both dietary patterns the highest quartile was not associated with the cardiometabolic score, and the association of the third quartile of the diet score was no longer significant after additional adjustment for body composition at the age of 6 years (Table 5.3.1).

In crude models (Supplement 5.3.1), higher adherence to the 'Western-like' PCA-derived pattern, and the 'high FMI' RRR-derived pattern, was associated with a higher cardiometabolic risk factor score. However, after adjustment for confounders, these associations strongly attenuated and were no longer statistically significant, except for the association of the third quartile of adherence to the 'Western-like' pattern with a higher cardiometabolic risk score (Table 5.3.1).

Associations between dietary patterns and individual cardiometabolic risk factors

Associations of the dietary patterns in early childhood with the individual cardiometabolic components at 6 years of age are presented in Table 5.3.2. Adherence to the PCA-patterns or the diet score was not associated with the individual cardiometabolic factors, except for an inverse association between the 'health-conscious' pattern (quartile 4) and TAG levels; and an inverse association between the third quartile of the 'Western-like' pattern and HDL cholesterol concentrations with the first quartile as reference. Furthermore, children with a higher score (quartiles 2, 3 and 4) on the 'high FFMI' RRR-derived dietary pattern had a lower DBP at the age of 6 years than children in the lowest quartile. A higher adherence to 'high FMI' dietary pattern was associated with lower TAG levels, but not with any of the other cardiometabolic risk factors (Table 5.3.2).

Additional analyses

We observed no interaction of child sex with any of the dietary patterns on the cardiometabolic risk factor score. There was a significant interaction between sex and the 'high FMI' RRR-derived pattern on HDL (p=0.012). In stratified analyses, we observed that the associations of this RRR-pattern with HDL went in opposite direction for boys and girls, but none of the associations were significant (boys: 0.06 (95% CI -0.03, 0.15), girls: -0.06 (95% CI -0.16, 0.03)). There was no significant interaction for any of the other cardiometabolic components.

In sensitivity analyses in which we excluded each component from the cardiometabolic score one by one, the association between the 'health-conscious' PCA-derived pattern with the cardiometabolic score remained significant in all analyses, suggesting that the association was not driven by a single cardiometabolic risk factor. The associations of the other dietary patterns with the cardiometabolic score also remained similar in these sensitivity analyses, except for the association of the 'Western-like' PCA-pattern with the cardiometabolic risk factor score, for which the effect estimate became larger and statistically significant after exclusion of insulin concentrations (0.07 (95% CI 0.01, 0.13)).

Table 5.3.1 Multivariable associations of dietary patterns at the age of 1 year with the cardiometabolic risk factor score at age 6 years

	Associations adjusted for confounders β (95%CI)	**Associations additionally adjusted for FMI and FFMI** β (95%CI)
Diet quality score		
Per SD	-0.025 (-0.075, 0.024)	-0.031 (-0.077, 0.016)
Q1 (low adherence)	*Reference*	*Reference*
Q2	-0.072 (-0.212, 0.069)	-0.062 (-0.193, 0.068)
Q3	**-0.155 (-0.219, -0.014)**	-0.121 (-0.252, 0.009)
Q4 (high adherence)	-0.078 (-0.219, 0.062)	-0.096 (-0.227, 0.035)
'Health-conscious' dietary pattern (PCA)		
Per SD	**-0.069 (-0.122, -0.015)**	**-0.068 (-0.118, -0.018)**
Q1 (low adherence)	*Reference*	*Reference*
Q2	-0.114 (-0.257, 0.028)	-0.093 (-0.226, 0.039)
Q3	**-0.206 (-0.348, -0.064)**	**-0.195 (-0.327, -0.063)**
Q4 (high adherence)	**-0.231 (-0.379, -0.082)**	**-0.233 (-0.371, -0.095)**
'Western-like' dietary pattern (PCA)		
Per SD	0.047 (-0.017, 0.111)	0.045 (-0.015, 0.104)
Q1 (low adherence)	*Reference*	*Reference*
Q2	0.059 (-0.081, 0.199)	0.072 (-0.058, 0.202)
Q3	**0.212 (0.065, 0.358)**	**0.182 (0.045, 0.318)**
Q4 (high adherence)	0.120 (-0.042, 0.282)	0.135 (-0.016, 0.286)
'High FMI' dietary pattern (RRR)		
Per SD	0.016 (-0.047, 0.079)	-0.016 (-0.075, 0.043)
Q1 (low adherence)	*Reference*	*Reference*
Q2	-0.002 (-0.143, 0.139)	-0.047 (-0.178, 0.084)
Q3	-0.054 (-0.195, 0.087)	-0.093 (-0.224, 0.039)
Q4 (high adherence)	0.050 (-0.097, 0.198)	-0.025 (-0.164, 0.113)
'High FFMI' dietary pattern (RRR)		
Per SD	-0.036 (-0.094, 0.022)	-0.016 (-0.070, 0.038)
Q1 (low adherence)	*Reference*	*Reference*
Q2	-0.136 (-0.282, 0.010)	-0.087 (-0.752, 0.048)
Q3	-0.068 (-0.214, 0.077)	-0.045 (-0.223, 0.091)
Q4 (high adherence)	-0.125 (-0.288, 0.038)	-0.090 (-0.181, 0.061)

Values are regression coefficients (95% confidence interval) that reflect the difference in cardiometabolic risk factor score (sum of 5 SD-scores) per 1 SD higher adherence to the dietary pattern; and for quartiles of dietary pattern adherence scores compared to the lowest quartile. **Bold** values indicate statistically significant results.

Models are adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child sex, breastfeeding in the first four months of life, timing of introduction of complementary feeding, age at dietary measurement, total energy intake at 1 year, television watching at age 2 years and height at age 6 years.

Abbreviations: PCA, principal component analysis; RRR, reduced rank regression; SDS, standard deviation score.

Table 5.3.2 Multivariable associations of dietary patterns at 1 year of age with individual cardiometabolic outcomes at age 6 years

	SBP (SDS) *n*=1,931	DBP (SDS) *n*=1,931	HDL-C (SDS) *n*=1,371	TAG (SDS) *n*=1,368	Insulin (SDS) *n*=1,364
Diet quality score					
Per SD	0.005 (-0.038, 0.049)	-0.030 (-0.072, 0.013)	0.013(-0.039, 0.065)	-0.044(-0.096, 0.009)	0.005(-0.047, 0.056)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.058 (-0.063, 0.180)	-0.036 (-0.154, 0.082)	0.027 (-0.123, 0.176)	-0.091(-0.241, 0.060)	-0.057(-0.204, 0.091)
Q3	0.000 (-0.122, 0.123)	**-0.122(-0.241, -0.004)**	0.020 (-0.128, 0.168)	-0.144(-0.293, 0.005)	-0.037(-0.183, 0.109)
Q4 (high adherence)	0.045 (-0.079, 0.168)	-0.051 (-0.171, 0.069)	0.063(-0.210, 0.210)	-0.131(-0.280, 0.018)	-0.022(-0.168, 0.124)
'Health-conscious' pattern (PCA)					
Per SD	-0.035 (-0.082, 0.012)	-0.032 (-0.077, 0.014)	0.046 (-0.010, 0.103)	-0.056 (-0.114, 0.001)	-0.018 (-0.074, 0.038)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	-0.029 (-0.151, 0.093)	-0.007 (-0.125, 0.111)	0.149(-0.002, 0.301)	-0.064 (-0.217, 0.089)	0.006 (-0.143, 0.156)
Q3	-0.066 (-0.189, 0.058)	-0.104 (-0.224, 0.016)	0.147 (-0.003, 0.297)	-0.129 (-0.284, 0.023)	-0.035 (-0.183, 0.114)
Q4 (high adherence)	-0.077 (-0.205, 0.052)	-0.067 (-0.192, 0.057)	0.126 (-0.031, 0.283)	**-0.208 (-0.367, -0.050)**	-0.072 (-0.227, 0.082)
'Western-like' pattern (PCA)					
Per SD	0.020 (-0.036, 0.077)	0.021 (-0.034, 0.075)	-0.050 (-0.118, 0.018)	0.065(-0.004, 0.134)	-0.038 (-0.105, 0.029)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	-0.028 (-0.150, 0.065)	0.113 (-0.005, 0.232)	-0.076 (-0.225, 0.073)	0.052 (-0.099, 0.202)	-0.022 (-0.169, 0.124)
Q3	-0.026 (-0.152, 0.100)	0.081 (-0.041, 0.203)	**-0.213 (-0.368, -0.059)**	0.154(-0.003, 0.310)	0.064 (-0.088, 0.216)
Q4 (high adherence)	0.024 (-0.124, 0.159)	0.085 (-0.052, 0.221)	-0.122 (-0.294, 0.050)	0.155 (-0.019, 0.328)	0.011 (-0.158, 0.180)

Table 5.3.2 (continued) Multivariable associations of dietary patterns at 1 year of age with individual cardiometabolic outcomes at age 6 years

	SBP (SDS) *n*=1,931	DBP (SDS) *n*=1,931	HDL-C (SDS) *n*=1,371	TAG (SDS) *n*=1,368	Insulin (SDS) *n*=1,364
'High FMI' dietary pattern (RRR)					
Per SD	0.023 (-0.032, 0.077)	0.007 (-0.046, 0.061)	0.001 (-0.065, 0.066)	**-0.078 (-0.145, -0.012)**	-0.003 (-0.068, 0.061)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.066 (-0.056, 0.187)	0.066 (-0.052, 0.184)	0.111 (-0.037, 0.259)	-0.035 (-0.185, 0.115)	-0.028 (-0.174, 0.118)
Q3	0.012 (-0.112, 0.135)	0.056 (-0.064, 0.176)	0.113 (-0.036, 0.262)	-0.043 (-0.193, 0.108)	-0.143 (-0.290, 0.005)
Q4 (high adherence)	0.107 (-0.024, 0.238)	0.061 (-0.066, 0.188)	-0.017 (-0.173, 0.139)	-0.153 (-0.311, 0.006)	-0.045 (-0.199, 0.108)
'High FFMI' dietary pattern (RRR)					
Per SD	-0.017 (-0.067, 0.033)	-0.039 (-0.088, 0.009)	0.034 (-0.027, 0.096)	0.021 (-0.042, 0.083)	0.004 (-0.057, 0.064)
Q1 (low adherence)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	-0.117 (-0.241, 0.007)	**-0.140 (-0.260, -0.020)**	0.114 (-0.039, 0.268)	-0.028 (-0.183, 0.128)	0.045 (-0.106, 0.197)
Q3	-0.099 (-0.227, 0.029)	**-0.141 (-0.265, -0.018)**	0.092 (-0.063, 0.247)	-0.018 (-0.175, 0.138)	0.137 (-0.016, 0.289)
Q4 (high adherence)	-0.122 (-0.263, 0.019)	**-0.193 (-0.330, -0.057)**	0.144 (-0.029, 0.316)	-0.051 (-0.226, 0.124)	0.139 (-0.031, 0.309)

Values are regression coefficients (95% confidence interval) that reflect the difference in outcome (age- and sex-specific SD-scores) per 1 SD higher adherence to the dietary pattern and for quartiles of pattern adherence compared to the lowest quartile, based on imputed data. **Bold** values indicate statistically significant results.

Models are adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child sex, breastfeeding in the first four months of life, timing of introduction of complementary feeding, age at dietary measurement, total energy intake at 1 year, television watching at age 2 years and height SDS at age 6 years.

Abbreviations: DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; PCA, principal component analysis; RRR, reduced rank regression; SBP, systolic blood pressure; SDS, standard deviation score; TAG, triacylglycerol.

DISCUSSION

In a large population-based cohort study in young children, we observed that higher adherence to a 'health-conscious' dietary pattern at 1 year of age is associated with a lower cardiometabolic risk factor score at 6 years of age. This 'health-conscious' pattern was characterized by a high intake of vegetables, fruit, potatoes, pasta and rice, fish, meat, legumes, and vegetable oil. A 'Western-like' dietary pattern was associated with poorer cardiometabolic health at 6 years of age, but not consistently so. This pattern was characterized by high intake of refined grains, savory snacks, confectionary, soups and sauces, and sugar-containing beverages. No clear association was observed between dietary patterns and individual cardiometabolic risk factors. In addition, our results suggest that associations between dietary patters in early childhood with cardiometabolic health at school age are not explained by differences in fat mass or fat-free mass index at the age of 6 years.

Interpretation and comparison with previous studies

We are one of the first to study associations of overall diet with cardiometabolic health in young children. A few previous studies examined the association of dietary patterns in older children or adolescents with cardiovascular or metabolic outcomes.[15-16, 18, 20] In a large cross-sectional study among 5,198 German adolescents aged 12 to 17 years, four different diet scores were evaluated and none of the scores was associated with total or HDL cholesterol.[15] The authors suggested that their diet scores, either developed for children or for the general population, were not specific enough to predict cardiovascular risk. In another study in 130 children aged 4 to 13 years, adherence to a dietary guideline index for children and adolescents was not associated with blood lipids.[16] A third study from Finland, in 1768 participants aged 3 to 18 years at baseline, identified two PCA-derived dietary patterns and several cardiometabolic risk factors were assessed 21 years later. A traditional dietary pattern, characterized by high consumption of potatoes, butter, sausages, and milk, was positively associated with total and LDL cholesterol concentrations, SBP, and insulin levels; whereas a dietary pattern reflecting more health-conscious food choices, such as high consumption of vegetables, legumes and nuts, was inversely associated with cardiovascular risk factors.[18] Finally, a study in 1419 Australian children observed that a higher adherence to Australian dietary guidelines at the ages of 14 and 17 years was associated with lower insulin and TAG levels but not with blood pressure or cholesterol levels.[20]

To our knowledge, only one previous study examined the relation between dietary patterns in the preschool period with cardiometabolic outcomes. This study from the U.K. had a design similar to our study, and measured diet in over 4,000 children at several points in time up to the age of 2 years and several health outcomes at the age of 7 to 8 years.[17,19] In this study, a higher adherence to complementary feeding guidelines at the age of 6 months was associated with a lower DBP, but not with SBP or with blood lipid levels in childhood.[17] In addition, in the same study it was shown that a so-called 'less-healthy' PCA-derived dietary pattern was associated with a higher SBP and a higher DBP.[19]

In our study, we did not find clear associations between dietary patterns in early life with any of the individual cardiometabolic risk factors, but we found associations only with the combined cardiometabolic score. It might thus be that the previous studies that did not observe clear associations with individual risk factors were also underpowered to detect small differences in individual cardiometabolic components, while diet could in fact affect overall cardiometabolic health.

Our secondary aim was to assess whether associations with cardiometabolic health were explained by differences in body composition. In the previously described cohort of young children from the U.K., the association of the 'less-healthy' pattern with SBP was no longer significant after additional adjustment for child height, BMI and waist circumference, but the association with a higher DBP remained.[19] These results suggest that body composition partially, but not fully, mediates the association between unhealthy diet and blood pressure. In our study, additional adjustment for child fat mass index and fat-free mass index did not change the results. Furthermore, the RRR-derived dietary patterns, which were constructed on the basis of variation in body composition, were not associated with cardiometabolic health. This suggests that changes in body composition may not fully mediate the association between overall diet in early childhood and cardiometabolic health

Methodological considerations

An important strength of our study is that we had a prospective study design with detailed information on a large number of potential confounders. Several family sociodemographic and lifestyle characteristics are related to child dietary patterns,[23-24] and may also be associated to child health. These factors should thus be taken into account when studying the relation between diet and cardiometabolic health. Previous studies were not always able to adjust for important confounders, such as parental BMI and lifestyle factors. Furthermore, we had a large study population and more than 80% of all children in our study who had data on dietary patterns, also participated in the follow-up measurements at the age of 6 years. However, blood samples were available in only 68% of these children. As described in more detail in Chapter 2.3, children with blood samples had on average slightly higher educated mothers than children without blood samples, but did not differ with respect to their BMI, body fat, or blood pressure at the age of 6 years.[5]

Even though not all participating children had blood drawn, the availability of blood levels is a great strength of our study. We had detailed measures of various cardiovascular and metabolic outcomes. This also enabled us to create a continuous cardiometabolic risk factor score which combines the individual risk factors. As compared to a dichotomous metabolic syndrome definition, the benefits of a continuous score are that more information is being used which makes it more sensitive to detect differences, and that the continuous score is less prone to errors.[29] Furthermore, we had information on fat and fat-free mass instead of only BMI, so we could examine potential mediation by body composition in more detail.

We used an FFQ to measured food intake which is known to be prone to measurement error. Nevertheless, an FFQ measures habitual diet and is considered an appropriate method to use for dietary pattern analysis.[37] Finally, the dietary pattern approach has important advantages over studying individual foods or nutrients, especially when studying multifactorial processes, such as in cardiometabolic risk factors.[6,7] In the current study we used several different approaches for dietary pattern analyses, while previous studies often only focused on one method only.

Conclusions

Higher adherence to a 'health-conscious' dietary pattern at the age of 1 year was associated with a lower combined cardiometabolic risk factor score at the age of 6 years. This association was independent of body composition of the children at the age of 6 years. In addition, dietary patterns constructed on the basis of variations in body composition at age 6 years were not associated with cardiometabolic health, further suggesting that there is an association of diet in early childhood with cardiometabolic health that is independent of body composition.

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Supplement 5.3.1 Associations of dietary patterns at 1 year of age with childhood cardiometabolic outcomes at age 6 years: crude, confounder adjusted, and body composition adjusted models

	Cardiometabolic risk factor score ***n*=1,297** β (95% CI)	**SBP (SDS)** ***n*=1,931** β 95% CI)	**DBP (SDS)** ***n*=1,931** β (95% CI)	**HDL-C (SDS)** ***n*=1,371** β (95% CI)	**TAG (SDS)** ***n*=1,368** β (95% CI)	**Insulin (SDS)** ***n*=1,364** β (95% CI)
Diet score (per SD)						
Crude	-0.028 (-0.078, 0.021)	0.004 (-0.040, 0.047)	-0.032 (-0.074, 0.009)	0.006 (-0.045, 0.057)	-0.037 (-0.089, 0.014)	0.009 (-0.041, 0.060)
Confounder adjusted (main model)	-0.025 (-0.075, 0.024)	0.005 (-0.038, 0.049)	-0.030 (-0.072, 0.013)	0.013 (-0.039, 0.065)	-0.044 (-0.096, 0.009)	0.005 (-0.047, 0.056)
Additionally adjusted for FMI & FFMI	-0.031 (-0.077, 0.016)	-0.001 (-0.044, 0.043)	-0.029 (-0.072, 0.013)	0.013 (-0.039, 0.065)	-0.046 (-0.099, 0.006)	0.000 (-0.051, 0.052)
'Health-conscious' pattern (PCA) (per SD)						
Crude	**-0.069 (-0.124, -0.015)**	-0.037 (-0.084, 0.010)	-0.030 (-0.075, 0.015)	0.040 (-0.017, 0.096)	-0.048 (-0.105, 0.008)	-0.015 (-0.071, 0.040)
Confounder adjusted (main model)	**-0.069 (-0.122, -0.015)**	-0.035 (-0.082, 0.012)	-0.032 (-0.077, 0.014)	0.046 (-0.010, 0.103)	-0.056 (-0.114, 0.001)	-0.018 (-0.074, 0.038)
Additionally adjusted for FMI & FFMI	**-0.068 (-0.118, -0.018)**	-0.039 (-0.086, 0.007)	-0.031 (-0.076, 0.015)	0.046 (-0.011, 0.103)	-0.057 (-0.115, 0.001)	-0.020 (-0.076, 0.036)
'Western-like' pattern (PCA) (per SD)						
Crude	**0.063 (0.001, 0.124)**	**0.054 (0.000, 0.108)**	**0.054 (0.003, 0.106)**	-0.030 (-0.094, 0.034)	0.047 (-0.017, 0.112)	-0.044 (-0.107, 0.019)
Confounder adjusted (main model)	0.047 (-0.017, 0.111)	0.020 (-0.036, 0.077)	0.021 (-0.034, 0.075)	-0.050 (-0.118, 0.018)	0.065 (-0.004, 0.134)	-0.038 (-0.105, 0.029)
Additionally adjusted for FMI & FFMI	0.045 (-0.015, 0.104)	0.020 (-0.036, 0.076)	0.022 (-0.033, 0.076)	-0.050 (-0.119, 0.018)	0.064 (-0.004, 0.133)	-0.040 (-0.107, 0.027)

Supplement 5.3.1 (continued) Associations of dietary patterns at 1 year of age with childhood cardiometabolic outcomes at age 6 years: crude, confounder adjusted, and body composition adjusted models

	Cardiometabolic risk factor score ***n*=1,297** β (95% CI)	**SBP (SDS)** ***n*=1,931** β 95% CI)	**DBP (SDS)** ***n*=1,931** β (95% CI)	**HDL-C (SDS)** ***n*=1,371** β (95% CI)	**TAG (SDS)** ***n*=1,368** β (95% CI)	**Insulin (SDS)** ***n*=1,364** β (95% CI)
'High FMI' dietary pattern (RRR) (per SD)						
Crude	**0.067 (0.006, 0.129)**	**0.068 (0.015, 0.122)**	0.047 (-0.005, 0.098)	-0.004 (-0.067, 0.059)	**-0.075 (-0.138, -0.011)**	0.011 (-0.052, 0.073)
Confounder adjusted (main model)	0.016 (-0.047, 0.079)	0.023 (-0.032, 0.077)	0.007 (-0.046, 0.061)	0.001 (-0.065, 0.066)	**-0.078 (-0.145, -0.012)**	-0.003 (-0.068, 0.061)
Additionally adjusted for FMI & FFMI	-0.016 (-0.075, 0.043)	0.005 (-0.050, 0.060)	0.004 (-0.049, 0.058)	0.002 (-0.064, 0.068)	**-0.086 (-0.152, -0.019)**	-0.013 (-0.078, 0.052)
'High FFMI' dietary pattern (RRR) (per SD)						
Crude	-0.056 (-0.113, 0.001)	-0.038 (-0.087, 0.011)	**-0.058 (-0.105, -0.011)**	0.02 (-0.038, 0.080)	0.026 (-0.034, 0.085)	0.006 (-0.053, 0.065)
Confounder adjusted (main model)	-0.036 (-0.094, 0.022)	-0.017 (-0.067, 0.033)	-0.039 (-0.088, 0.009)	0.034 (-0.027, 0.096)	0.021 (-0.042, 0.083)	0.004 (-0.057, 0.064)
Additionally adjusted for FMI & FFMI	-0.016 (-0.070, 0.038)	-0.018 (-0.068, 0.032)	-0.035 (-0.164, -0.083)	0.033 (-0.029, 0.094)	0.021 (-0.042, 0.083)	0.003 (-0.058, 0.064)

Values are regression coefficients that reflect the difference in outcome (age- and sex- specific SD-scores) per 1 SD higher adherence to the dietary pattern, based on imputed data. **Bold** values indicate statistically significant results.
Crude models are adjusted child sex, age at dietary measurement and total energy intake at 1 year.
Confounder adjusted models are adjusted for maternal age, BMI at enrollment, parity, folic acid supplement use, smoking and alcohol use during pregnancy; paternal smoking and education; household income; and child sex, breastfeeding in the first four months of life, timing of introduction of complementary feeding, age at dietary measurement, total energy intake at 1 year, television watching at age 2 years and height at age 6 years.
The last model is additionally adjusted for FMI SDS and FFMI SDS at the age of 6 years
Abbreviations: DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; PCA, principal component analysis; RRR, reduced rank regression; SBP, systolic blood pressure; SDS, standard deviation score; TAG, triacylglycerol.

Chapter 6

General discussion

The aim of the research described in this thesis was to study nutrition during early life and its associations with body composition and cardiometabolic health in childhood. Nutritional factors of interest were protein intake, fatty acid intake and blood levels, vitamin D status, and dietary patterns. Specific findings and discussion points from these analyses are described in the previous chapters. The current chapter provides a brief summary of the main findings and a general discussion of the major methodological considerations, followed by public health implications and directions for future research.

MAIN FINDINGS

Protein intake in early childhood

In a systematic review of the previously published literature, we concluded that prior studies reported no consistent associations between protein intake and cardiometabolic health in children (Chapter 2.1). However, most of these studies were of low methodological quality, for example because they did not adjust for key confounders such as energy intake or other dietary factors. In subsequent analyses on protein intake and cardiometabolic health among children participating in the Generation R Study, we aimed to better control for confounding factors.

First, we analyzed whether protein intake at the age of 1 year was related to measures of body composition at the age of 6 years (Chapter 2.2). In line with a few previous studies,[1-3] we observed that a higher protein intake in early childhood is associated with a higher body mass index (BMI) in later childhood. This relation remained present after adjustment for BMI at the age of 1 year. In addition, ours is the first large study to show that this association between protein intake and BMI was fully driven by a higher fat mass index (FMI) and not by differences in fat-free mass index (FFMI). Further analyses showed that associations with FMI were stronger for intake of animal protein than for intake of vegetable protein, and that associations were slightly stronger in girls than in boys.

Sex differences were also observed when we examined the relation of protein intake with other cardiometabolic health outcomes (Chapter 2.3). Higher protein intake at 1 year of age was associated with higher insulin levels at the age of 6 years in girls, but with lower triacylglycerol (TAG) concentrations in boys. In the whole group, a higher protein intake at the age of 1 year was also linked to a lower diastolic blood pressure, but not to systolic blood pressure. Furthermore, protein intake was not independently related to kidney size or function at the age of 6 years (Chapter 2.4). Associations with insulin, TAG and blood pressure did not change after additional adjustment for measures of body composition, indicating that these findings were not explained by the relation of protein intake with body fat.

The observation that protein intake at the age of 1 year was linked to higher insulin and body fat levels is in line with the 'early protein hypothesis'. This hypothesis postulates that high protein in early postnatal life triggers hormonal responses, such as increased release of insulin and insulin-like growth factor 1 (IGF-1), which subsequently increase adipogenesis.[1, 4] In the Generation R Study, we had no information on concentrations of growth hormones around the age of 1 year. However, previous studies suggested that intake of animal, but not vegetable protein is associated

with higher IGF-1 concentrations,[5] and that the IGF-1 axis response to high protein intake is stronger in girls than in boys.[6] Therefore, our observations that the relation between protein intake and FMI was stronger for animal protein than for vegetable protein intake, and that the relation was stronger in girls than in boys, support the hypothesis that these associations may be mediated by endocrine responses.

Fatty acids in early life

We systematically reviewed the previous literature on the associations of polyunsaturated fatty acids (PUFAs) in early life with obesity or cardiometabolic health in later life (Chapter 3.1). The 19 intervention studies and 28 observational studies that met all selection criteria were of overall high quality. However, after critically reviewing these studies, we concluded that the current literature provides insufficient evidence to support or refute a beneficial effect of PUFAs during pregnancy, lactation, or early childhood on cardiometabolic health. A few intervention studies reported suggestive evidence that omega-3 (*n*-3) PUFAs supplementation may have a favorable effect on measures of obesity,[7-9] but overall results were inconsistent. The inconsistency between studies may be explained by heterogeneity in several study characteristics, for instance the study design, the duration of supplementation, the timing of exposure and outcome measurements, or factors related to the study population, such as baseline fatty acids status. Another potential reason for the inconsistent findings, is that the studies did not all examine the same types of PUFAs, whereas different fatty acids may have different effects on cardiometabolic health. For example, it has been suggested that *n*-3 and omega-6 (*n*-6) fatty acids have opposing effects on adipocyte differentiation.[10-11] Also certain individual fatty acids may have contrasting effects.[12] Furthermore, effects of fatty acids can depend on levels of other fatty acids. Therefore, the relative amounts or ratios of certain fatty acids may be of importance, rather than only the absolute amounts.[13-14]

In order to take these potential interactions between different fatty acids into consideration, we performed a pattern analysis on plasma fatty acid concentrations of the women participating in the Generation R Study (Chapter 3.2). During mid-pregnancy, plasma concentrations of 22 individual fatty acids were determined in these women. With a PCA – a data reduction technique – we identified three distinct fatty acid patterns from these individual fatty acids. Our results suggest that a fatty acid pattern that was characterized by high concentrations of *n*-3 fatty acids in mid-pregnancy, which we named a 'high *n*-3 PUFA pattern', may be favorable for child body composition and metabolic health. We observed that children of women with a higher score on this pattern during pregnancy had a lower FMI and a higher FFMI at the age of 6 years than children whose mothers had a lower score on this pattern. Independent of body composition, a higher score on this 'high *n*-3 PUFA' pattern was also associated with higher HDL cholesterol and lower triacylglycerol concentrations, but not with blood pressure, insulin concentrations, or the combined cardiometabolic risk factor score in children. Patterns characterized by high levels of *n*-6 PUFAs; or by monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) were not consistently associated with cardiometabolic outcomes of the children. Intervention studies in adults have also suggested that higher intakes of *n*-3 FAs may decrease body fat percentage and TAG concentrations, but evidence is not conclusive.[15-17] Underlying pathways may include

increased fat oxidation, reduction of hepatic synthesis of TAG, or an increased lipoprotein lipase activity in response to higher circulating *n*-3 fatty acids.[10, 15, 18-20]

In addition to circulating fatty acids during pregnancy, we also examined the association of dietary intake of fatty acids in early childhood with cardiometabolic health (Chapter 3.3). In these analyses, we observed that intake of SFA, MUFA or PUFA at the age of 1 year was not consistently associated with body fat, blood lipids, insulin concentrations, or blood pressure at the age of 6 years. A higher MUFA or PUFA intake at the expense of SFA intake was also not related to cardiometabolic health. This suggests that fatty acid intake in early postnatal life may be less important for cardiometabolic health than fatty acid exposures during the prenatal period. However, because we did not have detailed information on dietary intake or blood levels of specific fatty acids during childhood we may have not been able to detect a potential association. Therefore, future studies with detailed fatty acid measurements should further examine the role of individual fatty acids and fatty acid patterns during early life on body composition and cardiometabolic health.

Vitamin D status

We measured concentrations of 25-hydroxyvitamin D (25(OH)D)) in the children participating in the Generation R Study when they were 6 years of age and we observed a high prevalence of vitamin D deficiency. Almost 30% of the examined children were vitamin D deficient – defined as a 25(OH)D concentration of <50 nmol/L (Chapter 4.1). The prevalence of deficiency was especially high among children with a non-Western ethnic background (54.5%). Differences in vitamin D levels between different ethnicities might be explained by higher levels of skin pigmentation, which limits cutaneous vitamin D synthesis,[22] or by other genetic or cultural differences. Previous studies among older children and adults also reported a high prevalence of vitamin D deficiency in several different populations worldwide. It has been suggested that there is a reemergence of vitamin D deficiency in developed countries, which has been attributed to changes in lifestyle such as spending more time indoors.[21-22] The importance of sun exposure for vitamin D levels was also apparent in other determinants of vitamin D status in our population; the prevalence of vitamin D deficiency was higher in winter than in summer, and vitamin D deficiency was associated with less time spent on playing outside and more time spent on television watching (Chapter 4.1).

Because vitamin D is required for calcium absorption from the gut, vitamin D deficiency in early childhood may adversely affect bone development. Severe vitamin D deficiency causes rickets in children and osteomalacia and osteoporosis in adults.[22] In addition to skeletal health, studies in adults have suggested that vitamin D is also important for other aspects of health, including body composition[23] and cardiometabolic health.[24] In cross-sectional analyses in our population of 6-year-old children, we observed an inverse association between circulating 25(OH)D levels with adiposity in girls, but not with adiposity in boys (Chapter 4.2). We found no consistent associations between vitamin D status and children's blood pressure, blood lipids, or the cardiometabolic risk factor score, but we did observe an inverse association with insulin concentrations (Chapter 4.3). Whether the associations of vitamin D deficiency with adiposity and insulin levels are causal or hampered by residual confounding from common causes such as a lack of physical activity needs further study. Furthermore, if causal, it is unclear whether vitamin D deficiency is cause or consequence.

In the case of obesity, for example, it is unknown whether children with lower vitamin D levels have an increased risk of adiposity or whether obesity increases the risk of vitamin D deficiency.[25] For both pathways, plausible mechanisms have been suggested. For instance, vitamin D has been shown to increase adipose tissue lipolysis and decrease adipogenesis,[26] but vitamin D is also known to be stored in adipose tissue leading to lower levels of circulating 25(OH)D levels.[27] In order to examine the direction and causality of the association with adiposity, we performed a bidirectional instrumental variable analysis in which we used established genetic correlates of 25(OH)D levels and BMI. Results of these analyses provided no clear evidence for a causal relation in either direction (Chapter 4.2). A previous study in adults that applied a similar instrumental variable analysis in several cohort studies suggested that a higher BMI may lead to lower vitamin D concentrations.[25] These effects may not be present in young children, or alternatively, our study may have been underpowered to detect an association.

Dietary patterns in early childhood

The first chapters of this thesis focus on nutrients. However, individuals do not consume single nutrients, but foods, combined into meals, comprised of a variety of macronutrients and micronutrients. These foods and nutrients in the diet may have synergistic or antagonistic effects on health and disease.[28] In Chapter 5 of this thesis, we therefore used another approach in nutritional epidemiology: dietary pattern analysis.[28-30] We examined dietary patterns of 1-year-old children with the use of three different methods: an *a priori*-defined diet quality score; *a posteriori*-derived patterns based on variation in food group intake; and *a posteriori*-derived patterns based on variations in both food intake and later body composition.[31]

To assess overall diet quality, we developed a novel food-based diet score for preschool children on the basis of dietary guidelines (Chapter 5.1). Subsequently, we used this score to evaluate diet quality of children participating in the Generation R Study at their ages of 1 and 2 years. In this population, we first assessed the score's construct validity, i.e., whether the diet score actually measures the construct being investigated: a healthy diet. Because the diet score was based on food groups, we used nutrient intakes as alternative indicators of healthy diet to assess construct validity. The diet score was indeed positively related to intake of nutrients considered to be healthy, including PUFAs, dietary fiber, and calcium; and inversely related to intakes of unhealthy nutrients, such as SFAs and sugars. Furthermore, the score was positively associated with the likelihood of achieving recommended nutrient intakes. These results indicate that the diet score has adequate construct validity and that the score may be used as an indicator of a healthy diet. In addition, we observed that a higher diet score was associated with several health-conscious behaviors of mothers, such as using folic acid supplements in early pregnancy, and with less television watching of the child. This indicates that a healthier diet of young children tends to cluster with other health-conscious behaviors.

In addition to this *a priori*-defined diet score, we also applied two other dietary pattern approaches to analyze the dietary data of the 1-year-old children. With the use of principal component analysis (PCA), we derived two dietary patterns – which we labeled 'health-conscious' and 'Western-like' –, that explained maximum variation in food group intake in our population.

With reduced rank regression (RRR) two other dietary patterns were extracted that explained maximum variation in children's FMI and FFMI at the age of 6 year. Based on their correlations with body composition outcomes we labeled these patterns 'high FMI' and 'high FFMI' dietary patterns. We examined the associations of all five dietary patterns with the children's body composition (Chapter 5.2) and cardiometabolic health (Chapter 5.3) at the age of 6 years. We observed that higher adherence to the diet quality score, the PCA-derived 'health-conscious' pattern, and the RRR-derived 'high FFMI' dietary pattern was related to a higher FFMI, but not to FMI (Chapter 5.2). These dietary patterns were all characterized by high intake of foods considered to be healthy, such as vegetables, grains, and vegetable oils. The 'Western-like' PCA-derived pattern was not related to body composition. The only pattern that was positively associated with FMI, was the 'high FMI' RRR-derived dietary pattern, which was characterized by high intake of refined grains, traditional dinner foods (i.e., potatoes, vegetables, and meat), and sugar-containing beverages.

When we subsequently examined the associations of these five dietary patterns with cardiometabolic health (Chapter 5.3), we observed that adherence to the 'health-conscious' dietary pattern was associated with a lower cardiometabolic risk factor score, but not clearly with any of the individual cardiometabolic components. These associations were not explained by differences in FMI or FFMI. Moreover, the RRR-derived dietary patterns based on variations in body composition were not associated with cardiometabolic health, suggesting that dietary patterns in early childhood may influence cardiometabolic health in later childhood independent of effects on body composition at this age.

METHODOLOGICAL CONSIDERATIONS

Strengths and limitations of the specific studies included in this thesis have been discussed in the corresponding chapters. This section addresses some general methodological issues related to systematic reviews, study design, assessment of dietary intake, assessment of body composition and cardiometabolic health, and confounding. These methodological aspects were assessed as part of the quality score that we used for the systematic reviews presented in this thesis, and should also be considered when interpreting the results of our analyses in the Generation R Study.

Systematic reviews

The main strength of systematic reviews compared to non-systematic or narrative reviews is the systematic approach to identify, select, and appraise relevant primary research. This systematic approach permits scientists to summarize all research objectively, in a reproducible manner, and minimizing bias. To systematically identify potentially relevant articles, we conducted extensive literature searches in several electronic databases, advised by a medical information specialist. No limits were set on language or on year of publication. We thereby aimed to identify all published literature that addressed the topic. However, not all research gets published, which results in publication bias. We aimed to reduce this problem of publication bias by contacting authors to identify additional unpublished studies. In order to systematically select relevant studies, we first

set predefined study eligibility criteria. Working in pairs, together with a second reviewer, we independently screened the identified abstracts to evaluate whether the studies satisfied the selection criteria. Finally, for systematic appraisal of the studies, we extracted data from the selected studies with a structured database created prior to the literature search.

Systematic reviews – either with or without a quantitative meta-analysis of the results – are often considered as the highest form of evidence, and hence positioned at the top of the hierarchy of evidence. However, they may not give definitive answers. The value of a systematic review depends on the quality of the review method, as described above, but also on the quality of the individual studies. For example, in the systematic review presented in Chapter 2.1, several studies were identified that investigated the relation between protein intake and cardiometabolic health in children. However, the majority of the included studies was of overall low quality, which made it difficult to draw conclusions regarding the absence or presence of the association evaluated.

As a measure of quality of the individual studies we applied a quality scoring method that we developed on the basis of previously used scoring systems[32-33] to assess the relative quality of studies with various study designs.[34] Although several methods exist to assess the quality of studies, most of these are developed to assess the quality of intervention studies only.[35] A few tools have been developed to assess quality of observational studies, but these are designed to measure the quality of only observational studies.[32, 36] However, in many systematic reviews, studies with different designs are included. Therefore, we developed a quality score that is applicable to both interventional and observational studies. Each study was scored on five aspects: 1) study design, 2) the size of the study population, 3) the quality of the exposure assessment or blinding, 4) the quality of the outcome assessment, and 5) adjustment for confounders or randomization. This score was used to, more objectively, distinguish between lower and higher quality studies and to guide interpretation of the results. Following the components of the quality score, some methodological considerations of the Generation R Study will be discussed in the subsequent paragraphs.

Study design

Most of the studies described in this thesis were performed in the Generation R Study, a prospective closed cohort study. A prospective cohort is a powerful design to identify potential risk factors for common diseases, although its observational nature does not allow to directly infer causality of the associations. A main strength of a prospective cohort study is that information on exposures such as dietary intake can be collected before certain outcomes of interest develop. This reduces recall bias and allows estimation of the temporality of the relation, i.e., whether the exposure precedes the outcome.[37] Ideally, one would like to have measurements of exposures and outcomes at different time-points, to further assess this temporality. Unfortunately, repeated measures of children's diet and cardiometabolic health were not yet available for the studies presented in this thesis. Only BMI was measured repeatedly during early childhood. In our analyses of protein intake and dietary patterns in relation to body composition, we adjusted for BMI at the age of 1 year. For both studies associations remained similar, which suggests that these dietary exposures predicted changes in body composition independent of baseline BMI. In order to be able to better estimate temporality of the associations, longer follow-up is needed during which both nutritional factors and measures

of cardiometabolic health and body composition are measured repeatedly over time. With data on repeatedly measured exposures and outcomes, we could for example adjust the observed associations for earlier measurements or we could use cross-lagged models. This would give us better indication of the potential causality of the associations.[37]

Another approach to explore causality in studies with an observational design, is the use of Mendelian randomization analysis.[38] We applied this method in the analyses on vitamin D status in relation to adiposity and insulin levels, for which we only had cross-sectional data available. Mendelian randomization analysis is an instrumental variable analysis that uses common genetics polymorphisms as instruments for exposures. To use this method, genes need to be available that are known to be associated with the exposure (e.g., vitamin D levels), and they need to affect the outcome (e.g., adiposity) only via that exposure.[38] This approach uses the fact that – in contrast to environmental exposures – genotypes are randomly assigned to persons and do not change over time. Mendelian randomization analysis thereby avoids the problems of reverse causation and confounding and can thus be used to examine whether observed associations are causal.[38-39]

Study population

Besides its prospective study design, another main strength of the Generation R Study is the large sample size. The Generation R Study started with 9,778 pregnant women, giving birth to 9,749 children. However, these are not all included in our analysis because of loss to follow-up, exclusion from specific follow-up visits, or missing data. The number of subjects in the studies presented in this thesis ranges from 2,026 to 4,167 children. This attrition may have caused selection bias. This type of bias occurs if the association between for example diet and cardiometabolic health is different in children who are included in the analysis compared to those who are not included but would be eligible. This can result from selective participation in the study at baseline or from selective loss to follow-up, which are both discussed below.

For the Generation R Study we observed that of all women eligible for the study, the overall response to participate was 61%.[40] In general, as compared to population figures of Rotterdam, women with a low socioeconomic status or with a non-Dutch ethnic background are slightly underrepresented in the Generation R Study. Also, the percentages of infants born preterm or with a low birthweight are smaller than expected.[40] This indicates a selection toward a relatively more healthy and affluent population. Although selective participation in cohort studies at baseline has been shown not to bias relative risks,[41] it may influence the observed prevalence rates and thereby the external validity of our results. For example, in Chapter 4 we described that almost 30% of the examined 4,167 children were vitamin D deficient. Because of both selective participation at baseline and selective follow-up, this number might not be generalizable to all 6-year-old children born in Rotterdam. We observed for instance a higher prevalence of vitamin D deficiency in children of mothers with a lower educational level or lower household income. Because of the selection toward a population with a higher socioeconomic status, the observed prevalence of vitamin D deficiency may be lower than in the general population.

Although selective non-response at baseline is only expected to influence external validity, selective loss to follow-up may also threaten internal validity. At several stages in our study, selective

inclusion or loss to follow-up may have occurred. For example, although almost 8,000 children were available for postnatal measurements, we sent the food-frequency questionnaire (FFQ) to measure child diet around the age of 1 year to only 5,088 participants. This selection was partly random as the questionnaire was introduced at a later stage in the study, but not completely random as we also selected mothers with sufficient mastery of the Dutch language. Of all mothers who received the FFQ, 72% returned the questionnaire. These mothers were generally more highly educated and had healthier lifestyle habits than mothers who did not return the questionnaire. When the children were around 6 years of age, children and their mothers were invited to participate in cardiometabolic and body composition follow-up measurements. The response rate at this follow-up was approximately 70%.[40] Again, mothers of children who visited the research center more frequently had healthy lifestyle habits and were more highly educated than the total study population. Another possible cause of selection bias is the differential availability of blood samples at the age of 6 years. We collected a blood sample in 67% of all children who visited the research center at the age of 6 years. This lower number was mainly caused by non-consent for venous puncture or crying of the child. Compared to children who visited the research center but did have their blood drawn, children with blood samples more often had mothers with a higher educational level or a higher household income.

Although in all these instances the differences were small, overall there seems to be a consistent selection in the studies in this thesis toward a more highly educated, healthier, and more affluent population. This may have consequences for external validity, as the observed results may not be directly generalizable to the general population. Nevertheless, it would result in selection bias only if the associations between nutrition and cardiometabolic health are different between children who are included in the analysis and children who are not. This is unlikely, but cannot be excluded.

Nutritional assessment

For many of the analyses described in this thesis, we used nutritional intake data assessed with an FFQ. This is the most widely used dietary assessment method in epidemiological studies for two main reasons. Firstly, because researchers are generally interested in average long-term dietary intake, rather than intake on one or a few specific days, and an FFQ measures this habitual dietary intake. Secondly, FFQs are relatively easy to complete for study participants and relatively easy to process in large quantities; these practical aspects make an FFQ the method of choice for large studies.[42] However, because FFQs assess average dietary intake over a longer time period, estimations are often less precise than those obtained with other approaches such as food records, 24-hour recalls, or recovery biomarkers. For that reason, FFQs are usually not suitable to measure absolute intakes, but can be used to rank subjects on the basis of their usual intakes.[42]

A challenge in using an FFQ is that it should be tailored to the specific study population. Food items should be chosen that are habitually consumed by subjects in the study population and/or that explain a large part of the variation in nutrients of interest in the population. The FFQ used for the 1-year-old children in the Generation R Study was developed specifically for this age group. In this FFQ we included foods that are frequently consumed by children aged 9-18 months and that contribute ≥0.1% to the total consumption of energy, protein, fat, carbohydrates, and dietary fiber

in this age group, according to a Dutch National Food Consumption Survey in 2002.[43] Furthermore, with 211 items, our FFQ was quite extensive, which has been shown to be important for the validity of FFQs.[44]

The main limitation of an FFQ is that it is a self-reported retrospective dietary assessment method and the reported dietary intakes are subject to substantial measurement error.[45-46] This measurement error is an important source of information bias in studies on dietary intake in relation to health or disease. This measurement error, or misclassification of exposure, is assumed to be mainly non-differential, meaning that it is random and not related to the outcome under study.[45] Non-differential measurement error of the exposure may result in attenuation of the observed associations and in wider confidence intervals. Hence, it leads to underestimation of associations and reduces statistical power to detect associations. As a consequence, it could be suggested that for some of the analyses in which we found no relation, e.g., fatty acid intake and cardiometabolic health, we might not have had enough power to be able to detect an existing association. However, errors in dietary intake assessment may also be differential, i.e., related to the outcome. In adults, for example, it has been shown that obese people are more likely to underreport their food intake than people with a normal weight.[47] When one examines the relation between diet and obesity, this underreporting could therefore lead to differential misclassification of exposure. Not much is known about potential differential measurement error in parent-reported dietary intake data of children. However, we expect that the dietary measurement errors in our study are unlikely to be strongly related to the outcomes, because we assessed dietary intake a few years before we assessed most of the outcomes. However, differential measurement error cannot be ruled out.

There are methods to reduce the influence of measurement error of reported nutrient intakes in the statistical analyses. In all our analyses with nutrient intakes, the nutrient of interest was adjusted for total energy intake, either with the residual method,[48] or with the nutrient-density method within a macronutrient substitution model.[49] Energy adjustment addresses not only confounding by energy, but also the measurement error that is related to energy intake.[45-46] Another method to reduce measurement error in dietary intake data is regression calibration.[50] However, to apply this method, an adequate reference instrument is needed to calibrate against. Our FFQ was evaluated against three 24-h recalls in 32 children and intraclass correlation coefficients for nutrient intakes ranged from 0.36 to 0.74. Although these correlations are not optimal, they are similar to coefficients obtained in other validation studies of dietary measurements.[44] However, because of correlated measurement errors between FFQs and 24-h recalls and because of the small sample size, we chose to not apply regression calibration of our FFQ data against the data from this validation study. We were not able to validate our FFQ against a 'golden standard' that would have been suitable for regression calibration, such as multi-day weighed food records, doubly labeled water for energy intake, or urine nitrogen levels for protein intake.[46]

For some of our analyses we used nutritional biomarkers, such as plasma fatty acids and vitamin D concentrations. Although biomarkers can usually be measured more objectively and precisely than dietary intake, these measures also have some disadvantages. At several stages of collection, processing, storage and laboratory assessment of the biological samples, measurement errors can be introduced.[42] But foremost, it is important to note that most nutritional biomarkers, including

blood concentrations of 25(OH)D and fatty acids are concentration biomarkers, – not recovery biomarkers. This means that they reflect nutrient status, but not necessarily nutrient intake. Nutrient intake is just one determinant of nutrient status and sometimes – as we have shown in Chapter 4 for vitamin D – only plays a minor role in explaining biomarker concentrations. Also plasma fatty acid concentrations are not necessarily directly related to intake of that particular fatty acid. Most fatty acids can be synthesized by the human body, and only *n*-6 and *n*-3 PUFAs are essential in the diet. Nevertheless, even for these essential PUFAs, blood concentrations of specific fatty acids depend for example also on intake of precursors of the fatty acid and variations in enzyme activities, which in turn depend on several genetic and metabolic factors.[51] As a consequence, results from concentration nutritional biomarkers cannot be directly translated into dietary advice.

In addition to individual nutrients, we also examined dietary patterns in the diets of the 1-year-old children participating in the Generation R Study. An advantage of examining dietary patterns is that the correlations between foods and nutrients in the diet are captured.[28, 52] In doing so, dietary pattern analysis overcomes problems with collinearity among individual foods and nutrients, and takes into consideration the interactions that foods and nutrients may have with respect to their effects on health.[28] Many different approaches have been proposed to construct dietary patterns,[31] of which several have been applied in this thesis. The main distinction between the different approaches is that dietary patterns can be defined *a priori*, for example on the basis of dietary guidelines, or they can be derived *a posteriori*, on the basis of food intake data of the study population.[31]

Advantages of an *a priori*-defined pattern are that they may better reflect a desirable dietary pattern because they are based on guidelines; and that dietary patterns of different populations can be more easily compared. However, disadvantages of *a priori*-defined patterns are that variation between subjects might be small and that there is often scientific debate on what constitutes the healthiest diet.[31, 52] In the development of an *a priori*-defined diet quality score for preschool children we were limited by the scarcity of quantitative dietary guidelines for children in this age group. For *a posteriori*-derived patterns, variation between subjects is larger because dietary data from that study population is used to construct the patterns. Two subtypes of *a posteriori*-derived patterns can be used. Firstly, patterns can be extracted on the basis of food intake data only and independent of the outcome, for example with the use of PCA. These patterns reflect which combinations of food explain the most variation in intake of foods and beverages and reflect actual dietary patterns in the population. Secondly, dietary patterns can be constructed to be outcome-dependent, for example with RRR. These patterns reflect what combinations of foods and beverages explain maximum variation in health markers. In the studies described in this thesis, we were for example interested in which dietary patterns best predicted variation in body composition, and therefore chose FMI and FFMI at the age of 6 years as health markers for the RRR analysis.

For the studies presented in this thesis, repeated nutritional data was not available yet. We could therefore not assess whether associations between early life nutrition and later health are independent of later diet. Recently, dietary intake assessment of the children participating in the Generation R Study at the age of 8 years has been completed. This measurement was followed by

detailed health assessment at the age of 9 years. These data will enable us to examine whether nutrition in early life is associated with later body composition and cardiometabolic health independent of diet in later childhood.

Body composition and cardiometabolic outcome assessment

The primary outcomes in the research presented in this thesis are body composition and cardiometabolic health at the age of 6 years. We used detailed measurements that were taken in the Generation R research center. We measured height and weight to calculate BMI, and in addition we used dual X-ray absorptiometry to measure body fat mass and fat-free mass. Because a certain BMI can encompass a wide range of fat mass in children, BMI is considered to be of limited use to measure adiposity in childhood.[53] Although many previous studies only examined child BMI, we have demonstrated in several studies in this thesis that child BMI can be a misleading measure of adiposity because no distinction is made between fat mass and fat-free mass. For example, a healthier dietary pattern in early childhood was associated with a higher BMI at the age of 6 years, but this difference in BMI was explained by a higher fat-free mass, and not by a higher fat mass (Chapter 5.2). This is important, as a higher relative fat mass has been associated with higher risk of cardiometabolic diseases, whereas a higher relative fat-free mass has been linked to better cardiometabolic health.[54-55]

To assess cardiometabolic health, we measured blood pressure and blood concentrations of several metabolic markers, such as insulin, cholesterol and triacylglycerol. The blood samples were not collected in a fasting state. According to a large study in adults, fasting time has little influence on HDL and LDL cholesterol levels.[56] However, concentrations of triacylglycerol[56] and insulin[57] varied more substantially with differences in fasting time. If we assume that fasting time of the children visiting the research center is randomly distributed and not related to early-life nutrition, this measurement error would have led to non-differential misclassification of these outcomes. This measurement error of the outcome may therefore have resulted in an underestimation of our effect estimates for associations with TAG and insulin concentrations.

In addition to these individual cardiometabolic risk factors, we used a combined cardiometabolic risk factor score to capture overall cardiometabolic health. In line with previous studies in children, we created a continuous score including body fat percentage, blood pressure, HDL cholesterol, triacylglycerol, and insulin concentrations.[58] Advantages of a continuous score over a dichotomous definition of the metabolic syndrome are that it is less prone to error and more sensitive to detect differences because more information is used.[58]

A limitation of our outcome measurements, is that for most outcomes we had measurements at only one point in time. We therefore could not examine whether early-life nutrition was associated with changes of cardiometabolic health over time. Only for BMI we had measurements at multiple points in time throughout childhood. For body composition outcomes, the associations that we observed for protein intake and dietary patterns persisted after additional adjustment for BMI at the time of dietary assessment. However, analyses of protein intake at the age of 1 year with repeatedly measured BMI as outcome showed that the relation between protein intake and BMI becomes smaller over time.[59] This suggests that in studies in which we did not observe an

association with body composition at the age of 6 years, there may have been an effect on body composition earlier, which is no longer visible at 6 years of age. Whether this is still relevant for later health needs further research. On the contrary, certain cardiometabolic risk factors may only become apparent at a later age. Studies with longer follow-up and repeated measurements, including future measurements in the Generation R Study, are crucial to examine associations between early life nutrition and cardiometabolic health over a longer time period. These follow-up studies will also enable us to investigate to what extent these cardiometabolic outcomes track to later adulthood and how they affect the risk of cardiometabolic disease in adulthood.

Confounding

As described earlier in this chapter, the Generation R Study is an observational study. A major limitation of observational studies is the high risk of bias due to confounding. Participants are not randomized to a certain exposure; they are only observed as having a certain exposure or not. Therefore, individuals that differ by exposure may also differ by other characteristics. If these other characteristics are also associated with the outcome, they may be responsible for the observed associations. This is particularly important for lifestyle variables such as dietary intake, because – as we have also reported in Chapter 5.1 – having a healthy diet tends to cluster with other health-conscious behaviors, such as more physical activity, less television watching, and less maternal smoking, and many of these factors are also associated with health. Therefore, these other lifestyle factors and sociodemographic correlates of diet quality are potential confounders in the association of diet with body composition and cardiometabolic health. Also, blood concentrations of nutrients are associated with several other variables. For example, vitamin D concentrations are strongly associated with playing outside, which may also protect against obesity. Playing outside could therefore be a confounder in the associations between vitamin D and obesity.

In addition to confounding by lifestyle variables and sociodemographic factors, there may also be confounding by other dietary factors. Intake of foods and nutrients are correlated with each other and hence may be confounded by each other. For example, a higher intake of animal protein is often related to a higher saturated fat intake, and a higher saturated fat intake has been suggested to adversely affect cardiometabolic health.[60] Saturated fat intake may consequently be a potential confounder in the relation between animal protein intake and cardiometabolic health. Because of these correlations within the diet, it is often difficult to attribute an observed change in a health outcome to the intake of one specific food or nutrient. As described previously, this correlation between foods and nutrients is one of the reasons to perform dietary pattern analysis, which we applied in the studies presented in Chapter 5 of this thesis.

Another approach that we applied to account for correlated nutrient intakes is the use of macronutrient substitution models.[48] Macronutrients, such as protein and fatty acids, provide energy. Because we are often interested in the effect of a macronutrient independent of its energy content, we can adjust for total energy intake. However, when keeping energy intake constant, a higher intake of one macronutrient involves a lower intake of at least one of the other macronutrients. In substitution models, one can examine relative macronutrient intakes by including total energy intake and all macronutrients except for one in the same model. With this

model the effect of increasing one macronutrient can be estimated while keeping energy and the other macronutrients included in the model constant – hence, at the expense of the macronutrient not included in the model. For example, in our analyses on child fat intake in relation to cardiometabolic health we included total energy intake, protein intake and saturated, monounsaturated, and polyunsaturated fat intake in one model, i.e., all macronutrients except for carbohydrate intake. The regression coefficient for PUFA intake can therefore be interpreted as the effect of a one unit increase in PUFA intake at the expense of a similar amount of energy from carbohydrates, because energy, protein, SFA, and MUFA intake are kept constant. Similarly, in a model including energy, protein, carbohydrate, PUFA, and MUFA intake, the coefficient for PUFA intake represents a higher PUFA intake at the expense of SFA. By including different macronutrients in the models, different contrasts can be examined.

In all our studies, we adjusted for several potentially confounding variables. However, residual confounding may still be present, for example because of unmeasured confounders, but also because of measurement error in the assessment of the confounding variables. Although we were able to adjust for many variables in our analyses, some potential confounders were not measured or were not sufficiently measured. For example, we had no detailed information on children's physical activity, but only proxies of physical activity, such as participation in sports and playing outside. Future measurements in the Generation R cohort using accelerometers will enable us to better adjust for confounding by physical activity.

IMPLICATIONS FOR PUBLIC HEALTH AND FUTURE RESEARCH

The results of the research presented in this thesis add to the existing literature on this topic. Combined, they can provide directions for future research and for public health strategies, for which some recommendations will be given in this paragraph. Overall, our results suggest that diet quality and vitamin D status in early childhood are suboptimal. Furthermore, although effect sizes were relatively small, our results show that several aspects of nutrition in early life may be associated with children's body composition and cardiometabolic health. More specifically, we observed that a lower protein intake in early childhood, a fatty acid pattern characterized by high levels of *n*-3 fatty acids in prenatal life, and an overall healthy dietary pattern in early childhood may be beneficial for later body composition and for certain cardiometabolic markers.

Due to the observational design of our study, we cannot establish causality of the observed associations. Nevertheless, for the association between a high protein intake in early childhood and later obesity, a randomized double-blind controlled trial already confirmed that the effect on BMI is causal.[2] However, a higher BMI in childhood does not necessarily reflect adiposity. In our study, we observed that the relation between protein intake and BMI was specifically explained by a higher fat mass, and not a higher fat-free mass (Chapter 2.2). Combined, results from our study and previous trial data[1-3] provide evidence that high protein intake in early childhood may increase the risk of adiposity. Therefore, a lower protein intake during infancy and early childhood should be considered as a strategy to lower adiposity risk in later life. One way to achieve this could be to lower the amount of protein in infant formula and toddler foods. However, a too low protein intake

may limit growth. Future studies should therefore explore what the optimal amounts of protein intake are for infants and young children for optimal growth and later health. Additionally, further studies, including future analyses in the Generation R Study, should examine whether the observed changes in body composition persist into later childhood and adulthood, and if the effects of a high protein intake in early childhood can be reversed with diet in later life. These studies should also examine the potential relation between protein intake and cardiometabolic and kidney health, for which we observed inconsistent associations (Chapters 2.1, 2.3 and 2.4).

In addition to the quantity of protein intake, previous studies and our studies suggest that the quality of protein may also be important. Detrimental effects of protein on obesity may be particularly driven by protein from animal sources. Therefore, studies should explore whether intake of certain types of protein or certain amino acids are specifically associated with adiposity, for example by studying the endocrinal response to different protein subtypes. Results from these analyses can further optimize recommendations regarding adequate intake of not only total protein, but also specific sources or types of protein in early childhood. Finally, although in our analyses we did not find clear differences for effects of protein in different macronutrient substitution models, other studies should further examine whether a lower protein intake is best compensated by a higher carbohydrate intake or by a higher fat intake, and what specific subtypes of carbohydrates or fatty acid.

Although previous research in adults suggests different health effects of different types of fatty acids, we observed no consistent associations for intake of PUFAs, MUFAs, or SFAs on cardiometabolic health among children in the Generation R cohort (Chapter 3.3). Furthermore, we performed a systematic review from which we concluded that the current literature does not support a clear effect of PUFA intake or circulating levels in early life on child obesity or cardiometabolic health (Chapter 3.1). This suggests that fatty acids in early life may not be of large importance for cardiometabolic health. However, results of previous studies were very inconsistent, possibly because there are many different fatty acids which may have distinct effects, or which may interact in their effects on health. Future studies should take these complexities into account. When we explored patterns of fatty acids in pregnant women participating in the Generation R Study, we found that patterns high in *n*-3 fatty acids were associated with a more favorable body composition and blood lipid profile of their children (Chapter 3.2). Other studies should replicate our studies and further examine the role of fatty acids both during pregnancy as well as during early childhood, on body composition and cardiometabolic health of the child. These studies should analyze individual fatty acids but also take account of potential interactions. If these studies confirm our findings that fatty acid profiles characterized by high *n*-3 fatty acids are associated with more favorable child health outcomes, further studies could examine whether these effects could be achieved by changing maternal diet during pregnancy. Concentrations of SFA and MUFA are also influenced by endogenous synthesis, but especially concentrations of circulating *n*-3 fatty acids depend more strongly on changes in dietary intake of *n*-3 fatty acids, for example from fish, nuts, or dietary supplements.

A nutrient for which its blood concentrations are not strongly influenced by diet, is vitamin D. We observed a high prevalence of vitamin D deficiency in 6-year-old children, with almost 30% of

the children having deficient 25(OH)D concentrations (Chapter 4.1). Although in our analyses, vitamin D status was not consistently associated with body composition or cardiometabolic health (Chapters 4.2 and 4.3), it is known from previous studies and from other analyses in the Generation R Study that vitamin D status in childhood is important for other health outcomes, such as bone health.[22, 61] Therefore, it is important to reduce the prevalence of vitamin D deficiency in children.

As expected, we observed that ethnicity was an important determinant of vitamin D deficiency. Of all children with a non-Western ethnic background, over 50% was vitamin D deficient. Vitamin D levels primarily depend on subcutaneous production in response to sun exposure.[22] Differences in vitamin D concentrations of children with different ethnicities might be explained by a higher levels of skin pigmentation, which inhibits cutaneous vitamin D synthesis.[22] In line with this, we also observed that vitamin D deficiency was more common in children who watch television more often, who play outside less often, and who bike to school less often. Hence, our results support the current recommendations to use vitamin D supplementation for children who have a dark skin or who have limited exposure to sunlight.[62] More efforts should be made to improve adherence to these guidelines. Health care providers should be made aware of the high prevalence of vitamin D deficiency among children and should promote spending more time outside. Furthermore, they should more actively advise the use of vitamin D supplements to children with a darker skin type and to children for whom exposure to sufficient sunlight is not possible.

More research is needed to define optimal concentrations of vitamin D. In line with several guidelines, we used a cut-off value of 50 nmol/L to define deficiency. However, several researchers suggest that optimal vitamin D concentrations may be higher.[22] Research should examine whether vitamin D concentrations above 50 nmol/L are related to better health outcomes in children. These studies should not only examine bone health, but also address potential non-skeletal health effects of vitamin D, including effects on body composition and cardiometabolic health. Especially for these non-skeletal outcomes, studies should address causality, for example with the use of large-scale Mendelian Randomization analyses or randomized controlled trials. These studies will provide more information on whether higher vitamin D concentrations improve non-skeletal health, or whether vitamin D status is merely a proxy of being healthy or having a healthy lifestyle, that confounds the observed associations.

Although the current recommendations for vitamin D supplement use for young children are clear, and breastfeeding is actively promoted for the first months of life, recommendations for overall dietary intake of young children after the lactation period are scarce. When we constructed a diet quality score for preschool children, we were limited by the scarcity of dietary guidelines for this age group.[63] More research is needed on what constitutes the optimal diet for preschool children. However, more effort should also be made to summarize the already available scientific evidence into dietary guidelines for this age group. Furthermore, public health initiatives to improve diet quality should start earlier, and also target parents of infants in order to provide advice on appropriate diet for the children after lactation. These recommendations should be particularly targeted at parents whose children are at risk of having a lower diet quality, such as mothers with a low-socioeconomic background, mothers with overweight, mothers who smoke, and mothers with multiple children (Chapter 5.1).[64] It is very important to start early in life, because dietary behaviors

develop already in early childhood and remain relatively stable throughout life.[65] On top of that, diet in early childhood may have a lasting impact on growth and later health.[66-67] We observed for example that dietary patterns at the age of 1 year were associated with body composition and cardiometabolic health at the age of 6 years (Chapters 5.2 and 5.3). Future studies should replicate our analyses in other populations and further examine the optimal dietary pattern in early childhood for health during childhood and for health in later life.

In most of the studies presented in this thesis, we evaluated body composition and cardiometabolic health of children at the age of 6 years. At this young age, trajectories toward health and disease are still developing and deviations from health may not yet be measurable.[68] Therefore, it is important to continue to follow these children to evaluate the associations with cardiometabolic health at a later age. In addition, although previous studies suggested that body composition and cardiometabolic health factors remain stable from childhood to later life, it is important to study whether early life nutrition indeed predicts adult health; and to what extent the effects of adverse dietary exposures during early childhood can be mitigated by a healthier lifestyle in later life. Currently, within the Generation R Study, detailed health outcome measurements are being completed on children around their age of 9 years. In addition, dietary intake data have been collected around the age of 8 years. All these measurements will enable us to study to what extent body composition and cardiometabolic health track and whether nutrition in early life is associated with these outcomes independent of diet in later childhood. Longer follow-up and repeatedly measured exposures and outcomes, in the Generation R Study and in other population-based cohort studies are needed to establish to what extent early life nutrition affects cardiometabolic risk in adulthood.

To conclude, findings from this thesis suggest that nutrition in early childhood is suboptimal and that this may affect body composition and cardiometabolic health. More specifically, we observed that many children were vitamin D deficient and that adherence to dietary guidelines was relatively low. Furthermore, we found that a lower protein intake and a healthy overall dietary pattern in early childhood may be beneficial for body composition and for certain cardiometabolic markers. Although effect sizes were small, our findings may be important for early prevention of obesity and cardiometabolic diseases on a population level. Public health interventions and future scientific research should therefore put more focus on nutrition quality in early childhood and examine its long-term health effects.

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Appendices

Summary

Nutrition is vital for health throughout life, and may be particularly important during early life. In early childhood, dietary behaviors develop that tend to remain stable, but moreover, nutrition during early life may have important consequences for health in adulthood. The origins of several non-communicable disorders, such as cardiovascular diseases and type 2 diabetes, have been linked to nutritional factors in early life. Risk factors for these cardiometabolic disorders, including overweight, high blood pressure, insulin resistance, and dyslipidemia, are already present in childhood. A healthy diet may reduce these risk factors already from early life onward. Several studies, for example, have reported beneficial effects of breastfeeding on later health. However, studies on diet quality of young children shortly after the breastfeeding and weaning period are scarce.

In the research described in this thesis we studied nutrition in early life, particularly in early childhood, and its association with body composition and cardiometabolic health. We studied several nutritional factors that previous studies suggested to be important in early life, but for which research gaps remain. These include protein intake, which has been suggested to be too high in the first years of life; fatty acids, for which the composition in the diet may not be optimal; and vitamin D status, for which a high prevalence of deficiency has been reported. Finally, we evaluated overall diet quality and dietary patterns of 1-year-old children. In order to study the health effects of these nutritional factors, we performed systematic reviews of the literature and we carried out analyses in the Generation R Study, a population-based prospective cohort from fetal life onward in Rotterdam, the Netherlands.

The Generation R Study is designed to identify early determinants of normal and abnormal growth, development, and health over the life course. Child food intake around the age of 1 year was assessed with a 211-item food-frequency questionnaire, which was developed for this age group. When the children were 6 years of age, they were invited to our research center in the Sophia Children's Hospital in Rotterdam where different health outcomes were assessed. We measured the children's height and weight, their fat and fat-free mass, and their blood pressure. In addition, we collected blood samples in which we determined concentrations of triacylglycerol, cholesterol, and insulin.

Protein intake

The studies on child protein intake are described in Chapter 2. First, we showed in a systematic review of the literature that previous studies reported no consistent associations between protein intake and cardiometabolic health in children. Most of these studies were of low methodological quality, for example because they did not adjust for key confounders such as energy intake or other dietary and lifestyle factors. Subsequently, we assessed the association between protein intake at the age of 1 year and body composition, kidney health, and cardiometabolic health at the age of 6 years in children participating in the Generation R Study. In line with a few previous studies, we observed that a higher protein intake in early childhood is associated with a higher body mass index (BMI) in later childhood. In addition, we showed that this association between protein intake and BMI

was explained by a higher fat mass, but not by a difference in fat-free mass index. Additional analyses showed that the associations with BMI and fat mass index were stronger for intake of animal protein than intake of vegetable protein. Furthermore, we observed that the associations with fat mass index were slightly stronger among girls than among boys. These sex differences were also observed when we examined cardiometabolic health outcomes: higher protein intake was associated with higher insulin levels in girls, but with lower triacylglycerol concentrations in boys. We further found that a higher protein intake at the age of 1 year was associated with a lower diastolic blood pressure, but not with kidney size or kidney function at the age of 6 years. Because our study is observational, we cannot establish causality of the observed associations. However, our finding that protein intake at the age of 1 year is associated with a higher body fat mass is consistent with results from a large randomized controlled intervention study, that showed that a lower protein intake during infancy leads to a lower body weight in childhood. Therefore, a lower protein intake during infancy and early childhood, for example via a reduction of protein in infant formula and toddler foods, may be a strategy to decrease later adiposity risk. However, a very low protein intake may limit growth. Future research should therefore explore the optimal range of protein intake and the optimal composition of dietary protein in early childhood for growth and later health.

Fatty acids

In Chapter 3 our research on fatty acids is described. We systematically reviewed the literature on the associations of polyunsaturated fatty acids (PUFAs) – both intake and blood levels – in early life with obesity and with cardiometabolic health in later life. Many of the previous studies were of high methodological quality, however, overall the combined literature provides no consistent evidence for an effect of PUFAs during pregnancy, lactation, or early childhood on later cardiometabolic health. One possibility for the inconsistent findings between studies is that there are many different types of PUFAs, which may have different effects on cardiometabolic health. Also, effects of certain fatty acids may depend on levels of other fatty acids. To take these potential interactions into consideration, we examined fatty acid patterns during pregnancy in the women participating in the Generation R Study. Using principal component analyses, we identified three fatty acid patterns from plasma levels of 22 individual fatty acids. We observed that a high score on a pattern characterized by high concentrations of *n*-3 PUFAs may be beneficial for body composition and metabolic health of the children. Children of women with a higher score on this pattern had a lower fat mass index, a higher fat-free mass index, higher HDL cholesterol concentrations and lower triacylglycerol concentrations at their age of 6 years than children of mothers with a lower score on this pattern. However, when we subsequently examined the associations of dietary intake of fat in early childhood with cardiometabolic health, we observed no consistent association between intake of saturated, monounsaturated or polyunsaturated fatty acids at the age of 1 year with body composition or cardiometabolic health at the age of 6 years. Future studies with detailed fatty acid measurements are needed to further examine the role of fatty acids in early life and potential interactions between these fatty acids on later body composition and cardiometabolic health.

Vitamin D status

In Chapter 4 vitamin D status of the 6-year old children participating in the Generation R Study is described. We observed that almost 30 percent of the examined children was vitamin D deficient, defined as 25-hydroxyvitamin D concentrations below 50 nmol/L. Of the children with a non-Western ethnic background, – including children originating from Cape Verde, Morocco, Indonesia, the Dutch Antilles, Suriname, and Turkey – almost 55 percent were vitamin D deficient. Furthermore, more than 6 percent of all children and almost 20 percent of non-Western children had a severe vitamin D deficiency (concentrations below 25 nmol/L). One of the potential explanations for the higher prevalence of vitamin D deficiency in children with non-Western ethnic backgrounds could be a difference in skin color. Vitamin D concentrations mainly depend on subcutaneous production in response to sunlight, and higher levels of skin pigmentation inhibit vitamin D synthesis. The importance of sun exposure for vitamin D levels was also apparent in other determinants of vitamin D status in our population; the prevalence of vitamin D deficiency was higher in winter than in summer, and vitamin D deficiency was associated with less time spent on playing outside and more time spent on television watching. In subsequent studies, we observed that children with higher vitamin D levels had lower insulin concentrations. Furthermore, we observed that girls, but not boys, with vitamin D deficiency had a higher body fat percentage than those with an optimal vitamin D status. The direction of the potential relation between vitamin D and adiposity is unclear. Future studies should examine whether children with low vitamin D levels have an increased risk of adiposity, if obesity increases the risk of vitamin D deficiency, or if there is no causal relation. Although potential cardiometabolic health effects of vitamin D are not clear yet, we know from previous studies that vitamin D is important for bone development. Therefore, it is important to reduce the prevalence of vitamin D deficiency among children. The Health Council of the Netherlands advises to give vitamin D supplements to all children up to the age of 4 years; and to continue using vitamin D supplements after the age of 4 years if exposure to sufficient sunlight is not possible and for children with a darker skin type. The results of our study support these recommendations.

Dietary patterns

In Chapter 5, we examined dietary patterns of 1-year-old children using different approaches. Firstly, we developed a novel food-based diet score for preschool children on the basis of dietary guidelines. We used this score to rate diet quality of children participating in the Generation R Study. Subsequently, we constructed dietary patterns on the basis of food intake of children in the Generation R Study using principal component analysis; and we identified two patterns that best predicted their body composition at the age of 6 years using reduced rank regression. We observed that children with a higher diet quality score or a higher score on a so-called 'health-conscious' dietary pattern had a higher BMI at the age of 6 years than children with a lower score on these patterns. However, this association was explained by a higher fat-free mass, and not by a difference in fat mass. Higher scores for these two dietary patterns were also associated with a lower cardiometabolic risk factor score, but not clearly with any of the individual cardiometabolic components. Of the two patterns that were developed to predict body composition, one dietary

pattern was associated with a higher fat-free mass index, whereas the other was associated with a higher fat mass index. The pattern that predicted a fat-free mass index, as well as the diet score and the 'health-conscious' pattern, were all characterized by high intake of vegetables, grains, and vegetable oils. The pattern that predicted a higher FMI was characterized by intake of refined grains, potatoes, vegetables, meat, fish, sugar-containing beverages, and soups and sauces. Overall, the results of these studies suggest that dietary patterns of young children are linked to later body composition and cardiometabolic health. In order to prevent obesity and cardiometabolic disease it might therefore be important to improve overall diet already in early childhood.

Lastly, Chapter 6 provides a general discussion of the studies described in this thesis. Overall, findings from this thesis suggest that nutrition in early childhood is suboptimal and that certain nutritional factors may affect body composition and cardiometabolic health. Although the effect estimates were small, they may be relevant on a population level in predicting later cardiometabolic health in later life. In chapter 6 the methodological considerations of our studies, and the implications of our findings for public health policy and future research are addressed.

Samenvatting

Goede voeding is belangrijk voor de gezondheid gedurende het hele leven, maar goede voeding in de vroege kindertijd is mogelijk van extra belang. In deze periode is goede voeding niet alleen cruciaal voor de groei en gezondheid van het kind en voor het aanleren van smaken en gezonde voedingsgewoontes, maar ook voor de gezondheid in het latere leven. Voeding in het vroege leven kan bijvoorbeeld invloed hebben op het ontstaan van latere cardiometabole ziekten, zoals hart- en vaatziekten en diabetes. Verschillende studies hebben aangetoond dat risicofactoren voor deze cardiometabole ziekten al op jonge leeftijd aanwezig zijn. Voorbeelden van deze risicofactoren zijn overgewicht, een hoge bloeddruk, insulineresistentie en hoge cholesterolwaarden. Een gezonde leefstijl kan deze risicofactoren helpen voorkomen en kan daarin al vanaf het vroege leven belangrijk zijn. Er is al veel onderzoek gedaan naar de gezondheidseffecten van borstvoeding en flesvoeding, maar er is nog weinig bekend over de gezondheidseffecten van voeding van jonge kinderen na deze periode.

In dit proefschrift hebben we verschillende aspecten van de voeding van jonge kinderen bestudeerd en gekeken of deze geassocieerd zijn met hun lichaamssamenstelling en met hun cardiometabole gezondheid. De focus lag hierbij op eiwitten, vetzuren, vitamine D en voedingspatronen. Eerdere onderzoeken hebben namelijk gesuggereerd dat verhoogde eiwitinname door jonge kinderen tot overgewicht kan leiden, dat verzadigde vetzuren slecht zijn voor de cardiometabole gezondheid en onverzadigde vetzuren juist goed, en dat een vitamine D tekort vaak voorkomt. Wij hebben dit onderzocht door middel van systematisch literatuuronderzoek en met behulp van gegevens die we hebben verzameld in het Generation R onderzoek, een langlopend bevolkingsonderzoek in Rotterdam.

In het Generation R onderzoek worden de gezondheid, groei en ontwikkeling van kinderen onderzocht vanaf het begin van de zwangerschap. Toen de kinderen ongeveer 1 jaar oud waren, hebben de ouders een uitgebreide voedselfrequentievragenlijst ingevuld over wat hun kinderen eten. Op de leeftijd van 6 jaar werden de kinderen uitgenodigd voor een bezoek aan het onderzoekscentrum van Generation R, waar onder andere hun lengte, gewicht, bloeddruk, vetmassa en vetvrije massa werden gemeten. Ook werd er bloed geprikt waarin we concentraties van cholesterol, triglyceriden en insuline hebben bepaald. Verdere achtergrond van dit proefschrift is beschreven in hoofdstuk 1.

Eiwitinname

In hoofdstuk 2 presenteren we de onderzoeken naar eiwitinname van kinderen. Ten eerste hebben we in een systematische analyse van de voorgaande wetenschappelijke literatuur laten zien dat eerdere onderzoeken geen duidelijk verband hebben aangetoond tussen eiwitinname van kinderen en aspecten van hun cardiometabole gezondheid, zoals bloeddruk, insulinewaarden en cholesterolwaarden in het bloed. We concludeerden echter ook dat de methodologische kwaliteit van de meeste onderzoeken relatief laag was en dat beter onderzoek nodig is. Vervolgens hebben we binnen het Generation R onderzoek de associaties tussen eiwitinname op 1-jarige leeftijd en verschillende gezondheidsuitkomsten op 6-jarige leeftijd bekeken. In overeenstemming met eerder onderzoek zagen we dat kinderen met een hogere eiwitinname op de leeftijd van 1 jaar een hoger

BMI op 6-jarige leeftijd hebben. Bovendien zagen we in ons onderzoek dat dat dit verband tussen eiwitinname en BMI vooral werd verklaard door een hogere vetmassa en niet door een hogere vetvrije massa. Deze associatie tussen eiwit en vetmassa was sterker voor eiwit uit dierlijke voedselbronnen, zoals zuivel en vlees, dan voor eiwit uit plantaardige bronnen, zoals granen en peulvruchten. Daarnaast vonden we dat het verband iets sterker was voor meisjes dan voor jongens. Deze verschillen tussen jongens en meisjes zagen we ook toen we naar cardiometabole factoren keken: een hogere eiwitinname was geassocieerd met hogere insulinewaarden in meisjes, maar met lagere triglyceridewaarden in jongens. Ook zagen we dat een hogere eiwitconsumptie gerelateerd was aan een lagere bloeddruk, maar niet aan niergrootte of nierfunctie op 6-jarige leeftijd. Concluderend ondersteunen onze resultaten eerder bewijs dat een hoge eiwitinname in het eerste levensjaar kan leiden tot een hoger gewicht. Daarom zou een vermindering van de eiwitinname van jonge kinderen een strategie kunnen zijn om overgewicht te voorkomen. Dit zou bijvoorbeeld gerealiseerd kunnen worden door de hoeveelheid eiwit in flesvoeding te verlagen. Te weinig eiwit is echter slecht voor de groei dus vervolgonderzoek is nodig om de optimale hoeveelheid en samenstelling van eiwit in het vroege leven te onderzoeken voor adequate groei en latere gezondheid.

Vetzuren

In hoofdstuk 3 hebben we ons onderzoek naar vetzuren beschreven. We hebben een systematisch literatuuronderzoek gedaan naar de effecten van meervoudig onverzadigde vetzuren tijdens het vroege leven op cardiometabole gezondheid. De meeste voorgaande onderzoeken waren van hoge kwaliteit, maar desondanks was er geen overtuigend bewijs dat de concentraties of inname van deze vetzuren tijdens de zwangerschap, borstvoedingsperiode of de vroege kindertijd een effect hebben op overgewicht of cardiometabole gezondheid van kinderen. Een mogelijke reden voor de inconsistente resultaten is dat er veel verschillende vetzuren zijn. Individuele vetzuren kunnen verschillende effecten hebben en er zouden wisselwerkingen op kunnen treden tussen de verschillende vetzuren in hun effecten op cardiometabole factoren. Om rekening te houden met deze mogelijke wisselwerkingen hebben we in het Generation R onderzoek gekeken naar verschillende vetzuurpatronen tijdens de zwangerschap. Hiervoor hebben we drie verschillende vetzuurpatronen afgeleid bij zwangere vrouwen, op basis van de concentraties van 22 vetzuren in hun bloed. We zagen dat een hoge score op een vetzuurpatroon dat gekenmerkt werd door veel omega-3 vetzuren goed zou kunnen zijn voor de cardiometabole gezondheid van de kinderen. Kinderen van moeders die hoog scoorden op dit omega-3 patroon hadden namelijk een lagere vetmassa en een hogere vetvrije massa, hogere HDL-cholesterolwaarden en lagere triglycerideconcentraties vergeleken met kinderen van moeders met een lagere score op dit patroon. We vonden echter geen duidelijk verband tussen de inname van verzadigde, enkelvoudig onverzadigde, of meervoudig onverzadigde vetzuren inname van de kinderen zelf op 1-jarige leeftijd met hun lichaamssamenstelling of de cardiometabole gezondheid op 6-jarige leeftijd. Toekomstige onderzoeken met gedetailleerde vetzuurmetingen moeten verder onderzoek doen naar de relatie tussen vetzuren in het vroege leven en lichaamssamenstelling en cardiometabole gezondheid in de kindertijd en het latere leven.

Vitamine D status

In hoofdstuk 4 is ons onderzoek beschreven naar de vitamine D status van de 6-jarige kinderen in het Generation R onderzoek. We vonden dat bijna 30 procent van de kinderen een vitamine D tekort had, dat wil zeggen een concentratie onder de referentiewaarde van 50 nmol/L in het bloed. Van de kinderen met een niet-westerse achtergrond, bijvoorbeeld met ouders uit Kaapverdië, Marokko, Indonesië, de Nederlandse Antillen, Suriname en Turkije, had maar liefst 55 procent een vitamine D tekort. Bij ruim zes procent van alle kinderen en bijna 20 procent van de niet-westerse kinderen ging het zelfs om een ernstig vitamine D tekort, met concentraties onder de 25 nmol/L. Het verschil tussen de Nederlandse kinderen en kinderen met een andere etnische afkomst zou kunnen liggen aan een verschil in huidskleur. Het lichaam maakt namelijk zelf vitamine D aan wanneer de huid wordt blootgesteld aan zonlicht, maar dit gaat minder snel bij een getinte of donkere huid. Dat zonlicht een belangrijke bron is, bleek ook uit de verschillen tussen de seizoenen: er werd vaker een vitamine D tekort vastgesteld bij kinderen die in de winter werden onderzocht dan bij kinderen die in de zomer werden onderzocht. Ook leefstijlfactoren waren belangrijk. Kinderen met een vitamine D tekort keken vaker televisie, speelden minder vaak buiten en fietsten minder vaak naar school dan kinderen zonder vitamine D tekort. In vervolgstudies zagen we dat een lager vitamine D gehalte bij de kinderen samenhing met hogere insulinewaarden. Ook zagen we dat meisjes met een vitamine D tekort vaker een hoog lichaamsvetpercentage hadden, maar dit zagen we niet bij jongens. We kunnen echter nog niet concluderen of er een oorzakelijk verband is tussen vitamine D en lichaamsvet en insulinewaarden. We weten bijvoorbeeld niet of een vitamine D tekort de oorzaak is van de ongunstige insulinewaarden en lichaamsvet, of overgewicht mogelijk een vitamine D tekort veroorzaakt, of dat andere factoren een rol spelen in dit verband. Vervolgonderzoek moet dit verder uitwijzen. Wel weten we uit eerder onderzoek dat vitamine D belangrijk is voor een goede botopbouw. Het is dus belangrijk om het vitamine D tekort tegen te gaan. In Nederland adviseert de Gezondheidsraad om aan kinderen tot vier jaar dagelijks 10 microgram extra vitamine D te geven en dit na deze vier jaar te blijven doen bij kinderen met een donkere huid of bij kinderen met onvoldoende blootstelling aan zonlicht. De resultaten van ons onderzoek bevestigen dat dit advies nodig is.

Voedingspatronen

In de tot dusver besproken hoofdstukken hebben we gekeken naar voedingsstoffen in relatie tot gezondheidseffecten. In hoofdstuk 5 hebben we ons gericht op het gehele voedingspatroon van de kinderen op 1-jarige leeftijd. Deze voedingspatronen zijn op verschillende manieren bestudeerd. Eerst hebben we een score ontwikkeld om de kwaliteit van het eetpatroon van peuters volgens de huidige voedingsrichtlijnen te meten. We zagen dat de gemiddelde score van de kinderen niet hoog was en dat de voedingskwaliteit dus niet optimaal was. Vervolgens hebben we geanalyseerd welke voedingspatronen veel voorkwamen bij de kinderen in het Generation R onderzoek en welke voedingspatronen het meest voorspellend waren voor lichaamssamenstelling op 6-jarige leeftijd. We zagen dat kinderen wiens voedingsinname op 1-jarige leeftijd meer in lijn was met de voedingsrichtlijnen of met een 'gezondheidsbewust' eetpatroon, een hogere BMI hadden op 6-jarige leeftijd. In verdere analyses zagen we echter dat deze hogere BMI niet verklaard werd door

een hogere vetmassa, maar door een hogere vetvrije massa, wat mogelijk gunstig is voor de gezondheid. Deze twee voedingspatronen waren ook gerelateerd aan een lagere cardiometabole risicofactor-score, maar niet specifiek met de individuele cardiometabole uitkomsten. We vonden ook twee voedingspatronen die specifiek lichaamssamenstelling op 6-jarige leeftijd voorspelden, waarvan het ene patroon geassocieerd was met een hogere vetvrije massa en het andere met een hogere vetmassa. Het patroon dat voorspellend was voor een hogere vetvrije massa, en ook het 'gezondheidsbewuste' patroon en de score volgende de richtlijnen, werden allemaal gekenmerkt door een hoge inname van granen, groenten en fruit, en plantaardige oliën. Het voedingspatroon dat voorspellend was voor een hoger vetpercentage werd gekenmerkt door een hoge inname van suikerrijke dranken, vlees, aardappels, vis, soepen en sauzen, en geraffineerde graanproducten zoals wit brood. De resultaten van deze studies suggereren dat voedingspatronen op jonge leeftijd al belangrijk kunnen zijn voor latere lichaamssamenstelling en cardiometabole gezondheid. Om obesitas en cardiometabole ziekten te voorkomen kan het dus belangrijk zijn om het voedingspatroon al op jonge leeftijd te verbeteren.

Samenvattend suggereren de resultaten van het onderzoek dat beschreven is in dit proefschrift dat voeding van jonge kinderen niet optimaal is en dat sommige aspecten van voeding in het vroege leven invloed kunnen hebben op lichaamssamenstelling en cardiometabole gezondheid. In hoofdstuk 6 worden een aantal belangrijke methodologische aspecten van het onderzoek bediscussieerd en worden de implicaties van deze bevindingen voor verder volksgezondheidsbeleid en voor vervolgonderzoek besproken.

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Trudy Voortman was born on October 29th 1985, and grew up in Enter, the Netherlands. Because of her interest in the role of nutrition and lifestyle in health, she decided to study nutritional sciences. She completed her Bachelor of Science degree in Nutrition & Health *cum laude* in 2009 at Wageningen University, the Netherlands. At the same university, she continued with a two-year master's program, with a specialization in Molecular Nutrition. As part of this program, she conducted her thesis research at the TNO research institute in Zeist, the Netherlands, where she was involved in the development of an *ex vivo* model to study the release of gastrointestinal hormones. Afterwards, she did a six-month research internship on the regulation of lipolysis at the Department of Nutritional Science & Toxicology at the University of California, Berkeley, USA. She graduated *cum laude* from the Master of Science program in Nutrition & Health in 2011. After her graduation from Wageningen University, she worked at Unilever Research & Development in Vlaardingen, the Netherlands, in the field of blood cholesterol regulation.

In 2012, Trudy began her PhD program at the Department of Epidemiology at Erasmus University Medical Center in Rotterdam. She performed her research within ErasmusAGE, a research center focusing on the role of lifestyle and nutrition in health throughout the life course. The results of these projects are presented in this thesis. In addition to performing research, she is actively involved in supervising students and teaching at Erasmus Medical Center and at other universities. Furthermore, she completed a postgraduate Master of Health Sciences in Epidemiology at the Netherlands Institute of Health Sciences (NIHES). She graduated from this program in 2014 and won the NIHES Award for best master's thesis. In 2015, she worked as a visiting researcher at the Nestlé Research Center in Lausanne, Switzerland, and as a visiting postdoctoral scientist at the Harvard School of Public Health in Boston, Massachusetts, USA.

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16. Verhoeckx KMC, **Voortman T**, Balvers MGJ, Hendriks HF, M Wortelboer H, Witkamp RF. Presence, formation and putative biological activities of N-acyl serotonins, a novel class of fatty-acid derived mediators, in the intestinal tract. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2011; 1811:578–86.

17. Tielemans MJ, Garcia AH, Peralta Santos AP, Bramer WM, Luksa N, Luvizotto MJ, Moreira EM, Topi G, de Jonge E, Visser TL, **Voortman T**, Felix JF, Steegers EAP, Kiefte-de Jong JC, Franco OH. Macronutrient composition and gestational weight gain: a systematic review. *Submitted for publication.*

18. **Voortman T**, Tielemans MJ, Stroobant W, Schoufour JD, Kiefte-de Jong JC, Steenweg-de Graaff J, van den Hooven, Tiemeier H, Jaddoe VWV, Franco OH. Plasma fatty acids patterns during pregnancy and child body composition and cardiometabolic health: the Generation R Study. *Submitted for publication.*

19. Leermakers ETM*, **Voortman T***, Jaddoe VWV, Franco OH, Kiefte-de Jong JC, van den Hooven EH. *A priori* and *a posteriori* dietary patterns in toddlers and cardiometabolic health at age 6 years: The Generation R Study. *Submitted for publication.*

20. Tielemans MJ, **Voortman T***, Schoufour JD*, Tiemeier H, Jaddoe VWV, Hofman A, Franco OH, Kiefte-de Jong JC. Novel circulating fatty acid patterns during pregnancy and gestational weight gain. *Submitted for publication.*

21. **Voortman T,** van den Hooven EH, Vitezova A, Jaddoe VWV, Franco OH. Vitamin D status and cardiometabolic health in childhood: The Generation R Study. *Submitted for publication.*

22. Ars CL, **Voortman T**, Schneider N, El Marroun H, Franco OH, Jaddoe VWV, Tiemeier H, White T. Macronutrient intake in infancy and hippocampal volume and memory performance at age 6: The Generation R Study. *Submitted for publication.*

23. de Barse, LM, Tiemeier H, Leermakers ETM, **Voortman T**, Jaddoe VWV, Edelson L, Franco OH, Jansen PW. Longitudinal association between preschool fussy eating and body composition at 6 years of age. The Generation R Study. *Submitted for publication.*

24. Garcia AH, **Voortman T***, Baena C*, Chowdhurry R, Muka T, Jaspers L, Warnakula S, Tielemans MJ, Troup J, Bramer WM, Franco OH, van den Hooven EH. Maternal weight status and maternal diet as determinants of breastfeeding outcomes and timing of complementary feeding: a systematic review and meta-analysis. *Submitted for publication.*

25. Braun KVE, Erler NS, van den Hooven EH, Kiefte-de Jong JC, Jaddoe VWV, Franco OH, **Voortman T**. Protein intake in early childhood and growth: The Generation R Study. *Submitted for publication.*

26. Miliku K, **Voortman T**, Tiemeier H, Franco OH, Jaddoe VWV. Maternal vitamin D and child kidney outcomes. *Submitted for publication.*

27. Vitezova A*, Muka T*, Zillikens MC, **Voortman T**, Uitterlinden AG, Hofman A, Rivadeneira F, Kiefte-de Jong JC, Franco OH. Vitamin D and body composition in the elderly: The Rotterdam Study *Submitted for publication.*

28. **Voortman T***, Leermakers ETM*, Jaddoe VWV, Hofman A, Franco OH, van den Hooven EH, Kiefte-de Jong JC. *A priori* and *a posteriori* dietary patterns at the age of 1 year and body composition at the age of 6 years: The Generation R Study. *Submitted for publication.*

29. **Voortman T**, Braun KVE, Kiefte-de Jong JC, Hofman A, Jaddoe VWV, Franco OH, van den Hooven EH. Protein intake in early childhood and body composition at the age of 6 years: the Generation R Study. *Submitted for publication.*

30. Tielemans MJ, Steegers EAP*, **Voortman T***, Jaddoe VWV, Hofman A, Rivadeneira F, Franco OH, Kiefte-de Jong JC. Maternal protein intake during pregnancy and childhood body composition at the age of six years. *Submitted for publication.*

31. Tideman JW, Polling JR, **Voortman T**, Tiemeier H, Uitterlinden AG, Jaddoe VWV, Hofman A, Vingerling JR, Franco OH, Klaver CCW. Low serum Vitamin D is associated with axial length and risk of myopia in young children. The Generation R Study. *Submitted for publication.*

32. Vidakovic AJ, Gishti O, **Voortman T**, Tiemeier H, Hofman A, Jaddoe VWV, Gaillard R. Maternal polyunsaturated fatty acid levels during pregnancy and childhood adiposity: The Generation R Study. *Submitted for publication.*

33. Dashti HS, Zuurbier LA, de Jonge EAL, **Voortman T**, Jacques PF, Lamon-Fava S, Scheer FAJL, Kiefte-de Jong JC, Hofman A, Ordovas JM, Franco OH, Tiemeier H. Actigraphic sleep fragmentation, efficiency, and duration associate with dietary intake: the Rotterdam Study. *Submitted for publication.*

34. van den Hooven EH, **Voortman T**, Heijboer AC, Hofman A, Jaddoe VWV, Rivadeneira F, Franco OH. Vitamin D status and bone health in a multiethnic cohort of school-age children: the Generation R Study. *Submitted for publication.*

35. Kocevska D*, **Voortman T***, Ghassabian A, van den Hooven EH, Schneider N, Jaddoe VWV, Hofman A, Tiemeier H, Franco OH. Macronutrient intake in infancy and sleep characteristics in early childhood: The Generation R Study. *Submitted for publication.*

36. Garcia AH, Franco OH, **Voortman T**, de Jonge EAL, Gordillo NG, Jaddoe VWV, Rivadeneira F, van den Hooven EH. Dietary acid load in early childhood and bone health at school age: The Generation R Study. *Submitted for publication.*

37. Tharner A, van den Hooven EH, **Voortman T**, Edelson LR, Hofman A, Jaddoe VWV, Tiemeier H, Franco OH. Fussy eating behavior and vitamin D status in school-aged children. *Submitted for publication.*

38. Muka T, **Voortman T**, Braun KVE, Dhana K, Bramer WM, Troup J, Chowdhury R, Dehghan A, Franco OH. DNA methylation and dyslipidemia: a systematic review. *Submitted for publication.*

39. **Voortman T*,** Mehra R*, Richmond RC, Rivadeneira F, Felix JF, Jaddoe VWV, van den Hooven EH, Franco OH. Vitamin D status and adiposity in a multiethnic cohort of school-aged children: The Generation R Study. *Submitted for publication.*

40. Stroobant W*, Braun KVE*, Kiefte-de Jong JC, Jaddoe VWV, Franco OH, **Voortman T**. Fatty acid intake in early childhood and cardiometabolic health at school age: The Generation R Study. *Submitted for publication.*

** Denotes equal contribution within a manuscript*

PhD portfolio

Summary of PhD training and teaching

PhD student:	Trudy Voortman
Erasmus MC Department:	Epidemiology
Research School:	Netherlands Institute of Health Sciences (NIHES)
PhD period:	May 2012 – November 2015
Promotors:	Prof.dr. O.H. Franco and Prof.dr. V.W.V. Jaddoe
Copromotor:	Dr. E.H. van den Hooven

TRAINING	Year	ECTS credits
Master of Science in Health Sciences, Epidemiology, NIHES		
Study Design (CC01)	2013	4.3
Biostatistical Methods I: Basic Principles (CC02)	2012	5.7
Methodologic Topics in Epidemiologic Research (EP02)	2013	1.4
Biostatistical Methods II: Classical Regression Models (EP03)	2012	4.3
Public Health Research Methods (HS02)	2013	5.7
Principles of Research in Medicine (ESP01)	2012	0.7
Introduction to Global Public Health (ESP41)	2012	0.7
Methods of Public Health Research (ESP11)	2012	0.7
Markers and Prognostic Research (ESP62)	2012	0.7
Primary and Secondary Prevention Research (ESP45)	2013	0.7
Social Epidemiology (ESP61)	2013	0.7
Principles of Genetic Epidemiology (ESP43)	2013	0.7
Health Economics (ESP25)	2014	0.7
History of Epidemiologic Ideas (ESP53)	2014	0.7
Principles of epidemiological data analysis (EWP25)	2013	0.7
Women's Health (EP19)	2014	0.9
Maternal and child health (HS09)	2013	0.9
Causal Inference (ESP48)	2014	0.7
Topics in Meta-analysis (ESP15)	2014	0.7
Causal Mediation Analysis (ESP69)	2014	0.7
General academic skill courses		
Integrity in scientific research, Erasmus MC	2012	2.0
Radiation protection level 5R, Erasmus MC	2012	0.5
Systematic Literature Search, Medical Library, Erasmus MC	2012	0.6
Endnote, Medical Library, Erasmus MC	2012	0.3
Biomedical English Writing and Communication, Erasmus MC	2013	2.0
Project Management, Postdoc Network, Erasmus MC	2015	0.1

Other courses

Exposure assessment in Nutrition Research, Graduate school VLAG, Wageningen University	2012	1.5
Nutrition and physical activity, University of Cambridge	2013	1.4
An introduction to the joint modelling of longitudinal and survival outcomes, with applications in R, Erasmus MC	2013	0.3
Advanced medical writing and editing, NIHES	2014	0.7
European Nutrition Leadership Platform (ENLP) seminar, Luxembourg	2015	3.0

Attended Erasmus MC meetings

Seminars at the department of Epidemiology	2012-2015	1.0
ErasmusAGE research meetings	2012-2015	1.0
Generation R maternal and child health meetings	2013-2015	1.0
2020 Epidemiology meetings	2012-2015	1.0
Generation R research meetings	2012-2015	1.0
Nutritional Epidemiology (SIGN-E) research meetings	2012-2015	1.0
Erasmus MC PhD Days	2012-2015	1.0

Attended conferences, seminars and workshops

Congress VoedingNederland, Nieuwegein	2012	0.3
Congress Nutrimenthe: Nutrition and Cognitive Function, Rotterdam	2012	0.1
ABCD symposium 'Diversity in growth and development of children', Amsterdam	2012	0.3
Discussion meeting Dutch nutrition guidelines, Dutch Health Council (Gezondheidsraad), The Hague	2012	0.1
ENGAGE conference 'From genetic discovery to future health', Rotterdam	2012	0.3
Workshop Nutritional Epidemiology, ErasmusAGE, Rotterdam	2012	0.6
NAV najaarsforum & voorjaarsforum, Utrecht	2012-2015	1.0
DoHAD conference, Rotterdam	2012	0.6
CHARGE investigators meeting, Houston	2012	1.0
Het Nationale Voedingscongres, Ede	2013	0.3
CHARGE investigators meeting, Rotterdam	2013	0.6
Workshop diet scores, Wageningen University, Wageningen	2013	0.3
International Congress of Nutrition (IUNS), Granada	2013	1.0
DRINK symposium, VU University, Amsterdam	2013	0.3
NWO Nutritional Science Days, Deurne	2013-2015	1.0
NAV public lecture 'Gezonde voeding – wie kan ik nog vertrouwen?', Utrecht	2014	0.1
International Conference on Nutrition and Growth, Barcelona	2014	1.0
The Power of Programming, Early Nutrition, Munich	2014	1.0
International Conference of Biomedical Sciences, Tirana	2014	1.0
Voedingscommunicatie anno nu, Maatschappelijk Café Schuttelaar & Partners, The Hague	2014	0.1
Congress Jong geleerd is oud gedaan, FrieslandCampina Institute, Leiden	2014	0.3
Meetings Werkgroep Voedingsgewoonten (WeVo), Utrecht	2014	0.1

ECRIN Nutrition Network Meeting, Paris	2014	0.3
NAV public lecture 'Voedingsonderzoek op een tweesprong: Voedingsstoffen of voedingsmiddelen?', Utrecht	2015	0.1
Seminar on sodium, potassium and blood pressure, Nestlé Research Center, Lausanne	2015	0.1
National Dietary Assessment Reference Database (NDARD) meeting, Wageningen	2015	0.1
Media Training, Voedingscentrum/NAV, The Hague	2015	0.1
European Congress of Epidemiology, Maastricht	2015	1.0
European Nutrition Conference (FENS), Berlin	2015	1.0

Presentations

International Congress of Nutrition, Granada Protein intake & cardiometabolic health in children: a review – poster presentation	2013	0.2
International Conference on Nutrition and Growth, Barcelona A diet quality score for preschool children – poster presentation	2014	0.2
Early Nutrition, Munich Protein intake in early life & cardiometabolic health – oral presentation Protein intake in early life & kidney health – poster with distinction	2014	0.2
Jong geleerd is oud gedaan, Leiden Diet quality in toddlers – invited speaker	2014	0.2
NWO Nutritional Science Days, Deurne Protein intake & body composition – oral presentation	2014	0.2
Promovendis, Amsterdam Effects of diet during pregnancy on child health – invited speaker	2014	0.2
Vitamin D workshop, Delft Vitamin D status & body composition in children – poster presentation	2015	0.2
Wetenschapsdag Nederlands Huisartsengenootschap, Rotterdam The cohort study: a powerful research design – invited speaker	2015	0.2
European Congress of Epidemiology, Maastricht Vitamin D status & cardiometabolic health in children – oral presentation Dietary patterns in early childhood & body composition – poster presentation	2015	0.2
Nutritional Science Days, Heeze Vitamin D status & cardiometabolic health in children – oral presentation	2015	0.2
European Nutrition Conference, Berlin Dietary patterns in early childhood and body composition – poster presentation Vitamin D status & cardiometabolic health in children –oral presentation	2015	0.2

Other

Peer review of articles for scientific journals: *American Journal of Clinical Nutrition, British Journal of Nutrition, BMC Public Health, Journal of Pediatrics, Nutrients, Public Health Nutrition, Appetite, Maternal and Child Nutrition, Journal of Nutrition, American Journal of Epidemiology*	2012-2015	1.0
Seminar committee: organization of weekly seminars at the department of Epidemiology, Erasmus MC	2012-2015	1.0

Board member Young NAV (Nationale Academie van Voedingswetenschappen), Dutch Academy of Nutrition Sciences	2013-2015	1.0
Visiting researcher Nestlé Research Center, Lausanne	2015	1.0
Visiting researcher Harvard School of Public Health, Boston	2015	1.0

TEACHING

Lectures

Lectures 'How to read a scientific article' to 1st year medical students, Erasmus MC	2012-2014	1.0
Lecture 'Conducting a systematic review', ErasmusAGE workshop, Erasmus MC	2013	0.5
Course coordinator and lecturer of the preconference course 'Understanding medical research for health professionals', Tirana	2014	2.0
Lectures on the epidemiological transition and non-communicable diseases in the Global Health minor, Erasmus MC	2014-2015	1.0
Lectures on nutrition & lifestyle in relation to health in the Global Health minor, Erasmus MC	2014-2015	1.0
Lecture on determinants of childhood obesity in the course Maternal and child health, Leiden University College	2015	0.5
Lecture and assignment on maternal and child health in the course Global Public Health, University of Cambridge	2015	0.5
Course coordinator and lecturer of the course Public Health in Low and Middle Income Countries, NIHES	2015	2.0

Supervision of practicals and exercises

Supervising exercises in the course Public Health in Low and Middle Income Countries, NIHES	2012-2014	0.5
Teaching working groups in the course Principals of Research in Medicine, NIHES	2013-2015	2.0
Supervisor SPSS practicals, Biostatistics I, NIHES	2014	0.5
Teaching assistant in the course Fundamentals of Epidemiology, Harvard School of Public Health	2015	2.0

Supervision of students' thesis work

Nellija Luksa, BSc thesis Nutrition and Health, Riga Stradina University, Latvia	2013	1.0
Kim Braun, MSc thesis Nutrition and Health, VU University, Amsterdam	2013	2.0
Kozeta Miliku, MSc thesis Clinical Epidemiology, NIHES, Rotterdam	2013	2.0
Ruchika Mehra, MSc thesis Public Health, NIHES, Rotterdam	2013	2.0
Kim Braun, MSc thesis Epidemiology, NIHES, Rotterdam	2014	2.0
Wendy Stroobant, MSc thesis Nutrition and Health, VU University, Amsterdam	2014	2.0
Audry Garcia, DSc thesis Epidemiology, NIHES, Rotterdam	2015	2.0
Rafaelle van Gijssel, MSc thesis Nutrition and Health, VU University, Amsterdam	2015	2.0
Vincent Jen, research internship Medicine, Erasmus MC, Rotterdam	2015	2.0
Kim Braun, DSc thesis Genetic Epidemiology, NIHES, Rotterdam	2015	2.0
Anh Nhi Nguyen, MSc internship Nutrition and Health, VU University, Amsterdam	2015	2.0