DETERMINANTS OF GROWTH, ADIPOSITY AND BONE MASS IN EARLY LIFE

THE GENERATION R STUDY

Denise H.M. Heppe

The general design of the Generation R Study was made possible by financial support from the Erasmus Medical Center, Rotterdam, the Erasmus University Rotterdam, the Ministry of Health, Welfare and Sport, the Ministry of Youth and Families, the Netherlands Organization of Scientific Research (NWO) and the Netherlands Organization for Health Research and Development (ZonMw).

The work presented in this thesis was conducted in the Generation R Study Group, in close collaboration with the Departments of Epidemiology, Obstetrics and Gynecology and Pediatrics, Erasmus Medical Center, Rotterdam, the Netherlands. Publication of this thesis was supported by the Department of Epidemiology and the Erasmus University Rotterdam, the Netherlands. Additional financial support was kindly provided by Nutricia Research, De Nederlandse Zuivelorganisatie, Chipsoft B.V., AbbVie B.V. and Pfizer B.V.

ISBN:978-94-6169-807-0
Cover photo: Vladimir Zotov, Nizhny Novgorod, Russia
Thesis layout and printing: Optima Grafische Communicatie

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Determinanten van groei, vetzucht en botmassa in het vroege leven
Het Generation R Onderzoek

Proefschrift

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

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
vrijdag 15 januari 2016 om 13.30 uur

door

Denise Helena Maria Heppe
geboren te Vlaardingen

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren	Prof.dr. V.W.V. Jaddoe
	Prof.dr. E.A.P. Steegers
Overige leden	Prof.dr. I.K. Reiss
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CHAPTER 1

GENERAL INTRODUCTION

This thesis focuses on early determinants of fetal growth, and of childhood adiposity and bone development. In this introduction I provide a review of its main context and definitions, and present the general aims and research questions.

FETAL ORIGINS OF ADULT BODY COMPOSITION

The developmental-origins hypothesis suggests that various diseases in adulthood originate from fetal life and early infancy.[1] Exposure to poor nutrition in early life may lead to endocrine and metabolic adaptations to preserve development of key body organs.[2] These adaptations permanently change its physiology and metabolism to enhance survival in short-term, but increase the susceptibility to disease in long-term.[3] This hypothesis has been extensively studied in epidemiologic and animal intervention studies.[4] From these studies, we learned that poor fetal nutrition modulates adult body composition and increases susceptibility to obesity and low bone mineral density, which may lead to metabolic diseases and osteoporosis, respectively.[4] The underlying mechanisms are not yet fully understood.[3] Impaired fetal growth may lead to increased allocation of nutrients to adipose tissue.[5] These alterations may accelerate weight gain during childhood, even more so when growing up in today's obesogenic environment.[4] Similarly, impaired fetal growth may lower bone mineralization in utero and during later life.[6]

Maternal malnutrition during pregnancy appeared not to be the only environmental determinant leading to poor fetal nutrition and altered offspring body composition.[7] Lifestyle-related factors, such as smoking during pregnancy, known to impair placental function and thus fetal nutrient supply, were also suggested to affect offspring body composition.[8] Besides, also maternal diabetes or overweight, indicators of increased fetal nutrient supply, showed to play a role.[7] Since fetal growth did not always mediate the observed associations of early environmental factors with later body composition, several mechanisms, independent of fetal growth, have been proposed. This thesis focuses on identification of early environmental determinants of fetal growth, and of childhood adiposity and bone mass. In particular, it focuses on the role of maternal diet and smoking behavior during pregnancy.

FETAL GROWTH

The fetal period is a crucial phase in the development of human life. It is characterized by rapid growth and continued tissue and organ differentiation.[8] Tissues of the body grow during specific periods of rapid cell division, the so-called critical periods.[9] The timing of critical periods differs across tissues. Fetal growth velocity depends on fetal

oxygen and nutrient availability. Nutrient availability is mainly determined by maternal dietary nutrient intake during pregnancy and the transport of these nutrients across the placenta.[10] Maternal malnutrition affects fetal growth by slowing down the rate of cell division, especially in tissues undergoing a critical period.[8] This reduction in cell division is either direct or indirectly mediated through altered concentrations of growth factors or hormones, in particular, insulin, growth hormone, insulin-like-growth-hormones (IGF), and cortisol, which may also affect growth in later life.

Fetal growth assessment

Most previous studies assessing determinants of fetal growth, such as maternal diet during pregnancy, used birth size as a proxy for fetal development.[11] However, birth weight, a one-time measure, does not reflect the longitudinal trajectory of fetal growth. Besides, different patterns of fetal growth may underlie the same birth weight. Fetal growth restriction in early or late pregnancy may affect different tissues. Also, certain fetal organs essential to immediate survival, like the brain, may be differentially protected.[10] By measuring different fetal growth characteristics across pregnancy, critical periods can be identified for fetal growth and specific organ systems.

Maternal dietary factors and fetal growth

The relationship between maternal diet during pregnancy and fetal growth has been studied in different settings and designs.[12] For example, the consequences of maternal malnutrition largely depend on its timing. Data from the Dutch Hunger Winter of 1944-1945 showed that exposure to famine during late gestation severely affected birth weight, whereas during early gestation it did not.[13] Contrary to exposure during late gestation, exposure during early gestation led to an increased risk of obesity in later life.[14] Evidence relating specific maternal dietary factors to birth size is partly based on randomized supplementation trials in developing countries. The strongest evidence exists for balanced maternal protein-energy supplementation reducing the prevalence of low birth weight and small for gestational age (SGA) births among malnourished women.[15] Maternal iron, calcium and folic acid supplementation may contribute very modestly to a higher mean birth weight, although results are conflicting.[16-19] Supplementation of *n*-3 poly unsaturated fatty acids (PUFA) seems to increase birth weight through its effect on prolonging gestation.[20] Yet, given the highly interrelated nature of supplemented and dietary nutrients, studying single nutrients may provide less valuable information than studying whole foods.[12] Evidence on the effects of dietary products or patterns consumed by Western women on fetal growth mainly comes from observational studies. Dietary pattern studies, also performed within the Generation R Study, showed that pregnant women consuming diets rich in fish, vegetables, fruit, whole grains and (low-fat) dairy had infants with higher birthweight.[12,21] Interestingly, these patterns represent a diet high

in protein, fiber, minerals, *n*-3 PUFA and folate. Studies on individual dietary products have been less conclusive.

As fish is the most common dietary source of *n*-3 PUFA, maternal fish consumption during pregnancy has long been of great interest.[22] Through their influence on eicosanoid synthesis, *n*-3 PUFA have been hypothesized to prolong gestation and enhance fetal growth.[23,24] Although, maternal *n*-3 PUFA supplementation may have benefits, results on maternal fish consumption have been inconsistent. Inconsistent results may arise from the fact that fish not only contain nutrients considered beneficial to fetal growth, but also pollutants that may adversely affect fetal growth.[25] Further assessment of consumption of different fish types may help to separate potential harm from benefits.

Also maternal protein intake from dairy was first attributed growth promoting potential for the unborn child long ago.[26] More recently, an Indian study showed that pregnant women consuming more cow's milk, vegetables and fruit had larger infants at birth.[27] Currently, the potential benefit of maternal cow's milk consumption in promoting fetal growth has been studied and reported by more studies,[28-30] but not all.[31] Cow's milk contains various nutrients potentially beneficial to fetal growth.[32] It has become widely available is relatively cheap. Traditionally, it has been a commonly consumed product in the Netherlands.[33] Therefore, assessing the potential growth promoting effect of maternal milk consumption, and identifying the responsible nutrient, may help to clarify its value for daily practice.

CHILDHOOD ADIPOSITY

Early life influences, beginning with the fetal environment and continuing throughout the first few years of life, shape the trajectory of weight gain and body fatness throughout the life-course.[7] Despite of the growing understanding of the origins of overweight, its prevalence continues to increase across the globe. According to the World Health Organization (WHO), at least 2.8 million people die each year as a result of being overweight or obese, making it the fifth leading risk for global deaths.[34] The worldwide prevalence of overweight and obesity in preschool children increased from 4.2% in 1990 to 6.7% in 2010, a relative increase of 60%.[35] As a result, the global number of overweight children under the age of 5 in 2013 was estimated to be over 42 million.[35] In 2009 in the Netherlands, overweight affected 8.3% and 12.5% of boys and girls under the age of 5.[36]

Adiposity assessment

Adipose tissue consists of adipocytes and connective tissue. Between 14 and 28 gestational weeks, the fetal adipose tissue is generated and the number of fat lobules

determined.[37] Thereafter, an increase in adipose tissue results in enlargement of the lobules (hypertrophy). Yet, in the presence of obesity, the number of fat cells may increase. Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ.[38] By producing hormones, or adipokines, such as leptin, adiponectin, estrogen, resistin, interleukine-6 and TNFa, adipocytes are key in regulating free fatty acid levels, insulin sensitivity, satiety, inflammation and vascular functions. As adipose tissue is involved in so many biological processes its excess in overweight and obese individuals affects almost every organ system.[39] Particularly *visceral fat*, including the mesenteric, omental, perirenal, retroperitoneal, and pericardial fat stores, rather than *subcutaneous fat*, primarily located on the buttocks, thighs, and abdomen, is associated with disease like insulin resistance, hyperglycemia, dyslipidemia, hypertension, and prothrombotic and proinflammatory states.[40]

Obesity or overweight is generally defined based on a person's body size using the body mass index (BMI). BMI, a person's weight divided by its squared height, is a globally applied, simple and inexpensive tool to estimate adiposity at individual or population level.[41] However, as BMI does not differentiate fat from fat-free mass, BMI was found to be an inaccurate measure of adiposity among children who are not severely overweight.[42] Moreover, BMI does not separate subcutaneous from visceral fat, the strongest predictor of disadvantageous health outcomes.[40] Despite the disadvantages, BMI has been widely used in studies assessing risk factors of childhood adiposity.[42] In contrast to BMI, dual-energy X-ray absorptiometry (DXA), which uses a very low dose of X-ray, can estimate a person's actual fat mass.[43] DXA has also been shown to accurately measure fat mass in pediatric populations.[44] Furthermore, ultrasound has been validated against MRI, a very accurate, but expensive and invasive method, to measure visceral and subcutaneous abdominal fat mass. Use of DXA and ultrasound may help to further elucidate risk factors of childhood overweight.

Early determinants of childhood adiposity

Changes in the early life environment during critical periods of the developing fetus or infant could have irreversible, lifelong consequences. Childhood overweight not only tends to track into adulthood, but overweight adults who were overweight in childhood are also at higher risk of metabolic syndrome than overweight adults who were normal weight as children.[45] Therefore, prevention should focus on risk factors for overweight from early life onwards.[46] Numerous studies have assessed individual fetal and infant risk factors for early overweight.[47] Although results have not always been conclusive, frequently reported maternal risk factors are related to reduced or excessive fetal nutrient supply; maternal smoking during pregnancy, gestational diabetes, high BMI, and excessive gestational weight gain, or low socio-economic status.[48-53] Rapid weight gain during infancy was a frequently reported postnatal risk factor for preschool overweight.[49-54]

Results were inconsistent on the influence of breast feeding and birth size.[50,51,54] As overweight is a complex multifactorial trait, the development of childhood overweight results from an interaction between (epi)genetic, behavioral and environmental risk factors.[55] Although these risk factors operate at different times during the life-course, they have additive or synergistic effects and must be considered together when trying to estimate the impact of these risk factors in the development of overweight.[49] Few studies have considered the relative impact of these individual risk factors by simultaneous assessment.[49-54] None of these have considered the effect of fetal growth. Simultaneous assessment of risk factors, including fetal growth, may further clarify which are most important to overweight development.

CHILDHOOD BONE MASS

Early life influences during fetal life and infancy may not only shape fat mass of the body in later life, but have also shown to play a pivotal role in bone mass acquisition throughout the life-course.[8] Bone mass acquisition is known to start differing between individuals as early as infancy and tracks thereafter according to the influence of hereditable and environmental factors.[56] The risk of developing osteoporosis in later life is largely determined by the peak bone mass achieved during childhood and adolescence.[57] Osteoporosis is characterized by bone fragility and susceptibility to fracture,[58] and therefore is a major cause of morbidity and mortality.[59] According to the International Osteoporosis Foundation (IOF), osteoporosis affects more than 27.5 million people in Europe; 22% of postmenopausal women, leading to 3.5 million fractures and costing €37 billion yearly.[60] With an ageing population these numbers are expected to increase. In Rotterdam, the Netherlands, the study area of the studies included in this thesis, the age-adjusted prevalence of osteoporosis among elderly was 12% for men and 29% for women (mean age 68 years).[61]

Bone mass assessment

Bone is a complex tissue with a number of mechanical and physiological functions.[62] The skeleton protects vital organs and provides structural support for the body. Bone also serve as a mineral reservoir for calcium homeostasis, a reservoir of growth factors and cytokines, and takes part in the control of acid–base balance.[62] There are two main types of bone: the *cortical bone*, which forms the dense and solid outer surface of most bones, and the *trabecular bone,* which forms the inner network of spongy bone. The fetal skeleton develops through intramembranous (skull and facial bones) and endochondral ossification.[8] The latter involves ossification of cartilage by osteoblast to create the long bones. In total, the fetus accumulates approximately 30 g of calcium, the majority during

the third trimester.[8] In children, bone growth takes place inside the bone at trabecular and endocortical surfaces, making it denser, as well as outside the bone at the growth plates and periosteum, making it longer and wider.[62] Throughout life bone undergoes remodeling, which takes place inside the bone on the trabecular surface. It comprises the process of bone resorption and subsequent formation. In children, bone formation exceeds bone resorption leading to bone mass acquisition, whereas in adults bone resorption exceeds bone formation leading to net bone loss. By far the most common and convenient method to measure an individual's current bone mass, or bone mineral density, is DXA.[63] A more precise, but expensive method would be quantitative computed tomography (QCT), a low dose CT scan.[63] It differentiates trabecular from cortical bone mass. Nowadays, peripheral QCT (pQCT) is becoming more widely used in pediatric research, however, at the time it was not available yet in the Generation R Study.

Early determinants of childhood bone mass

The rapid rate of mineral gain during fetal life and infancy, along with the plasticity of the skeleton, makes it especially at this stage susceptible to interactions with environmental influences.[64] Infant and childhood growth measures, obtained from birth and school health records, were associated with adult bone mineral content, but not density.[65,66] Not only postnatal growth but also fetal growth, leading to a low birth weight were found to lead to lower bone mass accrual during childhood and lower peak bone mass attainment.[67] Whether this effect was to be attributed specifically to fetal growth or growth during infancy or childhood, or both, remained uncertain. Studies actually measuring fetal growth, instead of using birth weight as a proxy, are scarce.[68] Besides, identification of a critical period of growth is challenged by the correlation between repeatedly measured growth measures.[69] In fact, "early size" adjusted for "later size" in regression analysis is a measure of change in size, rather than a measure of absolute growth.[69] To handle this issue *conditional change modelling* has been used previously.[68] It aims to distinguish an actual change in growth velocity from an increase or decrease in growth that was expected based on prior growth. Using this technique to simultaneously assess repeatedly measured growth measures may help to establish the period of growth most critical to bone development.

As fetal growth largely depends on nutrient availability, also the maternal diet during pregnancy has been assessed in relation to bone mass acquisition during later life.[70-73] Previous results from birth cohort studies suggested that maternal intake of certain minerals was positively associated with bone mass in their offspring, and that vitamins involved in homocysteine metabolism may have potential benefit. [70-73] Also, maternal smoking during pregnancy, known to lead to lower calcium absorption,[74] impaired placental function[75] and low birth weight,[76] may impair fetal bone formation. However, results of studies assessing the relation between maternal smoking during pregnancy and offspring

bone mass acquisition have been less consistent.[77-79] Smoking is highly correlated with socio-demographic and lifestyle-related factors. Therefore, previously described associations may have been subject to residual confounding. A comparative analysis of both exposure to maternal and paternal smoking may help to disentangle intrauterine from confounding factors.[80]

AIMS

The overall aim of this thesis is to identify early determinants of fetal growth and childhood adiposity and bone mass, mainly by focusing on the potential role of (sub)optimal fetal nutrient supply.

The specific aims were to study:
1. Maternal dietary factors in relation to fetal growth and neonatal complications.
2. Parental, fetal and infant determinants of childhood adiposity.
3. Parental, fetal and infant determinants of childhood bone mass.

STUDY DESIGN

The studies presented in this thesis are embedded in The Generation R Study, a population-based prospective cohort study from fetal life onwards. As previously described in detail, the Generation R Study was designed to identify early environmental and genetic causes of normal and abnormal growth, development and health during fetal life, childhood and adulthood.[81] In short, all mothers who lived in Rotterdam and had an expected delivery date between April 2002 and January 2006 were eligible. Enrolment was aimed in the first trimester of pregnancy, but was allowed until birth of their child. Also, partners were invited to participate. As shown in **Figure 1.1**, 9,778 mothers enrolled in the study, of whom 8,880 (91%) during pregnancy, and 6,347 (71%) fathers. During pregnancy, visits were planned in early pregnancy (gestational age<18 weeks), mid-pregnancy (gestational age 18–25 weeks) and late pregnancy (gestational age >25 weeks) in two dedicated research centers. Before each visit mothers were asked to fill out postal questionnaires. The food frequency questionnaire was send out in early pregnancy, or as soon as possible thereafter. Physical and fetal ultrasound examinations were carried out and blood samples collected during each visit. Fathers participated in one postal questionnaire and one physical examination.

Of all mothers enrolled in the study, 9,749 live born children were eligible for follow-up in the preschool period, which in the Netherlands refers to 0 to 4 years of age. Due

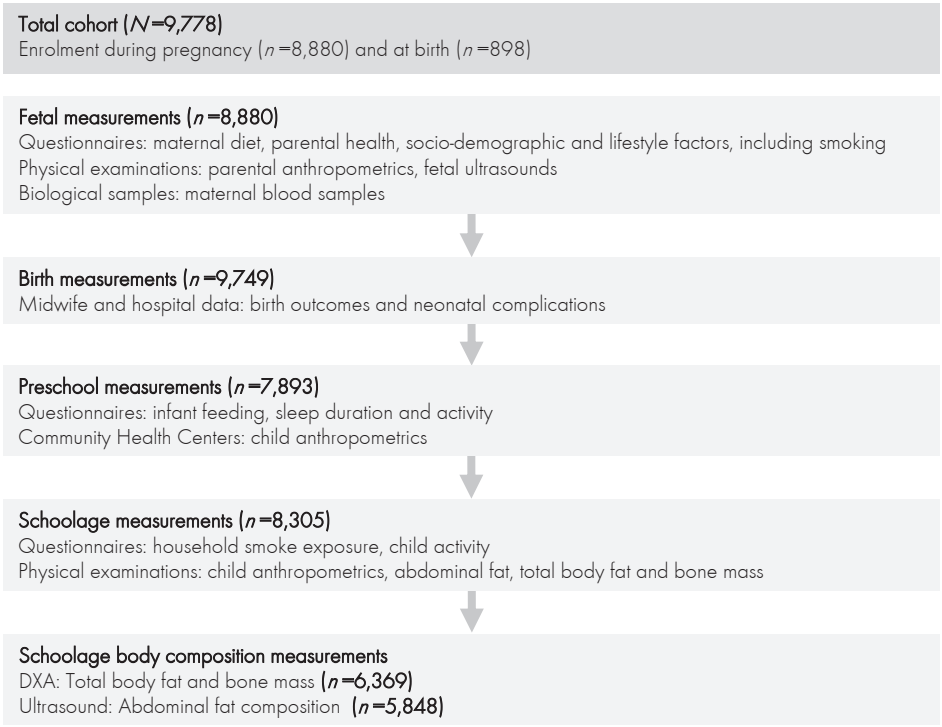

Figure 1.1 Design and data collection in the Generation R Study

to logistical constraints, data collection was restricted to the Northern part of Rotterdam. As a result, 1,166 children living in the South of Rotterdam, were not approached for follow-up. Of the remaining 8,583 children, 7,893 (81% of the original study population) children were eligible following their parents' consent. During the preschool period parents were regularly asked to fill out postal questionnaires. Childhood growth was routinely measured at the Community Health Centers at the ages of 6, 11, 24, 36 and 46 months. Regardless of their participation in the preschool period, all children were invited to visit a dedicated research center in the Erasmus Medical Center—Sophia Children's Hospital around the age of 6 years. Again, their parents were asked to fill out questionnaires. Of the 8,305 (85% of the original study population) children eligible for follow-up, 6,690 children visited the research center and underwent growth measurements, an ultrasound to measure abdominal fat mass, and a DXA scan to estimate total body fat mass and bone mass.

OUTLINE

This thesis describes results of studies performed to identify early determinants of fetal growth, childhood adiposity and bone mass. In **Chapter 2,** two studies on maternal dietary factors and their influence on fetal growth are presented. First, **Chapter 2.1** describes the associations of maternal fish consumption during pregnancy with fetal growth and the risk of adverse birth outcomes. In **Chapter 2.2**, the relation of maternal milk consumption with repeatedly measured fetal growth characteristics and adverse birth outcomes were evaluated. **Chapter 3** presents studies focused on the potential role of various maternal, paternal, fetal and infant determinants of childhood adiposity. In **Chapter 3.1**, risk factors for preschool overweight were identified by simultaneous analysis of 34 putative maternal, paternal, fetal or infant risk factors. In more detail, the associations of parental smoking during pregnancy with childhood fat distribution were examined in **Chapter 3.2**. **Chapter 4** focuses on potential maternal, paternal, fetal and infant determinants of healthy bone mass development. In **Chapter 4.1**, repeatedly measured fetal and childhood growth measures were simultaneously examined in order to identify the period of growth most critical to childhood bone mass. In **Chapter 4.2**, associations of maternal macro- and micronutrient intake and levels of folate, vitamin B12 and homocysteine with childhood bone mass were studied. Furthermore, **Chapter 4.3** shows the associations of parental smoking during and after pregnancy with childhood bone mass. Finally, **Chapter 5** provides an overall discussion of the main findings of this thesis in the context of previous studies on early determinants of fetal growth and childhood body composition. It includes methodological considerations and suggestions for underlying mechanisms, directions for future research and clinical implications.

REFERENCES

1. Barker DJ. The fetal and infant origins of adult disease. *BMJ*. 1990,301(6761):1111.
2. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. 1992,35(7):595-601.
3. Hales CN, Barker DJ. The thrifty phenotype hypothesis. *Br Med Bull*. 2001,60:5-20.
4. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*. 2008,359(1):61-73.
5. Barker DJ, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Trajectories of growth among children who have coronary events as adults. *N Engl J Med*. 2005,353(17):1802-9.
6. Devlin MJ, Bouxsein ML. Influence of pre- and peri-natal nutrition on skeletal acquisition and maintenance. *Bone*. 2012,50(2):444-51.
7. Fall CH. Evidence for the intra-uterine programming of adiposity in later life. *Ann Hum Biol*. 2011,38(4):410-28.
8. Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Review: developmental origins of osteoporotic fracture. *Osteoporos Int*. 2006,17(3):337-47.
9. Barker DJ. Fetal origins of coronary heart disease. *BMJ*. 1995,311(6998):171-4.
10. Barker DJ. The malnourished baby and infant. *Br Med Bull*. 2001,60:69-88.
11. Bergmann RL, Bergmann KE, Dudenhausen JW. Undernutrition and growth restriction in pregnancy. *Nestle Nutrition workshop series Paediatric programme*. 2008,61:103-21.
12. Grieger JA, Clifton VL. A review of the impact of dietary intakes in human pregnancy on infant birthweight. *Nutrients*. 2015,7(1):153-78.
13. Stein AD, Ravelli AC, Lumey LH. Famine, third-trimester pregnancy weight gain, and intrauterine growth: the Dutch Famine Birth Cohort Study. *Human biology*. 1995,67(1):135-50.
14. Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: an overview. *Reproductive toxicology*. 2005,20(3):345-52.
15. Imdad A, Bhutta ZA. Maternal nutrition and birth outcomes: effect of balanced protein-energy supplementation. *Paediatr Perinat Epidemiol*. 2012,26 Suppl 1:178-90.
16. Haider BA, Olofin I, Wang M, Spiegelman D, Ezzati M, Fawzi WW, *et al*. Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis. *BMJ*. 2013,346:f3443.
17. Pena-Rosas JP, De-Regil LM, Dowswell T, Viteri FE. Daily oral iron supplementation during pregnancy. *The Cochrane database of systematic reviews*. 2012,12:CD004736.
18. Buppasiri P, Lumbiganon P, Thinkhamrop J, Ngamjarus C, Laopaiboon M, Medley N. Calcium supplementation (other than for preventing or treating hypertension) for improving pregnancy and infant outcomes. *The Cochrane database of systematic reviews*. 2015,2:CD007079.
19. Lassi ZS, Salam RA, Haider BA, Bhutta ZA. Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. *The Cochrane database of systematic reviews*. 2013,3: CD006896.
20. Makrides M, Collins CT, Gibson RA. Impact of fatty acid status on growth and neurobehavioural development in humans. *Matern Child Nutr*. 2011,7 Suppl 2:80-8.
21. Timmermans S, Steegers-Theunissen RP, Vujkovic M, den Breeijen H, Russcher H, Lindemans J, *et al*. The Mediterranean diet and fetal size parameters: the Generation R Study. *Br J Nutr*. 2012,108(8):1399-409.
22. Olsen SF, Joensen HD. High liveborn birth weights in the Faroes: a comparison between birth weights in the Faroes and in Denmark. *J Epidemiol Community Health*. 1985,39(1):27-32.
23. Olsen SF, Hansen HS, Sorensen TI, Jensen B, Secher NJ, Sommer S, *et al*. Intake of marine fat, rich in (n-3)-polyunsaturated fatty acids, may increase birthweight by prolonging gestation. *Lancet*. 1986,2(8503):367-9.

24. Andersen HJ, Andersen LF, Fuchs AR. Diet, pre-eclampsia, and intrauterine growth retardation. *Lancet*. 1989,1(8647):1146.
25. Mahaffey KR. Fish and shellfish as dietary sources of methylmercury and the omega-3 fatty acids, eicosahexaenoic acid and docosahexaenoic acid: risks and benefits. *Environ Res*. 2004,95(3): 414-28.
26. Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *BMJ*. 1996,312(7028):410-4.
27. Rao S, Yajnik CS, Kanade A, Fall CH, Margetts BM, Jackson AA, *et al*. Intake of micronutrient-rich foods in rural Indian mothers is associated with the size of their babies at birth: Pune Maternal Nutrition Study. *J Nutr*. 2001,131(4):1217-24.
28. Ludvigsson JF, Ludvigsson J. Milk consumption during pregnancy and infant birthweight. *Acta Paediatr*. 2004,93(11):1474-8.
29. Mannion CA, Gray-Donald K, Koski KG. Association of low intake of milk and vitamin D during pregnancy with decreased birth weight. *CMAJ*. 2006,174(9):1273-7.
30. Olsen SF, Halldorsson TI, Willett WC, Knudsen VK, Gillman MW, Mikkelsen TB, *et al*. Milk consumption during pregnancy is associated with increased infant size at birth: prospective cohort study. *Am J Clin Nutr*. 2007,86(4):1104-10.
31. Mitchell EA, Robinson E, Clark PM, Becroft DM, Glavish N, Pattison NS, *et al*. Maternal nutritional risk factors for small for gestational age babies in a developed country: a case-control study. *Archives of disease in childhood Fetal and neonatal edition*. 2004,89(5):F431-5.
32. Nevo: Dutch food composition database 2006 [Internet]. Netherlands Nutrition Centre, The Hague, The Netherlands. 2006.
33. Voedingscentrum. Zo eten jongvolwassenen in Nederland. Resultaten van de Voedselconsumptiepeiling 2003. . Den Haag, The Netherlands: 2011.
34. Organization WH. World Health Statistics 2010. Geneva2010.
35. de Onis M, Blossner M, Borghi E. Global prevalence and trends of overweight and obesity among preschool children. *Am J Clin Nutr*. 2010,92(5):1257-64.
36. Schonbeck Y, Talma H, van Dommelen P, Bakker B, Buitendijk SE, Hirasing RA, *et al*. Increase in prevalence of overweight in Dutch children and adolescents: a comparison of nationwide growth studies in 1980, 1997 and 2009. *PLoS One*. 2011,6(11):e27608.
37. Stupin JH, Arabin B. Overweight and Obesity before, during and after Pregnancy: Part 1: Pathophysiology, Molecular Biology and Epigenetic Consequences. *Geburtshilfe und Frauenheilkunde*. 2014,74(7):639-45.
38. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*. 2004,89(6): 2548-56.
39. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology*. 2004,145(5):2273-82.
40. Grundy SM, Hansen B, Smith SC, Jr., Cleeman JI, Kahn RA, American Heart A, *et al*. Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association conference on scientific issues related to management. *Circulation*. 2004,109(4):551-6.
41. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ*. 2000,320(7244):1240-3.
42. Freedman DS, Sherry B. The validity of BMI as an indicator of body fatness and risk among children. *Pediatrics*. 2009,124 Suppl 1:S23-34.
43. Kaul S, Rothney MP, Peters DM, Wacker WK, Davis CE, Shapiro MD, *et al*. Dual-energy X-ray absorptiometry for quantification of visceral fat. *Obesity*. 2012,20(6):1313-8.
44. Helba M, Binkovitz LA. Pediatric body composition analysis with dual-energy X-ray absorptiometry. *Pediatr Radiol*. 2009,39(7):647-56.

45. Gunnell DJ, Frankel SJ, Nanchahal K, Peters TJ, Davey Smith G. Childhood obesity and adult cardiovascular mortality: a 57-y follow-up study based on the Boyd Orr cohort. *Am J Clin Nutr*. 1998,67(6):1111-8.
46. Bayer O, Kruger H, von Kries R, Toschke AM. Factors Associated With Tracking of BMI: A Meta-Regression Analysis on BMI Tracking. *Obesity*. 2010.
47. Monasta L, Batty GD, Cattaneo A, Lutje V, Ronfani L, Van Lenthe FJ, *et al*. Early-life determinants of overweight and obesity: a review of systematic reviews. *Obes Rev*. 2010,11(10):695-708.
48. Oken E, Levitan EB, Gillman MW. Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J Obes (Lond)*. 2008,32(2):201-10.
49. Gillman MW, Rifas-Shiman SL, Kleinman K, Oken E, Rich-Edwards JW, Taveras EM. Developmental origins of childhood overweight: potential public health impact. *Obesity*. 2008,16(7):1651-6.
50. Beyerlein A, Toschke AM, von Kries R. Risk factors for childhood overweight: shift of the mean body mass index and shift of the upper percentiles: results from a cross-sectional study. *Int J Obes (Lond)*. 2010,34(4):642-8.
51. Kitsantas P, Gaffney KF. Risk profiles for overweight/obesity among preschoolers. *Early Hum Dev*. 2010,86(9):563-8.
52. Huus K, Ludvigsson JF, Enskar K, Ludvigsson J. Risk factors in childhood obesity-findings from the All Babies In Southeast Sweden (ABIS) cohort. *Acta Paediatr*. 2007,96(9):1321-5.
53. Dubois L, Girard M. Early determinants of overweight at 4.5 years in a population-based longitudinal study. *Int J Obes (Lond)*. 2006,30(4):610-7.
54. Jouret B, Ahluwalia N, Cristini C, Dupuy M, Negre-Pages L, Grandjean H, *et al*. Factors associated with overweight in preschool-age children in southwestern France. *Am J Clin Nutr*. 2007,85(6):1643-9.
55. Han JC, Lawlor DA, Kimm SY. Childhood obesity. *Lancet*. 2010,375(9727):1737-48.
56. Cowell CT, Tao C. Nature or nurture: determinants of peak bone mass in females. *Journal of pediatric endocrinology & metabolism : JPEM*. 2002,15 Suppl 5:1387-93.
57. Winsloe C, Earl S, Dennison EM, Cooper C, Harvey NC. Early life factors in the pathogenesis of osteoporosis. *Curr Osteoporos Rep*. 2009,7(4):140-4.
58. Kanis JA, Melton LJ, 3rd, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res*. 1994,9(8):1137-41.
59. Center JR, Nguyen TV, Schneider D, Sambrook PN, Eisman JA. Mortality after all major types of osteoporotic fracture in men and women: an observational study. *Lancet*. 1999,353(9156):878-82.
60. Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, *et al*. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Archives of osteoporosis*. 2013,8(1-2):136.
61. Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, *et al*. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. *Bone*. 2004,34(1):195-202.
62. Clarke B. Normal bone anatomy and physiology. *Clinical journal of the American Society of Nephrology : CJASN*. 2008,3 Suppl 3:S131-9.
63. Leonard MB, Shults J, Elliott DM, Stallings VA, Zemel BS. Interpretation of whole body dual energy X-ray absorptiometry measures in children: comparison with peripheral quantitative computed tomography. *Bone*. 2004,34(6):1044-52.
64. Cooper C, Westlake S, Harvey N, Dennison E. Developmental origins of osteoporotic fracture. *Adv Exp Med Biol*. 2009,639:217-36.

65. Baird J, Kurshid MA, Kim M, Harvey N, Dennison E, Cooper C. Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis. *Osteoporos Int.* 2011,22(5): 1323-34.
66. Schlussel MM, Dos Santos Vaz J, Kac G. Birth weight and adult bone mass: a systematic literature review. *Osteoporos Int.* 2010,21(12):1981-91.
67. Steer CD, Tobias JH. Insights into the programming of bone development from the Avon Longitudinal Study of Parents and Children (ALSPAC). *Am J Clin Nutr.* 2011,94(6):1861S-4S.
68. Harvey NC, Mahon PA, Kim M, Cole ZA, Robinson SM, Javaid K, *et al.* Intrauterine growth and postnatal skeletal development: findings from the Southampton Women's Survey. *Paediatr Perinat Epidemiol.* 2012,26(1):34-44.
69. Lucas A, Fewtrell MS, Cole TJ. Fetal origins of adult disease-the hypothesis revisited. *BMJ.* 1999,319(7204):245-9.
70. Ganpule A, Yajnik CS, Fall CH, Rao S, Fisher DJ, Kanade A, *et al.* Bone mass in Indian children--relationships to maternal nutritional status and diet during pregnancy: the Pune Maternal Nutrition Study. *J Clin Endocrinol Metab.* 2006,91(8):2994-3001.
71. Tobias JH, Steer CD, Emmett PM, Tonkin RJ, Cooper C, Ness AR, *et al.* Bone mass in childhood is related to maternal diet in pregnancy. *Osteoporos Int.* 2005,16(12):1731-41.
72. Yin J, Dwyer T, Riley M, Cochrane J, Jones G. The association between maternal diet during pregnancy and bone mass of the children at age 16. *Eur J Clin Nutr.* 2010,64(2):131-7.
73. Jones G, Riley MD, Dwyer T. Maternal diet during pregnancy is associated with bone mineral density in children: a longitudinal study. *Eur J Clin Nutr.* 2000,54(10):749-56.
74. Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. *Clinical science.* 2007,113(5):233-41.
75. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev.* 2007,83(11):699-706.
76. Jaddoe VW, Verburg BO, de Ridder MA, Hofman A, Mackenbach JP, Moll HA, *et al.* Maternal smoking and fetal growth characteristics in different periods of pregnancy: the generation R study. *American journal of epidemiology.* 2007,165(10):1207-15.
77. Jones G, Hynes KL, Dwyer T. The association between breastfeeding, maternal smoking in utero, and birth weight with bone mass and fractures in adolescents: a 16-year longitudinal study. *Osteoporos Int.* 2012.
78. Jones G, Riley M, Dwyer T. Maternal smoking during pregnancy, growth, and bone mass in prepubertal children. *J Bone Miner Res.* 1999,14(1):146-51.
79. Macdonald-Wallis C, Tobias JH, Davey Smith G, Lawlor DA. Parental smoking during pregnancy and offspring bone mass at age 10 years: findings from a prospective birth cohort. *Osteoporos Int.* 2011,22(6):1809-19.
80. Smith GD. Assessing intrauterine influences on offspring health outcomes: can epidemiological studies yield robust findings? *Basic Clin Pharmacol Toxicol.* 2008,102(2):245-56.
81. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, *et al.* The Generation R Study: design and cohort update 2012. *Eur J Epidemiol.* 2012,27(9): 739-56.

CHAPTER 2

MATERNAL DIETARY FACTORS DURING PREGNANCY AND FETAL GROWTH

CHAPTER 2.1

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Br J Nutr. 2011 Mar;105(6):938-49.

MATERNAL FISH CONSUMPTION, FETAL GROWTH AND THE RISK OF NEONATAL COMPLICATIONS

ABSTRACT

Maternal fish consumption during pregnancy has been suggested to affect birth outcomes. Previous studies mainly focused on birth outcomes and did not study fetal growth during pregnancy. In a prospective cohort study from early pregnancy onwards in the Netherlands, we assessed the associations of first trimester maternal total, lean, fatty and shellfish consumption with fetal growth characteristics in second and third trimester, growth characteristics at birth and the risks of neonatal complications, including preterm birth, low birth weight and small for gestational age. In total, 3,380 mothers completed a 293-items semi-quantitative food frequency questionnaire to obtain information about fish consumption during the first trimester of pregnancy. Head circumference, femur length and fetal weight were estimated in the second and third trimester by ultrasound. Information about birth anthropometrics and neonatal complications was available from hospital and midwife registries. Maternal older age, higher educational level, folic acid supplement use, alcohol use and not smoking were associated with higher fish consumption ($P<0.01$). After adjustment, we observed no consistent associations of maternal total fish consumption or specific consumption of lean fish, fatty fish or shellfish with fetal growth characteristics in the second and third trimester, and at birth. Likewise, total fish consumption or specific consumption of any type of fish was not consistently associated with the risks of neonatal complications. These findings suggest that in a population with a relatively low fish intake, consumption of lean, fatty or shellfish in first trimester is not associated with fetal growth or the risks of neonatal complications.

INTRODUCTION

Maternal fish consumption during pregnancy has been suggested to affect pregnancy and birth outcomes.[1-4] Fish contains various nutrients considered to be beneficial for fetal growth and development, including polyunsaturated *n*-3 fatty acids, protein, selenium, iodine and vitamin D.[5-7] In particular, *n*-3 fatty acid docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been associated with higher birth weight in both randomized controlled trials and observational studies.[8-9] *N*-3 fatty acids are hypothesized to affect eicosanoid synthesis. Down regulation of prostaglandin (PG_2) production, which is related to initiation of the parturition process, has been suggested to increase pregnancy duration.[10-11] A shift of the prostacyclin/thromboxane balance to a more anti-aggregatory and vasodilator state might increase placental flow and as a consequence fetal growth.[12-13] However, fish consumption is also a well-known route of exposure to pollutants such as methyl mercury, dioxins and polychlorinated biphenyls (PCB's) which may adversely affect fetal growth and birth outcomes.[14-17] In a large study among Danish pregnant women, high maternal fish consumption was associated with lower birth weight, smaller birth length and head circumference.[18] Thus far, results from studies focused on the associations between maternal fish consumption and birth outcomes have not been consistent.[9,19-22] Differences in results may be explained by specific effects of different types of fish, such as lean fish, fatty fish, and shellfish. Fatty fish contains larger amounts of beneficial *n*-3 fatty acid, and, like shellfish, higher levels of contaminants.[16,23] Besides, previous studies mainly focused on birth outcomes as measure of fetal growth and development. However, similar birth weights might be the result of different fetal exposures or growth patterns. Assessing fetal growth characteristics in different trimesters of pregnancy may provide information about specific critical periods. Therefore, we examined the associations of first trimester maternal lean fish, fatty fish and shellfish consumption with fetal growth characteristics in second and third trimester and at birth and the risks of neonatal complications in a population-based prospective cohort study among 3,380 mothers and their children.

METHODS

Study design

The present study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in the city of Rotterdam, the Netherlands. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood, and has been described in detail previously.[24-25] Assessments during pregnancy included

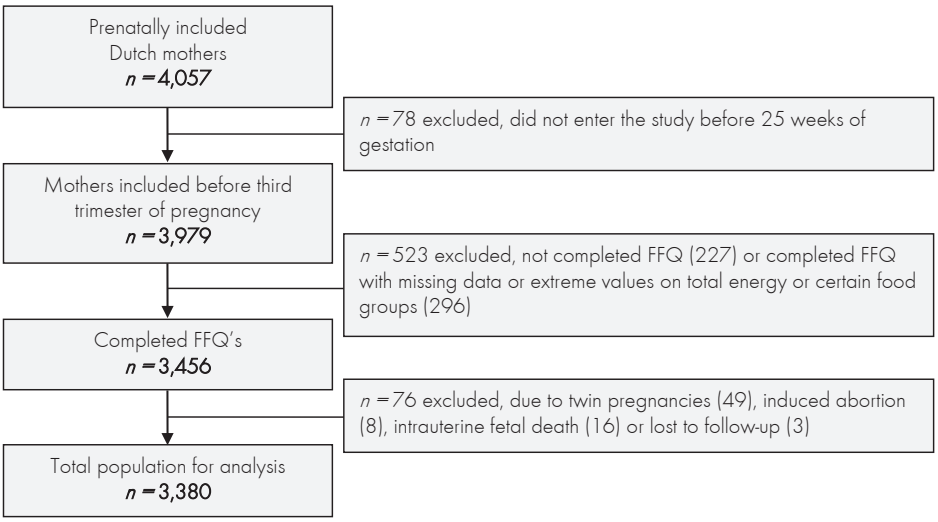

Figure 2.1.1 Flowchart of study participants

physical examinations, fetal ultrasounds, biological samples and questionnaires, and were planned in first, second and third trimester to collect information about fetal growth and its main determinants. The present study was performed in Dutch participants. Of the total group of Dutch mothers (*n*=4,057), 98% (*n*=3,979) enrolled in the first or second trimester of pregnancy, and 87% (*n*=3,456) fully completed the food frequency questionnaire including all questions referring to fish consumption. Twin pregnancies (*n*=49), pregnancies leading to intrauterine death (*n*=24), or without known birth outcomes (*n*=3) were excluded from the study population. The analyses were performed in the remaining 3,380 subjects (Figure 2.1.1). The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents. This study was conducted according to the guidelines laid down in the Declaration of Helsinki.

Dietary assessment

We assessed maternal dietary intake, including fish consumption, at enrolment in the study (median 13.5 weeks of gestation, total range 5.4-24.9) using a modified version of the validated semi-quantitative food frequency questionnaire (FFQ) of Klipstein-Grobusch *et al.*[26] This FFQ considered food intake over the prior three months, thereby covering dietary intake within first trimester of pregnancy. The FFQ consists of 293 items structured to meal pattern. Questions include consumption frequency, portion size, preparation method, and additions. Portion sizes were estimated using Dutch household measures and colored photographs of foods showing different portion sizes.[27] To calculate average daily nutritional values, the 2006 version of the Dutch food composition table

was used.[28] Frequency of fish consumption was assessed for total fish consumption and different types of fish. Based on nutrient content and information from previous studies we assessed consumption of different fish types by 7 categories: lean fish (codfish, plaice, catfish, sole fish, tuna, whiting, haddock), moderately fatty fish (trout, anchovy and gurnard), fatty fish (salmon, herring, mackerel, eel, sardines, halibut and bloater), shellfish (crab, lobster, shrimps and mussels), processed fish (fish fingers, fish burgers, crumbed and fried fish), fish derived from liver (haddock liver) and roe (soft and hard roe).[18] For the analysis of total fish we aggregated all fish consumed, and for the analysis of fatty fish we aggregated moderately fatty fish and fatty fish. Processed fish, roe and fish derived from liver were not further analyzed separately.

Fetal growth characteristics

Fetal ultrasound examinations were carried out at one of the two research centers in each trimester of pregnancy. Median (total range) of these visits were 12.9 (7.7-18.0), 20.5 (18.0-25.0) and 30.4 (25.8-37.0) weeks of gestation for first, second and third trimester respectively. These fetal ultrasound examinations were used for both establishing gestational age and assessing fetal growth characteristics.[24] Since gestational age was established by the first fetal ultrasound examination, these ultrasounds were not used to assess fetal growth In the second and third trimesters of pregnancy, we measured head circumference (HC), abdominal circumference (AC) and femur length (FL) to the nearest millimeter using standardized ultrasound procedures.[29] Estimated fetal weight (EFW) was calculated by means of the formula from Hadlock using head circumference, abdominal circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[30]

Neonatal complications

Information about offspring sex, gestational age, weight, length, and head circumference at birth was obtained from medical records and hospital registries. Since head circumference and length at birth were not routinely measured at birth, missing birth measures were completed with data from the first month visit at the routine child health center. Of all measurements, 25% and 16% were based on the first month visit for head circumference and birth length, respectively. No differences in mean maternal fish consumption was observed between children with measurements at birth and those without measurements at birth (Student's T-tests: P=0.46 for head circumference and P=0.94 for birth length). The regression models with neonatal head circumference and length as outcome were adjusted for postconceptional age (gestational age for measurements at birth or gestational age + postnatal age for measurement from the child health centers) and for the method of measurement (birth or child health center).[31] Preterm birth was defined as a gestational age of less than 37 weeks at delivery. Low birth weight was defined as

birth weight below 2,500g. Small size for gestational age at birth was defined as a gestational age and sex adjusted birth weight below the 5th percentile in the study cohort. We assessed both low birth weight and small for gestational age since it is important to differentiate between newborns having a low birth weight, independent of gestational age, and those who are fetal growth restricted. Both outcomes are independent risk factors of neonatal complications and development of diseases in adulthood.[32-33]

Covariates

Information about educational level, parity and periconceptional folic acid supplement use was obtained by a questionnaire at enrolment in the study. Maternal smoking and alcohol habits were assessed by questionnaires in each trimester. Maternal and paternal anthropometrics, including height (m) and weight (kg), were measured without shoes and heavy clothing and body mass index was calculated (weight/height^2 (kg/m^2)) in first, second and third trimester during visits at the research center. Information about maternal weight just before pregnancy was obtained by questionnaires. As enrolment in our study was in pregnancy, we were not able to measure maternal weight before pregnancy. However, in our population for analysis, 56% and 85% of all women enrolled before a gestational age of 14 and 18 weeks respectively. Correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was 0.97 (P<0.001). Since using weight measured at enrolment instead of pre-pregnancy weight obtained by questionnaire did not change our results,[34] and considering the better data quality, we decided to use weight measured at enrolment in the analyses. Maternal age was registered at enrolment.

Statistical analyses

Based on the distribution of fish consumption and number of subjects, we created five categories of total fish consumption (0, 1-69, 70-139, 140-209 and >210 g/week), four categories of lean fish consumption (0, 1-34, 35-69 and >70 g/week), four categories of fatty fish consumption (0, 1-34, 35-69 and >70 g/week) and three categories of shell fish consumption (0, 1-13 and >14 g/week). We used the category of "no fish consumption" as the referent for all analyses. Analysis of total fish consumption and consumption of lean fish, fatty fish and shellfish in quartiles and quintiles did not change the results. We used t-tests and chi-square tests to compare maternal characteristics in different categories of total weekly fish consumption. We analyzed the associations of total weekly fish consumption with fetal growth characteristics in second and third trimester and at birth using multiple linear regression models. In our first analyses, models were only adjusted for gestational age and fetal sex (Model A). Subsequently, we considered confounders based on previous studies.[15-16,18-19,21-22,31] These confounders included maternal total daily energy intake, age, body mass index, weight gain, parity,

marital status, educational status, smoking, alcohol use, coffee consumption, nausea, vomiting, periconceptional folic acid use and paternal height. Potential confounders were included in the models if the effect estimates changed more than 5% in exploratory analyses. Using this approach, weight gain and parity were not included in the final multiple analysis (Model B). We performed similar analyses (Model A and B) separately for consumption of lean fish, fatty fish and shellfish. We used logistic regression models to analyze the associations of total fish, lean fish, fatty fish and shellfish consumption categories with the risks of neonatal complications (preterm birth, low birth weight, small for gestational age). Tests for trends were performed by using weekly consumption of total fish, lean fish, fatty fish and shellfish as continuous variable in multiple linear and logistic regression analyses. Multiple imputation was used to complete missing data on the covariates body mass index (0.4%), weight gain (3.3%), educational status (1.9%), marital status (0.6%), parity (0.2%), smoking (7.6%), alcohol use (7.3%), nausea (7.8%), vomiting (8.1%) and folic acid supplement use (17.4%). Since there were no differences in the observed results between analyses with imputed missing data or complete cases only, solely results including imputed missing data are presented. All measures of association are presented with their 95% confidence intervals (95% CI). *P*-values are two-sided. Statistical analyses were performed using the Predictive Analytic Software version 17.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

Maternal age ranged from 15.7 to 46.3 years, with a mean of 31.4 years (**Table 2.1.1**). The median of weekly fish consumption was 75 g (total range 0-600 g). The median weekly consumption of lean, fatty, shellfish and processed fish, was 24 grams (total range 0-338 grams), 32 grams (total range 0-360 grams), 0 grams (total range 0-93 grams) and 0 grams (total range 0-261 grams) respectively.

Maternal older age, higher educational level, adequate folic acid supplement use, alcohol use, and not smoking were associated with higher fish consumption ($P<0.01$). In the total cohort, mean offspring birth weight was 3,489 g (556 SD) and the median gestational age at birth was 40.3 weeks (total range 26.7 to 43.4 weeks). Of all births, 4.7% were born preterm, 4.0% had a low birth weight and 6.1% were small for gestational age at birth. Fetal growth characteristics measured during second and third trimester of pregnancy were available in 97.8% and 97.9% of the mothers respectively. Results from the gestational age and sex adjusted regression models focused on the associations between fish consumption and fetal growth characteristics or neonatal complications (Model A) are given in **Supplement 2.1.1** to **2.1.5**. Higher consumption levels of total fish was associated with larger third trimester head circumference and birth length

Table 2.1.1 Maternal and infant characteristics according to maternal fish consumption during pregnancy

		All	Total weekly fish consumption					
Maternal characteristics		n=3,380	0 g n=668	1-69 g n=898	70-139 g n=1085	140-209 g n=503	>210 g n=226	*P*-value
Age	years	31.4 ± 4.4	30.1 ± 4.7	31.3 ± 4.4	31.8 ± 4.0	32.2 ± 4.1	31.8 ± 4.3	<0.01
Weight	kg	70.9 ± 12.6	73.2 ± 14.6	70.5 ± 12.4	70.0 ± 11.7	70.5 ± 12.1	70.2 ± 11.3	<0.01
Height	cm	171 ± 6.4	171 ± 6.7	171 ± 6.1	171 ± 6.4	171 ± 6.3	171 ± 6.5	0.05
Body mass index	kg/m^2	24.3 ± 4.1	25.2 ± 4.9	24.2 ± 3.9	23.9 ± 3.7	24.0 ± 4.0	23.8 ± 3.7	<0.01
Total energy intake	kJ	9007 ± 2148	8617 ± 2262	9013 ± 2125	9075 ± 2058	9322 ± 2147	9151 ± 2147	<0.01
Marital status	Married	45.0	46.6	45.2	46.3	42.1	40.3	0.19
	Living together	46.3	45.3	46.1	46.1	48.7	47.8	
	No partner	6.7	7.8	6.8	5.6	6.2	10.2	
	Missing	1.9	1.0	1.9	2.0	3.0	1.8	
Educational status	Primary school	3.2	6.4	3.5	1.9	1.8	1.3	<0.01
	Secondary school	37.3	50.7	40.1	32.5	28.4	29.5	
	Higher education	59.0	41.8	56.1	65.2	69.2	69.2	
	Missing	0.6	1.0	0.3	0.4	0.6	0.9	
Smoking	Never	69.7	64.2	68.5	70.9	74.8	73.9	<0.01
	Until pregnancy was known	8.0	6.1	8.5	8.4	8.2	9.7	
	Continued	14.7	22.2	15.0	13.3	9.5	9.3	
	Missing	7.6	7.5	7.9	7.5	7.6	7.1	
Alcohol use	Never	31.3	46.3	29.8	27.3	24.9	28.6	<0.01
	Until pregnancy was known	15.3	16.0	15.6	14.7	14.9	15.9	
	Continued	46.1	30.7	46.7	50.7	53.5	50.4	
	Missing	7.3	7.0	7.9	7.3	6.8	7.1	
Folic acid supplement use	Preconception start	46.4	44.3	45.7	47.2	49.9	44.7	

Table 2.1.1 (continued)

		All	Total weekly fish consumption					
		n=3,380	0 g n=668	1-69 g n=898	70-139 g n=1085	140-209 g n=503	>210 g n=226	*P*-value
	Postconception start	27.3	23.4	27.8	28.7	27.4	29.6	
	None	8.9	15.3	6.3	7.6	7.0	10.2	
	Missing	17.4	17.1	20.2	16.6	15.7	15.5	
Fetal characteristics								
Trimester 2								
Head circumference	mm	179 ± 13	179 ± 13	179 ± 13	179 ± 13	178 ± 14	180 ± 14	0.55
Femur length	mm	33.4 ± 3.3	33.4 ± 3.2	33.3 ± 3.3	33.4 ± 3.3	33.1 ± 3.3	33.5 ± 3.5	0.32
Fetal weight	g	379 ± 87	379 ± 84	379 ± 87	381 ± 85	372 ± 86	386 ± 93	0.26
Trimester 3								
Head circumference	mm	286 ± 12	284 ± 13	286 ± 12	286 ± 12	287 ± 12	286 ± 12	0.02
Femur length	mm	57.5 ± 2.9	57.3 ± 3.0	57.5 ± 3.0	57.5 ± 2.8	57.6 ± 2.8	57.3 ± 2.9	0.49
Fetal weight	g	1633 ± 256	1618 ± 274	1639 ± 264	1630 ± 241	1644 ± 249	1637 ± 255	0.57
Birth outcomes								
Males	%	50.5	51.8	51.2	50.0	49.1	49.1	0.86
Gestational age	weeks	39.9 ± 1.7	40.0 ± 1.8	39.9 ± 1.7	40.0 ± 1.7	40.1 ± 1.6	39.8 ± 1.8	0.27
Birth weight	g	3489 ± 556	3457 ± 569	3501 ± 563	3491 ± 546	3531 ± 551	3437 ± 534	0.12
Birth length	cm	51.2 ± 2.8	51.0 ± 2.7	51.3 ± 2.9	51.1 ± 2.9	51.4 ± 2.8	50.9 ± 3.0	0.06
Head circumference	cm	35.1 ± 2.3	34.9 ± 2.3	35.2 ± 2.3	35.1 ± 2.3	35.2 ± 2.3	35.1 ± 2.3	0.19
Preterm birth	%	4.7	5.1	5.0	4.3	4.0	5.3	0.76
Low birth weight	%	4.1	5.1	3.2	4.2	3.2	5.8	0.19
Small for gestational age	%	6.1	7.6	6.3	5.5	5.0	5.3	0.31

Values reflect the mean ± standard deviation for continuous variables and percentage for categorical variables. *P*-values are obtained by ANOVA for continuous variables and chi-square test for categorical variables.

and lower risk of low birth weight. Consumption of lean fish was positively associated with third trimester head circumference and birth weight and inversely associated with the risk of small for gestational age. Higher level of fatty fish consumption was also inversely associated with the risk of small for gestational age. A higher shellfish consumption level was associated with larger femur length in second trimester.

After additional adjustment for confounders most associations disappeared (Tables 2.1.2 to 2.1.5). The differences between Model A and Model B were largely explained by including smoking, educational status and folic acid supplement use in the regression

Table 2.1.2 Associations between maternal total fish consumption, fetal growth and growth characteristics at births

Total fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	653	Ref		641	Ref		541	Ref	
1-69 g/week	878	0.4	-0.3, 1.0	872	**1.3**	**0.3, 2.2***	742	0.2	-0.1, 0.5
70-139 g/week	1061	-0.4	-1.0, 0.2	1058	0.1	-0.8, 1.0	895	0.0	-0.2, 0.3
140-209 g/week	494	-0.2	-1.0, 0.5	487	**1.2**	**0.1, 2.3***	422	0.1	-0.2, 0.4
>210 g/week	221	0.2	-0.7, 1.2	218	0.9	-0.5, 2.3	175	-0.0	-0.4, 0.4
P for trend		*0.61*			*0.24*			*0.39*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	650	Ref		652	Ref		557	Ref	
1-69 g/week	877	0.1	-0.1, 0.2	879	0.1	-0.2, 0.2	758	0.3	-0.0, 0.5
70-139 g/week	1064	-0.1	-0.2, 0.1	1067	-0.0	-0.2, 0.2	911	-0.1	-0.3, 0.2
140-209 g/week	494	-0.1	-0.3, 0.1	492	-0.0	-0.3, 0.3	428	0.1	-0.3, 0.4
>210 g/week	221	0.1	-0.2, 0.3	220	-0.2	-0.5, 0.2	177	-0.2	-0.7, 0.2
P for trend		*0.87*			*0.61*			*0.26*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	647	Ref		651	Ref		667	Ref	
1-69 g/week	871	2.9	-1.6, 7.3	874	12.6	-6.4, 31.6	894	22.0	-22.5, 66.4
70-139 g/week	1061	-0.5	-4.8, 3.8	1062	-11.0	-29.5, 7.5	1080	5.1	-38.2, 48.4
140-209 g/week	491	-1.7	-6.8, 3.5	491	1.1	-21.1, 23.3	501	17.4	-34.6, 69.4
>210 g/week	221	3.6	-3.0, 10.3	220	2.1	-26.6, 30.9	225	-3.0	-70.4, 64.4
P for trend		*0.94*			*0.63*			*0.86*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of total fish consumption compared to the reference group. All models are adjusted for maternal energy intake, age, body mass index, marital status, education, smoking, alcohol use, nausea, vomiting, folic acid supplement use, gestational age at measurement, paternal height and fetal sex. ***P*-value <0.05**

models. As compared to no fish consumption, weekly maternal consumption of 0-69 and 140-209 grams of total fish was associated with larger head circumference measured in third trimester (both *P*-values < 0.05). The test for trend, however, was not significant. No associations were observed between maternal total fish consumption and other fetal growth characteristics in second and third trimester or at birth. Weekly lean and fatty fish consumption were not associated with fetal growth characteristics in second and third trimester or at birth. Weekly consumption of >14 grams of shellfish was associated with lower birth weight (*P*=0.04). Shellfish consumption was not associated with other fetal growth characteristics. Maternal consumption of total fish, fatty fish, lean fish or shellfish was not consistently associated with the risk of children born preterm, with a low birth weight or small size for gestational age (Table 2.1.6).

Table 2.1.3 Associations between maternal lean fish consumption, fetal growth and growth characteristics at birth

Lean fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	1239	Ref		1219	Ref		1017	Ref	
1-35 g/week	849	0.1	-0.5, 0.6	850	0.4	-0.5 1.2	724	-0.0	-0.2, 0.2
35-69 g/week	813	-0.0	-0.6, 0.5	804	0.6	-0.2, 1.4	697	0.1	-0.2, 0.2
>70 g/week	406	-0.0	-0.7, 0.7	403	-0.2	-1.2, 0.8	337	-0.1	-0.3, 0.2
P for trend		*0.78*			*0.40*			*0.76*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	1233	Ref		1234	Ref		1044	Ref	
1-35 g/week	853	0.1	-0.1, 0.2	857	0.1	-0.1, 0.3	742	0.2	-2.3, 2.7
35-69 g/week	810	-0.1	-0.2, 0.1	812	0.1	-0.2, 0.3	711	-0.1	-2.7, 2.4
>70 g/week	406	-0.1	-0.3, 0.1	407	-0.1	-0.4, 0.1	334	-1.5	-4.7, 1.8
P for trend		*0.49*			*0.41*			*0.56*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	1226	Ref		1230	Ref		1262	Ref	
1-35 g/week	849	0.7	-3.1, 4.5	852	4.2	-12.3, 20.7	869	13.2	-25.4, 51.8
35-69 g/week	810	-1.4	-5.4, 2.5	810	6.6	-10.3, 23.5	823	25.3	-14.3, 64.9
>70 g/week	406	-0.4	-5.3, 4.5	406	-5.9	-27.1, 15.2	413	-30.2	-79.7, 19.3
P for trend		*0.64*			*0.96*			*0.84*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of lean fish consumption compared to the reference group. All models are adjusted for maternal energy intake, age, body mass index, marital status, education, smoking, alcohol use, nausea, vomiting, folic acid supplement use, gestational age at measurement, paternal height and fetal sex.

Table 2.1.4 Associations between maternal fatty fish consumption, fetal growth and growth characteristics at birth

Fatty fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	1083	Ref		1064	Ref		912	Ref	
1-35 g/week	719	-0.0	-0.6, 0.6	725	0.6	-0.3, 1.4	610	0.0	-0.2, 0.3
35-69 g/week	911	-0.4	-1.0, 0.2	900	-0.3	-1.1, 0.6	764	-0.1	-0.3, 0.1
>70 g/week	594	-0.4	-1.0, 0.3	587	-0.4	-0.5. 1.4	489	-0.1	-0.3, 0.2
P for trend		*0.29*			*0.37*			*0.43*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	1079	Ref		1081	Ref		934	Ref	
1-35 g/week	723	-0.0	-0.2, 0.1	729	-0.0	-0.2, 0.2	626	0.1	-0.2,0.3
35-69 g/week	911	-0.1	-0.2, 0.1	908	-0.1	-0.3, 0.1	774	-0.1	-0.3, 0.2
>70 g/week	593	-0.1	-0.3, 0.1	592	-0.1	-0.3, 0.2	497	-0.1	-0.4, 0.2
P for trend		*0.57*			*0.45*			*0.29*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	1074	Ref		1077	Ref		1100	Ref	
1-35 g/week	719	-0.7	-4.7, 3.4	725	-1.3	-18.8, 16.2	737	-11.7	-52.8, 29.3
35-69 g/week	910	-1.7	-5.6, 2.2	907	-13.6	-30.3, 3.1	928	4.5	-34.8, 43.8
>70 g/week	588	-2.7	-7.2, 1.7	589	-11.5	-30.5, 7.6	602	-8.4	-53.3, 36.4
P for trend		*0.31*			*0.27*			*0.83*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of fatty fish consumption compared to the reference group. All models are adjusted for maternal energy intake, age, body mass index, marital status, education, smoking, alcohol use, nausea, vomiting, folic acid supplement use, gestational age at measurement, paternal height and fetal sex.

DISCUSSION

Main findings

In this cohort of pregnant women in the Netherlands, we found no consistent associations of total fish, lean fish or fatty fish consumption with fetal growth characteristics during second and third trimester and at birth, after adjustment for potential confounders. Shellfish consumption was not associated with fetal growth characteristics during second and third trimester, however, some evidence was found for an association between shellfish consumption and lower birth weight. No consistent associations were observed between fish consumption and the risks of neonatal complications. The median of weekly fish consumption in our study population was 75 g, which is higher than the median fish consumption presented in a Dutch cohort study (52g per week) and measured by the Dutch National Food Consumption Survey (63g per week) in a population of similar age.[35-36] These differences may be due to differences in time period, since data on fish

Table 2.1.5 Associations between maternal shellfish consumption, fetal growth and growth characteristics at birth

Shellfish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	2053	Ref		2027	Ref		1693	Ref	
1-13 g/week	604	0.2	-0.4, 0.8	609	0.8	-0.04, 1.64	521	-0.0	-0.3, 0.2
>14 g/week	650	-0.0	-0.6, 0.5	640	-0.3	-1.16, 0.48	561	-0.1	-0.3, 0.2
P for trend		*0.72*			*0.58*			*0.19*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	2049	Ref		2046	Ref		1747	Ref	
1-13 g/week	608	-0.0	-0.2, 0.1	618	0.0	-0.2, 0.2	528	-0.0	-0.3, 0.3
>14 g/week	649	-0.1	-0.3, 0.1	646	-0.2	-0.4, 0.0	556	-0.1	-0.4, 0.1
P for trend		*0.53*			*0.21*			*0.35*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	2042	Ref		2040	Ref		2090	Ref	
1-13 g/week	604	-1.0	-4.9, 3.0	615	4.1	-12.7, 20.8	620	-10.6	-50.1, 29.0
>14 g/week	645	-0.3	-4.2, 3.6	643	-14.8	-31.6, 2.1	657	**-41.7**	**-81.2, -2.2***
P for trend		*0.82*			*0.15*			*0.07*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of shellfish consumption compared to the reference group. All models are adjusted for maternal energy intake, age, body mass index, marital status, education, smoking, alcohol use, nausea, vomiting, folic acid supplement use, gestational age at measurement, paternal height and fetal sex. ***P*-value <0.05**

consumption was collected between 1993 and 1998. Mean fish consumption in our study was comparable to mean fish consumption in the Netherlands reported in a more recent study comparing fish consumption between European countries (13.4 g per day in our study and 12.6-14.8 g per day in the EPIC study).[37]

Interpretation of main findings

To our knowledge, only one previous study assessed the associations of total fish consumption with fetal growth characteristics in second and third trimester.[21] This study among 1,805 pregnant women in France did not show associations between total seafood consumption and fetal growth characteristics. Many studies assessed the associations of consumption of different types of fish with birth outcomes as measures for fetal development. In a large cohort in Denmark, no association was observed between lean fish consumption and the risks of adverse birth outcomes. However, associations were observed between frequent consumption of fatty fish with lower birth weight, and smaller birth length and head circumference.[18] Frequent fatty fish consumption was also associated with a higher risk of small size for gestational age at birth. Also, higher

Table 2.1.6 Associations between maternal fish consumption and risks of neonatal complications

	Preterm birth (*n*=159)				Low birth weight (*n*=138)				Small for gestational age (*n*=205)			
	n	cases	OR	95% CI	*n*	cases	OR	95% CI	*n*	cases	OR	95% CI
Total fish consumption												
0	668	34	Ref		667	34	Ref		667	51	Ref	
1-69 g/week	897	45	1.14	0.71, 1.82	894	29	**0.47**	**0.23, 0.95***	893	57	0.87	0.58, 1.30
70-139 g/week	1085	47	0.98	0.61, 1.57	1080	46	0.78	0.42, 1.44	1079	60	0.76	0.51, 1.14
140-209 g/week	502	20	0.92	0.51, 1.65	501	16	0.62	0.27, 1.40	501	25	0.68	0.41, 1.14
>210 g/week	226	13	1.21	0.61, 2.38	225	13	0.86	0.34, 2.17	225	12	0.67	0.34, 1.30
P for trend			*0.82*				*0.89*				*0.19*	
Lean fish consumption												
0	1267	75	Ref		1262	62	Ref		1260	91	Ref	
1-35 g/week	869	32	0.68	0.44, 1.05	869	27	0.66	0.35, 1.25	869	46	0.75	0.51, 1.10
35-69 g/week	828	38	0.84	0.55, 1.28	823	32	0.81	0.43, 151	823	37	0.67	0.44, 1.00
>70 g/week	414	14	0.61	0.33, 1.10	413	17	1.22	0.58, 2.54	413	31	1.15	0.73, 1.79
P for trend			*0.15*				*0.91*				*0.92*	
Fatty fish consumption												
0	1104	58	Ref		1100	50	Ref		1099	75	Ref	
1-35 g/week	739	26	0.70	0.43, 1.12	737	18	0.66	0.33, 1.31	737	51	1.00	0.68, 1.46
35-69 g/week	931	48	1.04	0.69, 1.57	928	47	1.23	0.69, 2.19	927	49	0.77	0.52, 1.14
>70 g/week	604	27	0.87	0.54, 1.42	602	23	1.00	0.50, 1.98	602	30	0.71	0.45, 1.12
P for trend			*0.87*				*0.81*				*0.06*	
Shellfish consumption												
0	2098	103	Ref		2090	87	Ref		2089	120	Ref	
1-13 g/week	620	24	0.84	0.53, 1.33	620	18	0.88	0.45. 1.72	620	44	1.21	0.85, 1.78
>14 g/week	660	32	1.15	0.75, 1.75	657	33	1.72	0.95. 3.07	656	41	1.20	0.83, 1.80
P for trend			*0.16*				*0.90*				*0.80*	

Values are based on multiple logistic regression models and reflect the odds ratio and 95% confidence interval for pregnancy complications for each level of fish consumption compared to the reference group. All models are adjusted for maternal energy intake, age, body mass index, marital status, education, smoking, alcohol use, nausea, vomiting, folic acid supplement use, gestational age at measurement, paternal height and fetal sex. ***P*-value <0.05**

total fish consumption levels were associated with lower birth weight and smaller head circumference, which, seems to be fully explained by consumption of fatty fish. In our study, we did not find consistent associations of total or fatty fish consumption with fetal growth characteristics. This difference in results might be due to differences in quality and quantity of fish consumption. In our population mean total fish consumption was much lower than in Denmark. A daily consumption of >40 or >60 g/day was reported by 14% and 6% in the Danish population, as compared to 3% and 1% in our study population.

Differences in the quantity of types of fish consumed complicate a direct comparison of results between countries.[37] A recent study in Spain showed an association between weekly consumption of ≥2 portions large oily fish and a higher risk of small for gestational age. The authors also described an association of high lean fish consumption with a lower risk of small for gestational age.[16] Mean consumption levels of large oily fish or lean fish were not presented, but the mean total fish consumption in this study was 65 g per day, compared to 13 g per day in our Dutch population. Another Spanish study showed that consumption of >1 portion crustaceans per week was associated with a higher risk of small for gestational age.[38] A French study showed the same association with consumption of >2 portions of shellfish per week.[23] In the Spanish study mean consumption of crustaceans was 6.3 g/day and 13.1 g/day for other types of shellfish, in the French study mean consumption of shellfish was 19.7 g/d. In our study population shellfish were barely consumed at all. Nevertheless, a tendency towards a negative association of shellfish consumption with birth weight was observed. This association has been reported before. It is therefore important to explore which type of shellfish contributes to this effect. It is most likely that this association is driven by large crustaceans (crabs, lobster, etc.) since they are known to contain more dioxins and PCB's. However, we were not able to separately analyze different types of shellfish, since we assessed shellfish by a predefined category. Therefore, this association needs to be studied in further detail.

Strong associations of higher fish consumption with increased growth measures at birth have mainly been described in ecological and cohort studies that collected data in the 1980s and 1990s in countries that are high in seafood consumption.[1-4,39-40] Results from large cohorts conducted more recently, have suggested absence of any association or inverse associations. Since not only in our study, but also in other cohort studies, high fish consumption was strongly related to a higher education level and healthier life style habits,[16,18,21,41] these positive associations between fish consumption and birth anthropometrics may be partly due to residual confounding by lifestyle related characteristics. However, in a Danish study conducted in 2002, the association of fish consumption with lower risk of preterm birth and having low birth weight remained significant after adjustment for confounders. Also, in a British study, the association of fish consumption with lower risk of fetal growth retardation remained significant after adjustment for maternal education.

Contamination of fish, has frequently been suggested as explanation for the inverse associations of fish consumption with birth outcomes.[16,18,38] In a study in Denmark, regular fatty fish consumption was associated with increased maternal plasma concentrations of PCB's.[17] These maternal plasma PCB concentrations were associated with lower birth weight in this Danish study, as well as in other studies conducted in Japan, Slovakia, United States and Sweden.[42-46] In the Netherlands, fish consumption contributes on

average to up to 26% of total PCB intake and 12% of dioxin intake.[47-48] In line with results from previous studies, PCB concentrations in maternal and cord blood have been associated with lower birth weight in the Netherlands as well.[49] This study, however, was conducted in 1998, when PCB levels were considerably higher. Contrary, in the study of Mendez et al, adjustment for blood levels of mercury and PCB did not change, and thus explain, the inverse associations between fish consumption and birth outcomes.[38] Some other studies also did not observe associations of maternal plasma PCB concentrations with birth weight.[50-52] Direct comparison of the effects of contamination by either mercury, dioxins or PCB's is complicated due to different levels of contamination between countries, area's and within fish species.[48] Dioxin and PCB concentrations in mussels, shrimps, mackerel and cod in the Netherlands are comparable to reported levels in other Northern European countries, but higher as compared to levels in Spain.[53] Furthermore, there seems to be a downward trend in concentrations of contaminants. A continuous decline, although leveling off, of dioxin and PCB concentrations has been shown in both the Dutch river systems, food and breast milk.[47-48,53] The latter might reflect concentrations in the human body.[48,54] Also, it appeared recently that when measuring plasma dioxin and PCB concentrations, timing of blood sampling around conception plays a an important role.[55] Furthermore, differences in results between studies may be explained by specific congeners assessed, since it is suggested that especially anti-estrogenic PCB's are associated with lower birth weight.[55]

Methodological considerations

The strength of this study is that we assessed fetal growth by actually measuring fetal growth characteristics in second and third trimester of pregnancy instead of using only birth outcomes as a proxy for fetal growth. We prospectively collected detailed information on consumption of different fish types which enabled us to separate the analyses. Also, from our questionnaires we were able to extensively collect information on many potential confounding variables. A limitation of the study is that we did not have biomarkers on mercury or other chemical exposures. Therefore, we were not able to separate harmful effects of pollutants from potential beneficial effects of fish consumption. Also, since we assessed fish consumption by categories of fish types, we were not able to assess separate effects of consumption of each different fish species or to recombine specific fish species. Furthermore, since 87% of eligible mothers completed the food frequency questionnaire, we may have introduced some bias by missing data. Effect estimates could be biased if the associations of fish consumption with fetal outcomes differ between mothers included and not included in the study population. This seems unlikely, but cannot be excluded.

Conclusion

In a population which is relatively low in exposure to fish consumption, we observed no consistent associations of total fish consumption and consumption of different types of fish with fetal growth characteristics or the risk of neonatal complications. Further studies are required focusing on the association between fish consumption and fetal growth measured by ultrasound. Monitoring contaminants in fish and analysis of additional contaminants, especially congeners having anti-estrogenic activity, might help to further elucidate potential associations.

REFERENCES

1. Olsen SF & Joensen HD (1985) High liveborn birth weights in the Faroes: a comparison between birth weights in the Faroes and in Denmark. *J Epidemiol Community Health* 39, 27-32.
2. Olsen SF, Hansen HS, Sorensen TI, *et al.* (1986) Intake of marine fat, rich in (n-3)-polyunsaturated fatty acids, may increase birthweight by prolonging gestation. *Lancet* 2, 367-369.
3. Olsen SF, Hansen HS, Sommer S, *et al.* (1991) Gestational age in relation to marine n-3 fatty acids in maternal erythrocytes: a study of women in the Faroe Islands and Denmark. *Am J Obstet Gynecol* 164, 1203-1209.
4. Olsen SF, Grandjean P, Weihe P, *et al.* (1993) Frequency of seafood intake in pregnancy as a determinant of birth weight: evidence for a dose dependent relationship. *J Epidemiol Community Health* 47, 436-440.
5. Philibert A, Vanier C, Abdelouahab N, *et al.* (2006) Fish intake and serum fatty acid profiles from freshwater fish. *Am J Clin Nutr* 84, 1299-1307.
6. Innis SM (2003) Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *J Pediatr* 143, S1-8.
7. Uauy R, Calderon F & Mena P (2001) Essential fatty acids in somatic growth and brain development. *World Rev Nutr Diet* 89, 134-160.
8. Szajewska H, Horvath A & Koletzko B (2006) Effect of n-3 long-chain polyunsaturated fatty acid supplementation of women with low-risk pregnancies on pregnancy outcomes and growth measures at birth: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* 83, 1337-1344.
9. Olsen SF, Osterdal ML, Salvig JD, *et al.* (2006) Duration of pregnancy in relation to seafood intake during early and mid pregnancy: prospective cohort. *Eur J Epidemiol* 21, 749-758.
10. Olsen SF (2004) Is supplementation with marine omega-3 fatty acids during pregnancy a useful tool in the prevention of preterm birth? *Clin Obstet Gynecol* 47, 768-774; discussion 881-762.
11. Hansen HS & Olsen SF (1988) Dietary (n-3)-fatty acids, prostaglandins, and prolonged gestation in humans. *Prog Clin Biol Res* 282, 305-317.
12. Sorensen JD, Olsen SF, Pedersen AK, *et al.* (1993) Effects of fish oil supplementation in the third trimester of pregnancy on prostacyclin and thromboxane production. *Am J Obstet Gynecol* 168, 915-922.
13. Olsen SF (1994) Further on the association between retarded fetal growth and adult cardiovascular disease. Could low intake or marine diets be a common cause? *J Clin Epidemiol* 47, 565-569.
14. Mahaffey KR (2004) Fish and shellfish as dietary sources of methylmercury and the omega-3 fatty acids, eicosahexaenoic acid and docosahexaenoic acid: risks and benefits. *Environ Res* 95, 414-428.
15. Ramon R, Murcia M, Ballester F, *et al.* (2008) Prenatal exposure to mercury in a prospective mother-infant cohort study in a Mediterranean area, Valencia, Spain. *Sci Total Environ* 392, 69-78.
16. Ramon R, Ballester F, Aguinagalde X, *et al.* (2009) Fish consumption during pregnancy, prenatal mercury exposure, and anthropometric measures at birth in a prospective mother-infant cohort study in Spain. *Am J Clin Nutr* 90, 1047-1055.
17. Halldorsson TI, Thorsdottir I, Meltzer HM, *et al.* (2008) Linking exposure to polychlorinated biphenyls with fatty fish consumption and reduced fetal growth among Danish pregnant women: a cause for concern? *Am J Epidemiol* 168, 958-965.
18. Halldorsson TI, Meltzer HM, Thorsdottir I, *et al.* (2007) Is high consumption of fatty fish during pregnancy a risk factor for fetal growth retardation? A study of 44,824 Danish pregnant women. *Am J Epidemiol* 166, 687-696.

19. Rogers I, Emmett P, Ness A, *et al.* (2004) Maternal fish intake in late pregnancy and the frequency of low birth weight and intrauterine growth retardation in a cohort of British infants. *J Epidemiol Community Health* 58, 486-492.
20. Buck GM, Tee GP, Fitzgerald EF, *et al.* (2003) Maternal fish consumption and infant birth size and gestation: New York State Angler Cohort Study. *Environ Health* 2, 7.
21. Drouillet P, Kaminski M, De Lauzon-Guillain B, *et al.* (2009) Association between maternal seafood consumption before pregnancy and fetal growth: evidence for an association in overweight women. The EDEN mother-child cohort. *Paediatr Perinat Epidemiol* 23, 76-86.
22. Oken E, Kleinman KP, Olsen SF, *et al.* (2004) Associations of seafood and elongated n-3 fatty acid intake with fetal growth and length of gestation: results from a US pregnancy cohort. *Am J Epidemiol* 160, 774-783.
23. Guldner L, Monfort C, Rouget F, *et al.* (2007) Maternal fish and shellfish intake and pregnancy outcomes: a prospective cohort study in Brittany, France. *Environ Health* 6, 33.
24. Jaddoe VW, van Duijn CM, van der Heijden AJ, *et al.* (2008) The Generation R Study: design and cohort update until the age of 4 years. *Eur J Epidemiol* 23, 801-811.
25. Jaddoe VW, Bakker R, van Duijn CM, *et al.* (2007) The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. *Eur J Epidemiol* 22, 917-923.
26. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, *et al.* (1998) Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. *Eur J Clin Nutr* 52, 588-596.
27. Donders-Engelen M vdHL, Hulshof K (2003) Tabel Maten en gewichten Zeist, the Netherlands: University of Wageningen.
28. Netherlands-Nutrition-Center (2006) Nevo: Dutch food composition database 2006 Netherlands Nutrition Center, The Hague, The Netherlands.
29. Verburg BO, Steegers EA, De Ridder M, *et al.* (2008) New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol* 31, 388-396.
30. Hadlock FP, Harrist RB, Sharman RS, *et al.* (1985) Estimation of fetal weight with the use of head, body, and femur measurements–a prospective study. *Am J Obstet Gynecol* 151, 333-337.
31. Bakker R, Steegers EA, Obradov A, *et al.* (2010) Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. *Am J Clin Nutr* 91, 1691-1698.
32. Pena IC, Teberg AJ & Finello KM (1988) The premature small-for-gestational-age infant during the first year of life: comparison by birth weight and gestational age. *J Pediatr* 113, 1066-1073.
33. Barker DJ (2006) Adult consequences of fetal growth restriction. *Clin Obstet Gynecol* 49, 270-283.
34. Ay L, Kruithof CJ, Bakker R, *et al.* (2009) Maternal anthropometrics are associated with fetal size in different periods of pregnancy and at birth. The Generation R Study. *Bjog* 116, 953-963.
35. de Goede J, Geleijnse JM, Boer JM, *et al.* (2010) Marine (n-3) fatty acids, fish consumption, and the 10-year risk of fatal and nonfatal coronary heart disease in a large population of Dutch adults with low fish intake. *J Nutr* 140, 1023-1028.
36. (1998) Basisrapportage en tabellen voedselconsumptiepeiling 1997-1998. Zeist: TNO.
37. Welch AA, Lund E, Amiano P, *et al.* (2002) Variability of fish consumption within the 10 European countries participating in the European Investigation into Cancer and Nutrition (EPIC) study. *Public Health Nutr* 5, 1273-1285.
38. Mendez MA, Plana E, Guxens M, *et al.* (2010) Seafood consumption in pregnancy and infant size at birth: results from a prospective Spanish cohort. *J Epidemiol Community Health* 64, 216-222.
39. Olsen SF & Secher NJ (2002) Low consumption of seafood in early pregnancy as a risk factor for preterm delivery: prospective cohort study. *Bmj* 324, 447.

40. Thorsdottir I, Birgisdottir BE, Halldorsdottir S, *et al.* (2004) Association of fish and fish liver oil intake in pregnancy with infant size at birth among women of normal weight before pregnancy in a fishing community. *Am J Epidemiol* 160, 460-465.
41. Donahue SM, Rifas-Shiman SL, Olsen SF, *et al.* (2009) Associations of maternal prenatal dietary intake of n-3 and n-6 fatty acids with maternal and umbilical cord blood levels. *Prostaglandins Leukot Essent Fatty Acids* 80, 289-296.
42. Konishi K, Sasaki S, Kato S, *et al.* (2009) Prenatal exposure to PCDDs/PCDFs and dioxin-like PCBs in relation to birth weight. *Environ Res* 109, 906-913.
43. Sagiv SK, Tolbert PE, Altshul LM, *et al.* (2007) Organochlorine exposures during pregnancy and infant size at birth. *Epidemiology* 18, 120-129.
44. Sonneborn D, Park HY, Petrik J, *et al.* (2008) Prenatal polychlorinated biphenyl exposures in eastern Slovakia modify effects of social factors on birthweight. *Paediatr Perinat Epidemiol* 22, 202-213.
45. Hertz-Picciotto I, Charles MJ, James RA, *et al.* (2005) In utero polychlorinated biphenyl exposures in relation to fetal and early childhood growth. *Epidemiology* 16, 648-656.
46. Rylander L, Stromberg U, Dyremark E, *et al.* (1998) Polychlorinated biphenyls in blood plasma among Swedish female fish consumers in relation to low birth weight. *Am J Epidemiol* 147, 493-502.
47. Baars AJ, Bakker MI, Baumann RA, *et al.* (2004) Dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs: occurrence and dietary intake in The Netherlands. *Toxicol Lett* 151, 51-61.
48. De Mul A, Bakker MI, Zeilmaker MJ, *et al.* (2008) Dietary exposure to dioxins and dioxin-like PCBs in The Netherlands anno 2004. *Regul Toxicol Pharmacol* 51, 278-287.
49. Patandin S, Koopman-Esseboom C, de Ridder MA, *et al.* (1998) Effects of environmental exposure to polychlorinated biphenyls and dioxins on birth size and growth in Dutch children. *Pediatr Res* 44, 538-545.
50. Grandjean P, Bjerve KS, Weihe P, *et al.* (2001) Birthweight in a fishing community: significance of essential fatty acids and marine food contaminants. *Int J Epidemiol* 30, 1272-1278.
51. Longnecker MP, Klebanoff MA, Brock JW, *et al.* (2005) Maternal levels of polychlorinated biphenyls in relation to preterm and small-for-gestational-age birth. *Epidemiology* 16, 641-647.
52. Weisskopf MG, Anderson HA, Hanrahan LP, *et al.* (2005) Maternal exposure to Great Lakes sport-caught fish and dichlorodiphenyl dichloroethylene, but not polychlorinated biphenyls, is associated with reduced birth weight. *Environ Res* 97, 149-162.
53. van Leeuwen SP, Leonards PE, Traag WA, *et al.* (2007) Polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls in fish from the Netherlands: concentrations, profiles and comparison with DR CALUX bioassay results. *Anal Bioanal Chem* 389, 321-333.
54. Polder A, Thomsen C, Lindstrom G, *et al.* (2008) Levels and temporal trends of chlorinated pesticides, polychlorinated biphenyls and brominated flame retardants in individual human breast milk samples from Northern and Southern Norway. *Chemosphere* 73, 14-23.
55. Murphy LE, Gollenberg AL, Buck Louis GM, *et al.* (2010) Maternal serum preconception polychlorinated biphenyl concentrations and infant birth weight. *Environ Health Perspect* 118, 297-302.

Supplement 2.1.1 Associations between maternal fish consumption, fetal growth and growth characteristics at birth

Total fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	653	Ref		641	Ref		541	Ref	
1-69 g/week	878	0.5	-0.1, 1.2	872	**1.7**	**0.8, 2.7***	742	0.3	-0.0, 0.5
70-139 g/week	1061	-0.1	-0.7, 0.5	1058	0.6	-0.3, 1.5	895	0.1	-0.1, 0.4
140-209 g/week	494	0.1	-0.6, 0.8	487	**1.8**	**0.8, 2.9***	422	0.2	-0.1 0.5
>210 g/week	221	0.5	-0.5, -1.4	218	0.9	-0.5, 2.3	175	0.1	-0.3 0.5
P for trend		*0.88*			*0.06*			*0.80*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	650	Ref		652	Ref		557	Ref	
1-69 g/week	877	0.0	-0.2, 0.2	879	0.1	-0.1, 0.3	758	**0.4**	**0.1, 0.7***
70-139 g/week	1064	-0.1	-0.3, 0.1	1067	0.0	-0.2, 0.2	911	0.1	-0.2, 0.4
140-209 g/week	494	-0.2	-0.4, 0.1	492	0.1	-0.2, 0.3	428	0.3	-0.1, 0.6
>210 g/week	221	0.0	-0.3, 0.3	220	-0.2	-0.6, 0.1	177	-0.1	-0.5, 0.4
P for trend		*0.52*			*0.72*			*0.92*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	647	Ref		651	Ref		667	Ref	
1-69 g/week	871	2.7	-1.6, 7.1	874	15.2	-3.8 34.2	894	34.8	-10.0, 79.5
70-139 g/week	1061	-0.1	-4.3, 4.1	1062	-8.1	-26.4, 10.1	1080	24.4	-18.6, 67.5
140-209 g/week	491	-0.9	-5.9, 4.1	491	7.5	-14.5 29.5	501	45.1	-6.7, 96.9
>210 g/week	221	4.9	-1.6, 11.5	220	-0.1	-10.2, 15.5	225	6.00	-28.6, 40.5
P for trend		*0.84*			*0.80*			*0.36*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of fish consumption compared to the reference group. **Models are only adjusted for gestational age at measurement and fetal sex.*P-value <0.05**

Supplement 2.1.2 Associations between maternal lean fish consumption, fetal growth and growth characteristics at birth

Lean fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	1239	Ref		1219	Ref		1017	Ref	
1-35 g/week	849	0.2	-0.3, 0.8	850	0.8	-0.0, 1.6	724	0.0	-0.1, 0.3
35-69 g/week	813	0.2	-0.3, 0.8	804	**1.1**	**0.3, 2.0***	697	0.1	-0.0, 0.4
>70 g/week	406	0.2	-0.5, 0.9	403	0.3	-0.8, 1.3	337	0.1	-0.2, 0.4
P for trend		*0.34*			*0.08*			*0.39*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	1233	Ref		1234	Ref		1044	Ref	
1-35 g/week	853	0.0	-0.2, 0.2	857	0.1	-0.1, 0.3	742	0.1	-0.1, 0.4
35-69 g/week	814	-0.1	-0.3, 0.0	812	0.1	-0.1, 0.3	711	0.2	-0.1, 0.4
>70 g/week	406	-0.2	-0.6, 0.1	407	-0.1	-0.4, 0.1	334	0.0	-0.3, 0.4
P for trend		*0.16*			*0.46*			*0.51*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	1226	Ref		1230	Ref		1262	Ref	
1-35 g/week	849	0.4	-3.4, 4.1	852	4.8	-11.7, 21.2	869	24.6	-14.1, 63.2
35-69 g/week	810	-1.4	-5.2, 2.4	810	8.8	-7.9, 25.5	823	**45.9**	**6.7, 85.2***
>70 g/week	406	0.0	-4.8, 4.8	406	-4.3	-25.4, 16.9	413	-11.4	-61.1, 38.4
P for trend		*0.74*			*0.88*			*0.58*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of lean fish consumption compared to the reference group. **Models are only adjusted for gestational age at measurement and fetal sex**. ***P*-value <0.05**

Supplement 2.1.3 Associations between maternal fatty fish consumption, fetal growth and growth characteristics at birth

Fatty fish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	1083	Ref		1064	Ref		912	Ref	
1-35 g/week	719	0.1	-0.5, 0.7	725	0.7	-0.1, 1.6	610	0.1	-0.2, 0.3
35-69 g/week	911	-0.2	-0.7, 0.4	900	-0.0	-0.8, 0.8	764	0.0	-0.2, 0.2
>70 g/week	594	-0.1	-0.7, 0.5	587	0.5	-0.4, 1.4	489	0.1	-0.2, 0.4
P for trend		*0.68*			*0.25*			*0.75*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	1097	Ref		1081	Ref		934	Ref	
1-35 g/week	723	-0.1	-0.2, 0.1	729	-0.0	-0.2, 0.2	626	0.1	-0.2, 0.4
35-69 g/week	911	-0.1	-0.3, 0.1	908	-0.1	-0.3, 0.1	774	0.1	-0.1, 0.4
>70 g/week	593	-0.1	-0.3, 0.1	592	-0.1	-0.3, 0.2	497	0.0	-0.2, 0.3
P for trend		*0.39*			*0.46*			*0.99*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	1074	Ref		1077	Ref		1100	Ref	
1-35 g/week	719	-0.1	-4.1, 4.0	725	0.1	-17.6, 17.8	737	-6.8	-48.6, 35.0
35-69 g/week	910	-0.6	-4.3, 3.2	907	-10.6	-27.3, 6.0	928	19.0	-20.2, 58.1
>70 g/week	588	-0.9	-5.2, 3.4	589	-8.6	-27.5, 10.3	602	6.4	-38.0, 50.8
P for trend		*0.72*			*0.38*			*0.40*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of fatty fish consumption compared to the reference group. Models are only adjusted for gestational age at measurement and fetal sex.

Supplement 2.1.4 Associations between maternal shellfish consumption, fetal growth and growth characteristics at birth

Shellfish consumption	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3307			Head circumference (mm) *n*=3276			Head circumference (cm) *n*=2775		
0	2053	Ref		2027	Ref		1693	Ref	
1-13 g/week	604	0.2	-0.3, 0.8	609	0.8	-0.0, 1.6	520	-0.0	-0.2, 0.2
>14 g/week	650	0.1	-0.4, 0.7	640	0.2	-0.6, 1.0	561	0.0	-0.2, 0.3
P for trend		*0.40*			*0.50*			*0.83*	
	Femur length (mm) *n*=3306			Femur length (mm) *n*=3310			Birth length (cm) *n*=2831		
0	2049	Ref		2046	Ref		1747	Ref	
1-13 g/week	608	-0.1	-0.2, 0.1	618	-0.0	-0.2, 0.2	528	0.0	-0.3, 0.3
>14 g/week	649	**-0.2**	**-0.3, -0.0***	646	-0.2	-0.4, 0.0	556	-0.0	-0.3, 0.2
P for trend		*0.13*			*0.21*			*0.60*	
	Estimated fetal weight (g) *n*=3291			Estimated fetal weight (g) *n*=3298			Birth weight (g) *n*=3367		
0	2042	Ref		2040	Ref		2090	Ref	
1-13 g/week	604	-0.8	-4.7, 3.1	615	1.9	-15.1, 18.9	620	-12.5	-52.8, 27.7
>14 g/week	645	-0.9	-4.7, 2.9	643	-12.6	-29.3, 4.1	657	-28.8	-68.1, 10.6
P for trend		*0.64*			*0.19*			*0.20*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of shellfish consumption compared to the reference group. Models are only adjusted for gestational age at measurement and fetal sex. **P*-value <0.05

Supplement 2.1.5 Associations between maternal fish consumption and risks of neonatal complications

	Neonatal complications											
	Preterm birth (*n*=334)				Low birth weight (*n*=138)				Small for gestational age (*n*=205)			
Fish consumption	*n*	cases	OR	95% CI	*n*	cases	OR	95% CI	*n*	cases	OR	95% CI
Total fish consumption												
0	668	34	Ref		667	34	Ref		667	51	Ref	
1-69 g/week	897	45	0.99	0.62, 1.56	894	29	**0.49**	**0.25, 0.95***	893	57	0.81	0.55, 1.20
70-139 g/week	1085	47	0.85	0.54, 1.33	1080	46	0.84	0.47, 1.51	1079	60	0.70	0.48, 1.03
140-209 g/week	502	20	0.78	0.44, 1.37	501	16	0.66	0.30, 1.43	501	25	0.63	0.38, 1.02
>210 g/week	226	13	1.14	0.59, 2.20	225	13	0.93	0.39, 2.21	225	12	0.67	0.35, 1.28
P for trend			*0.54*				*0.86*				*0.10*	
Lean fish consumption												
0	1267	75	Ref		1262	62	Ref		1260	91	Ref	
1-35 g/week	869	32	**0.61**	**0.40, 0.93***	869	27	0.67	0.37, 1.21	869	46	0.71	0.49, 1.02
35-69 g/week	828	38	0.77	0.51, 1.14	823	32	0.81	0.45, 1.44	823	37	**0.60**	**0.41, 0.89***
>70 g/week	414	14	0.56	0.31, 1.00	413	17	1.22	0.62, 2.40	413	31	1.03	0.68, 1.58
P for trend			*0.49*				*0.85*				*0.54*	
Fatty fish consumption												
0	1104	58	Ref		1100	50	Ref		1099	75	Ref	
1-35 g/week	739	26	0.66	0.41, 1.06	737	18	0.66	0.34, 1.30	737	51	1.00	0.70, 1.45
35-69 g/week	931	48	0.98	0.66, 1.46	928	47	1.23	0.72, 2.13	927	49	0.75	0.52, 1.09
>70 g/week	604	27	0.84	0.53, 1.35	602	23	1.04	0.55, 1.98	602	30	0.71	0.46, 1.09
P for trend			*0.35*				*0.80*				***0.04***	
Shellfish consumption												
0	2098	103	Ref		2090	87	Ref		2089	120	Ref	
1-13 g/week	620	24	0.78	0.50, 1.23	620	18	0.83	0.43, 1.57	620	44	1.24	0.87, 1.78
>14 g/week	660	32	0.99	0.66, 1.48	657	33	1.64	0.95, 2.83	656	41	1.08	0.75, 1.56
P for trend			*0.43*				*0.86*				*0.99*	

Values are bases on multiple logistic regression models and reflect the odds ratio and 95% confidence interval for neonatal complications for each level of fish consumption compared to the reference group. **Models are only adjusted for gestational age at measurement and fetal sex. **P*-value <0.05**

CHAPTER 2.2

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Am J Clin Nutr. 2011 Aug;94(2):501-9.

MATERNAL MILK CONSUMPTION, FETAL GROWTH AND THE RISK OF NEONATAL COMPLICATIONS

ABSTRACT

Background Maternal cow's milk consumption may increase birth weight. Previous studies did not assess the association of maternal milk consumption with trimester specific fetal growth.

Objective To assess associations of first trimester maternal milk consumption with fetal growth characteristics in different trimesters and the risk of neonatal complications.

Design In total, 3,405 mothers participating in a prospective cohort study completed a 293-item semi-quantitative food frequency questionnaire to obtain information about dairy consumption during the first trimester of pregnancy. Fetal head circumference, femur length, and weight were estimated in the second and third trimester by ultrasound.

Results Maternal milk consumption of >3 glasses/d was associated with greater fetal weight gain in the third trimester of pregnancy, leading to a 88 g (95% confidence interval (CI) 39, 135) higher birth weight, as compared to milk consumption of 0-1 glass/d. Also, head circumference tended to be 2.3 cm (95% CI -0.0, 4.6) larger when mothers consumed >3 glasses/d. Maternal milk consumption was not associated with length growth. Maternal protein intake (*P* for trend 0.01), but not fat or carbohydrate intake, from dairy products was associated with higher birth weight. This association appeared to be limited to milk (*P* for trend <0.01), whereas protein intake from non-dairy food or cheese was not associated with birth weight.

Conclusions Maternal milk consumption is associated with greater fetal weight gain. The association seems to be due to milk protein, or milk components closely associated with protein, rather than the fat or carbohydrate fraction of milk.

INTRODUCTION

Maternal nutrition is one of the major environmental exposures influencing fetal growth and development.[1-4] Maternal cow's milk consumption was associated with a higher neonatal weight, length, abdominal and head circumference in a Danish study.[5] However, results from earlier studies on the associations of maternal milk consumption with birth weight are not consistent.[6-12] Cow's milk contains various nutrients considered to be beneficial for fetal growth and development, including protein, B vitamins and minerals.[13] In both children and adults milk consumption increases blood concentration of insulin growth factor I (IGF-I), a peptide hormone and key regulator of postnatal growth.[14-20] Results from a recent randomized clinical trial suggested that, specifically the major protein fraction of milk, casein, increased IGF-I blood concentration.[21] In the Danish study, intake of protein from milk, but not from cheese, was associated with higher birth weight.[5] However, most previous studies assessing the associations of milk consumption and birth outcomes lacked information on specific intake of macronutrients from milk. Also, previous studies were not able to assess the associations between milk consumption and trimester specific fetal growth characteristics, but focused on birth weight as outcome. However, different fetal growth patterns may result in the same birth weight. Assessing fetal growth characteristics in different periods of pregnancy may give information about specific critical periods. Therefore, in a population-based prospective cohort study of 3,405 mothers and their children, we assessed the associations of first trimester maternal milk consumption and its constituents with fetal growth characteristics in different periods of pregnancy and the risks of neonatal complications.

METHODS

Study design

The present study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in the city of Rotterdam, the Netherlands.[22] The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all mothers. Mothers were enrolled during pregnancy between 2001 and 2005, and all children were born between April 2002 and January 2006. Of all eligible children in the study area, 61% participated at birth in the study.[22] Enrolment was aimed at early pregnancy (gestational age <18 weeks) at the routine fetal ultrasound examination in pregnancy, but was allowed until birth of the child. For the present study, we performed analyses in Dutch participants. Of the total group of enrolled Dutch mothers (*n*=4,057), 98% (*n*=3,979) were

enrolled in the first or second trimester of pregnancy. From these mothers 88% (*n*=3,482) fully completed the food frequency questionnaire. We excluded twin pregnancies 1.4% (*n*=50) and pregnancies leading to intrauterine death 0.7% (*n*=24) from the analyses and 0.1% (*n*=3) was lost to follow-up. We performed the present study in the remaining 3,405 mother-child pairs. (Flowchart is given in Supplement 2.2.1).

Milk consumption assessment

We assessed maternal dietary intake, including consumption of milk, at enrolment in the study (median 13.5 weeks of gestation, 95% range 10.8-21.1) using a modified version of the validated semi quantitative food frequency questionnaire (FFQ) of Klipstein-Grobusch *et al.*[23] This FFQ considered food intake over the prior three months, thereby covering dietary intake within first trimester of pregnancy. The FFQ consists of 293 items structured according to meal pattern. Questions include consumption frequency, portion size, preparation method, and additions. Portion sizes were estimated using Dutch household measures and photographs of foods showing different portion sizes.[24] To calculate average daily nutritional values we used the Dutch food composition table 2006.[13] For the assessment of milk consumption additional questions were asked about use of skimmed, semi-skimmed, and full-fat milk products, sweetened milk products, milk products with additional fruit and milk products enriched with vitamins or extra calcium. To obtain frequency measures of milk consumption we summed consumption of milk and milk drinks. According to the Dutch household measures, one glass of milk on average contains 150 mL milk.[24] For the analysis of dairy consumption, we added yoghurt, yoghurt drinks, cheese, butter, quark, pudding, ice cream (dairy cream based) and cream/creamers. Subsequently, we quantified daily intake of macronutrients from dairy consumption using the Dutch food composition table.[13]

Fetal growth characteristics

Fetal ultrasound examinations were carried out at one of the two research centers in each trimester of pregnancy. Medians (95% range) of these visits were 12.9 (11.0-16.8), 20.5 (19.0-22.6) and 30.4 (28.9-32.2) weeks of gestation for the first, second and third trimester respectively. Gestational age was established by the first fetal ultrasound examination, since using last menstrual period has several limitations, such as the large number of mothers who do not know the exact date of their last menstrual period or have irregular menstrual cycles.[25] In second and third trimesters of pregnancy, we measured fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) to the nearest millimeter using standardized ultrasound procedures.[25] In this study we considered femur length as a proxy for length growth. Estimated fetal weight (EFW) was calculated by means of the formula from Hadlock using head circumference, abdominal

circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[26]

Neonatal complications

Information about offspring sex, gestational age, weight, length, and head circumference at birth was obtained from medical records and hospital registries. Preterm birth was defined as gestational age of less than 37 weeks at delivery. Small size for gestational age at birth was defined as a gender and gestational age-adjusted birth weight below the 5th percentile in the study cohort (< -1.62 standard deviation score (SDS)). Large size for gestational age at birth was defined as a gender and gestational age-adjusted birth weight above the 95th percentile in the study cohort (>1.67 SDS).

Covariates

Maternal age was registered at enrolment. Information about educational level, marital status, parity and periconceptional folic acid supplement use was obtained by questionnaire at enrolment in the study. Frequency of nausea and vomiting and smoking and alcohol habits were assessed by questionnaires in each trimester. Maternal anthropometrics, including height and weight, were measured without shoes and heavy clothing in the first, second and third trimester at the research center. Information about maternal weight just before pregnancy was obtained by questionnaire. As enrolment in our study was in pregnancy, we were not able to measure maternal weight before pregnancy. Correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was 0.97 (P<0.01).

Statistical analysis

We analyzed milk consumption in four categories based on the distribution of milk consumption among mothers (0-1, >1-2, >2-3 and >3 glasses/d). We used the lowest category as the reference category in all models. First, we performed multiple linear regression analyses to assess the association of milk consumption with fetal growth characteristics. Second, to assess potential non-linear longitudinal effects, we used mixed effect models with unstructured residual covariance to longitudinally model fetal growth SDS from 20 weeks of pregnancy until birth by natural cubic splines.[27] We positioned interior knots of the spline based on moments of data collection (18, 23, 30, 37 and 43.4 weeks). The models include a separate spline models for each milk consumption category. We performed a multiple F-test to test for a difference between the splines of each milk consumption category as compared to the lowest reference category. Third, we used multiple logistic regression models to assess the association of milk consumption with the risk of neonatal complications (preterm birth, small or large for gestational age). Finally, we used multiple linear regression models to assess the association of macro-

nutrient (fat, protein, carbohydrates) intake from dairy products and protein intake from total diet, dairy products, non-dairy products, milk, and cheese with birth weight. We categorized macronutrient intake in quintiles and mutually adjusted the multiple analyses for the intake of other macronutrients. We performed trend tests by using milk, dairy or macronutrient consumption as continuous variable in the analyses. We adjusted models for potential confounders based on results from previous studies, including maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee and paternal height.[5,8-12,28-32] We used multiple imputations to complete missing data on the covariates maternal pre-pregnancy BMI (14% missing), parity (0.2%), marital status (2%), educational level (0.6%) smoking (8%), alcohol use (7%), folic acid supplement use (17%), nausea (0.1%), vomiting (0.1%) and paternal height (11%).[33] Imputations were based on the relations between all covariates in the study. All measures of association are presented with their 95% confidence intervals (95% CI). *P*-values are two-sided. Spline regression analyses were performed using the Statistical Analysis System (version 9.2; SAS Institute Inc., Cary NC) and other analyses were performed using the Predictive Analytic Software version 17.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

The median of reported milk consumption was 2.6 glasses/d (inter-quartile range 2.1 glass/d). As indicated in **Table 2.2.1**, mothers with higher milk consumption tended to have a higher energy intake, lower age and higher BMI, and more frequently smoked, but less frequently finished higher education (all $P<0.01$). Of all births, 4.7% were born preterm, 5.0% were small and 5.0% were large for gestational age at birth. Fetal growth characteristics measured during second and third trimester of pregnancy were available in 98.6% and 98.1% of the mothers respectively.

Milk consumption, fetal growth and neonatal outcomes

Results from the linear regression analyses, which are given in the **Table 2.2.2**, showed that some milk intake categories were associated with increased fetal head circumference or estimated fetal weight in second and third trimester of pregnancy. However, we did not find evidence for a dose response relationship, since the *P* for trend of these findings was not significant. Maternal milk consumption of >2-3 glasses was associated with a 2.2 cm (95% 0.2, 4.2) larger head circumference at birth (*P* for trend 0.03). Maternal milk consumption was not associated with fetal length characteristics or length at birth. Maternal milk consumption was positively associated with birth weight (*P* for

Table 2.2.1 Maternal and fetal characteristics according to maternal milk consumption during pregnancy

		Milk consumption (glasses/d)				
	All	0-1 (Ref)	>1-2	>2-3	>3	
	n=3,405	*n*=990	*n*=805	*n*=940	*n*=670	*P*-value
Maternal characteristics						
Age – years	31.4 ± 4.4	31.4 ± 4.4	31.8 ± 4.3*	31.4 ± 4.3	30.9 ± 4.6*	0.01
Height – cm	170.9 ± 6.4	170.5 ± 6.4	171.2 ± 6.4*	170.9 ± 6.3	171.0 ± 6.5	0.25
Body mass index – kg/m^2	23.2 ± 3.9	23.0 ± 3.9	23.1 ± 3.8	23.4 ± 3.9*	23.5 ± 4.2*	<0.01
Total energy intake – kJ	2145 ± 511	1953 ± 503	2101 ± 479*	2185 ± 471*	2424 ± 482*	<0.01
Parity ≥1 – %	39.8	38.3	36.9	43.9*	39.7	0.11
Missing	0.2	0.4	0.1	0	0	
Married / living together – %	91.3	90.0	92.3	93.1*	89.4	0.61
Missing	1.9	2.1	1.5	1.7	2.5	
High education – %	58.9	59.3	65.6*	58.6	50.6*	<0.01
Missing	0.6	0.5	0.2	1.0	0.4	
Smoking – %						
Never	69.6	71.2	69.9	71.7	63.7*	0.01
First trimester only	8.1	7.9	8.6	7.9	8.1	0.97
Continued	14.7	13.5	13.7	13.4	19.6*	<0.01
Missing	7.6	7.4	7.8	7.0	8.7	
Alcohol use – %						
Never	31.3	31.6	26.7*	33.0	33.9	0.08
First trimester only	15.4	16.0	14.0	14.4	17.5	0.52
Continued	46.0	45.2	52.2*	46.0	40.0	0.03
Missing	7.3	7.3	7.1	6.7	6.9	
Folic acid supplement use – %						
Preconception start	46.4	43.8	48.2	48.3	45.4	0.64
Post conception start	27.2	27.9	26.8	26.5	27.5	0.49
None	8.9	9.2	8.0	8.7	9.9	0.78
Missing	17.5	19.1	17.0	16.5	17.3	
Fetal characteristics						
Trimester 2						
Head circumference – mm	179 ± 13	179 ± 13	179 ± 13	180 ± 13	180 ± 14	0.48
Femur length – mm	33.4 ± 3.3	33.4 ± 3.3	33.3 ± 3.3	33.4 ± 3.3	33.3 ± 3.4	0.96
Estimated fetal weight – g	379 ± 87	377 ± 83	379 ± 86.9	381 ± 86	380 ± 92	0.42
Trimester 3						
Head circumference – mm	286 ± 12	285 ± 12	286 ± 12	287 ± 12*	285 ± 11	0.64
Femur length – mm	57.5 ± 2.9	57.5 ± 3.0	57.5 ± 2.8	57.5 ± 2.9	57.3 ± 2.9	0.47
Estimated fetal weight – g	1632 ± 256	1626 ± 254	1635 ± 253	1645 ± 256	1621 ± 262	0.84

Table 2.2.1 (continued)

		Milk consumption (glasses/d)				
	All	0-1 (Ref)	>1-2	>2-3	>3	
	n=3,405	*n*=990	*n*=805	*n*=940	*n*=670	*P*-value
Birth outcomes						
Males – %	50.5	48.5	49.4	53.3*	50.6	0.13
Gestational age – weeks	40.0 ± 1.7	40.0 ± 1.7	39.9 ± 1.7	40.0 ± 1.7	39.9 ± 1.7	0.71
Birth weight – g	3489 ± 556	3446 ± 554	3489 ± 558	3521 ± 548*	3508 ± 563*	<0.01
Birth length – cm	50.5 ± 2.4	50.4 ± 2.4	50.5 ± 2.5	50.5 ± 2.3	50.4 ± 2.9	0.46
Head circumference – cm	34.0 ± 1.6	33.9 ± 1.7	34.0 ± 1.7	34.1 ± 1.6	34.1 ± 1.7	0.12
Preterm birth – %	4.7	4.7	5.3	3.9	4.9	0.74
Small for gestational age – %	5.0	5.3	5.1	4.4	5.2	0.72
Large for gestational age – %	5.4	4.0	5.3	6.5*	6.1*	0.02

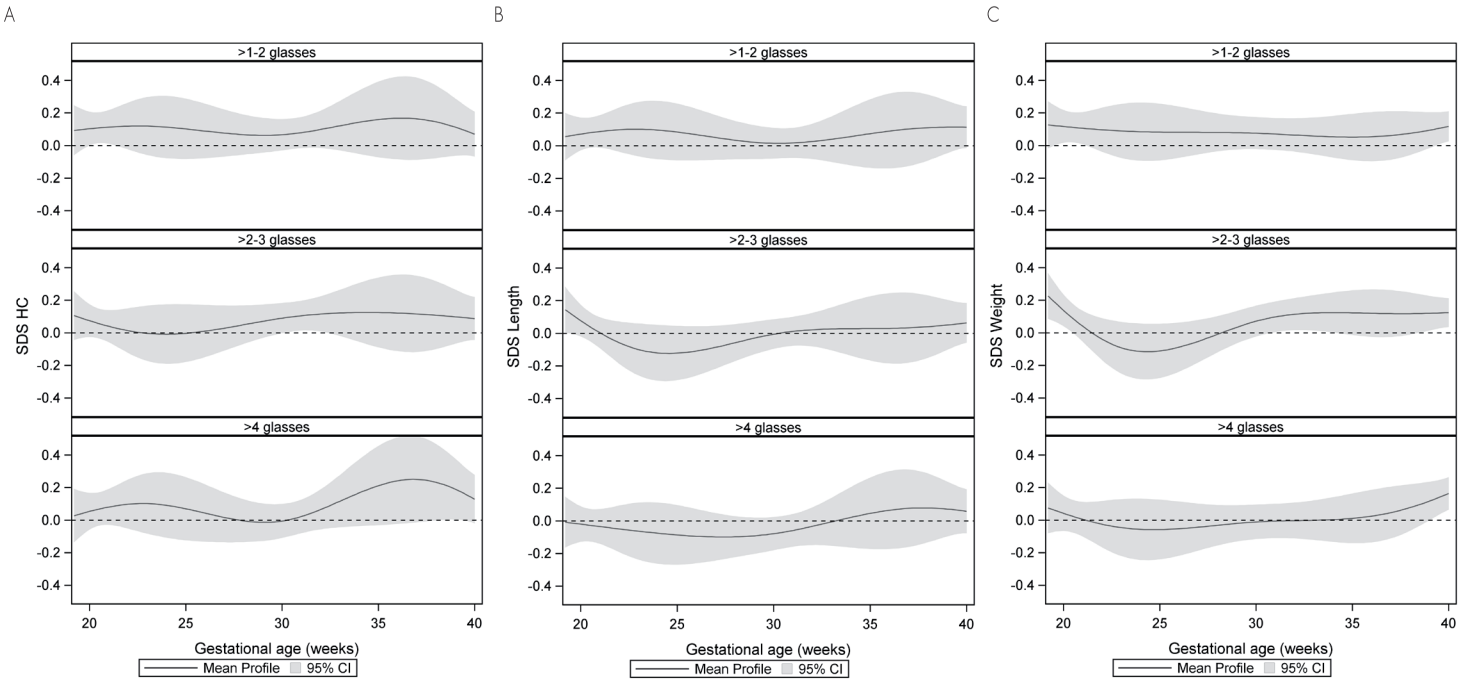
Values reflect the mean ± standard deviation for continuous variables and percentage for categorical variables. *P*-values are obtained by univariate regression models. **P*-value <0.05

trend < 0.01). The birth weight difference between the highest and lowest category of milk consumption was 88 g (95% CI 39, 135).

Figure 2.2.1.A-C shows the associations of maternal milk consumption with longitudinally measured fetal growth (head circumference, length and weight) between the gestational ages of 20 and 40 weeks. To easily compare effect estimates throughout pregnancy, results are presented as differences in gestational age–adjusted SD scores. Figure 2.2.1.A and 2.2.1.B show that we found no consistent associations of maternal milk consumption with longitudinally measured fetal head circumference or length. Figure 2.2.1.C shows that maternal milk consumption of >1-2 glasses/d (*P*<0.01), 2-3 glasses/d (*P*=0.01) and >3 glasses/d (*P*=0.06) was associated with increased fetal weight gain, as compared to the lowest reference category of consuming 0-1 glass/d. (The effect estimates of fetal growth differences at 40 weeks of pregnancy are presented in Supplement 2.2.2). Differences in fetal weight gain appeared from 20 weeks onwards, but became most evident in the last part of the third trimester.

Table 2.2.3 shows that maternal milk consumption was not associated with the risk of preterm birth or the risk of a small or large size for gestational age at birth in the offspring. Results of the unadjusted analyses are presented in Supplement 2.2.3 and 2.2.4. To exclude a potential effect of parity, we performed a sensitivity analysis by restricting the study population to nulliparous women. The effect estimates were only slightly changed. Also, performing complete case analysis did not significantly change our results. (Results not shown)

Figure 2.2.1 Associations of maternal milk consumption with longitudinally measured fetal growth



Figures are based on spline regression models of longitudinally measured **A) head circumference growth** (*n* = 8,542), **B) length growth** (*n* = 8,947) and **C) weight growth** (*n* = 10,025) for each level of milk consumption compared to the lowest reference group. *P*-values are based on multivariate *F*-tests and reflect the difference between the spline of each milk consumption category as compared to the lowest reference category. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height and fetal sex. Effect estimates of fetal growth differences at 40 weeks of pregnancy are given in **Supplement 2.2.2**

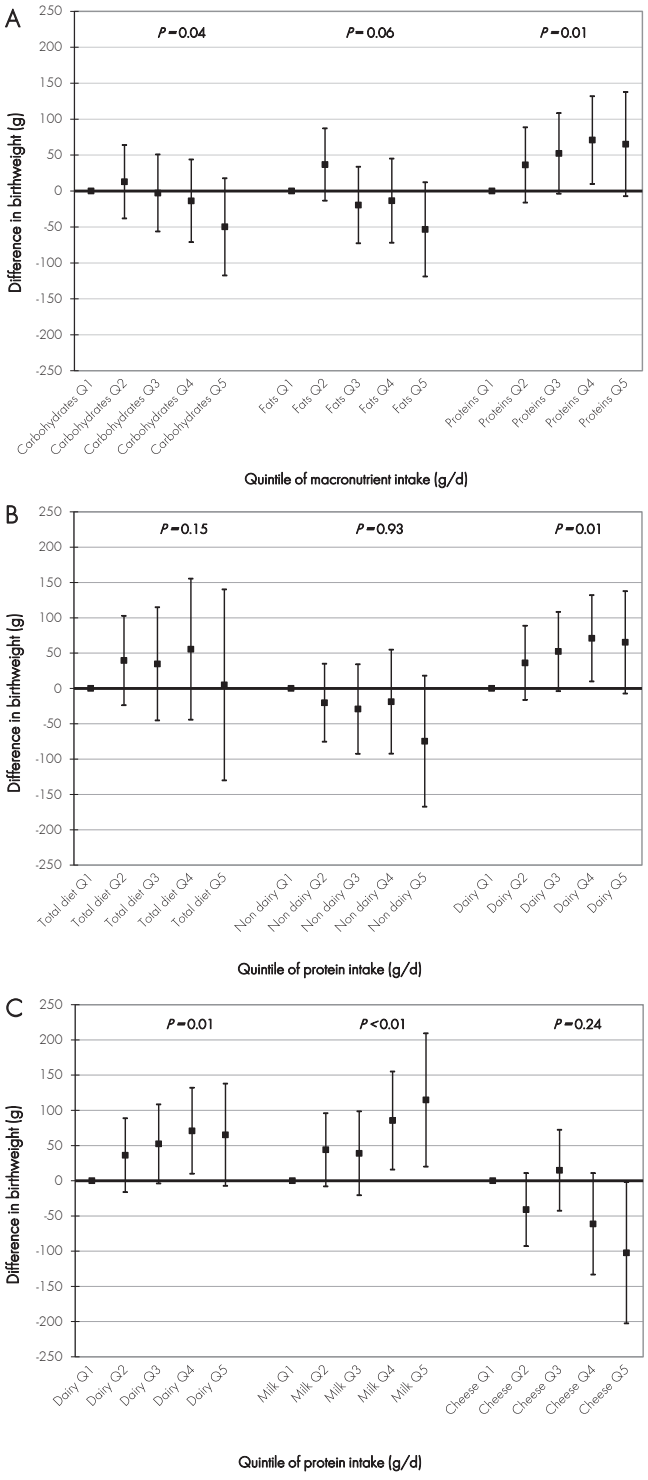


Figure 2.2.2 Associations of maternal macronutrient intake with offspring birth weight

Figures are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of **A) macronutrient intake from dairy products, B) protein intake from total diet, non-dairy products or dairy products, and C) protein intake from dairy products, milk, or cheese consumption** compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. Corresponding effect estimates are presented in Supplement **2.2.5**, **2.2.6** and **2.2.7**. **P*-value <0.05

Table 2.2.2 Associations of maternal milk consumption with fetal growth

Milk consumption (glasses/d)	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3331			Head circumference (mm) *n*=3301			Head circumference (mm) *n*=1936		
0-1	959	Ref		969	Ref		564	Ref	
>1-2	784	**0.8**	**0.1, 1.4***	773	**1.0**	**0.0, 1.9***	455	0.9	-1.2, 3.0
>2-3	926	0.3	-0.3, 0.9	911	**1.0**	**0.1, 1.9***	551	**2.2**	**0.2, 4.2***
>3	662	0.4	-0.3, 1.1	648	0.2	-0.8, 1.2	366	2.3	-0.0, 4.6
P for trend		*0.86*			*0.38*			***0.03***	
	Femur length (mm) *n*=3330			Femur length (mm) *n*=3334			Birth length (mm) *n*=2321		
0-1	965	Ref		974	Ref		663	Ref	
>1-2	783	0.1	-0.1, 0.3	784	-0.0	-0.3, 0.2	545	2.2	-0.3, 4.8
>2-3	922	0.1	-0.0, 0.3	919	0.0	-0.2, 0.3	653	1.7	-0.8, 4.2
>3	660	-0.1	-0.3, 0.1	657	-0.2	-0.4, 0.1	460	1.9	-0.9, 4.8
P for trend		*0.72*			*0.35*			*0.37*	
	Estimated fetal weight (g) *n*=3314			Estimated fetal weight (g) *n*=3322			Birth weight (g) *n*=3392		
0-1	961	Ref		969	Ref		987	Ref	
>1-2	779	**5.5**	**1.0, 9.9***	780	10.8	-8.4, 30.0	800	**63.8**	**20.3, 107***
>2-3	921	**5.2**	**0.9, 9.6***	917	15.0	-3.6, 33.6	939	**63.8**	**21.7, 106***
>3	653	2.4	-2.5, 7.3	656	-5.7	-26.9, 15.6	666	**87.5**	**39.3, 135***
P for trend		*0.36*			*0.57*			***<0.01***	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each level of milk consumption compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex and gestational age at measurement. ***P*-value <0.05**

Macronutrient intake from dairy products and birth weight

Figure 2.2.2.A shows the associations of macronutrient intake from dairy products with birth weight. Protein intake, but not fat or carbohydrates intake, from dairy products was associated with higher birth weight. (*P* for trend 0.01). Figure 2.2.2.B shows positive effect estimates for the association of maternal total protein intake with birth weight (*P* for trend 0.15). Since non-dairy protein intake shows negative effect estimates for the association with birth weight, the association of total protein intake seems more likely to be driven by protein intake from dairy products than by a general protein effect. Figure 2.2.2.C shows that the association of maternal protein intake from dairy products with birth weight was further restricted to protein intake from milk (*P* for trend < 0.01). Birth weight difference between lowest and highest quintile was 115 g (95% CI 20, 209). Protein intake from cheese was not associated with birth weight (the corresponding effect estimates are presented in Supplement 2.2.5, 2.2.6 and 2.2.7). We performed a sensitivity analysis to assess the possibility of a general effect of animal protein. Maternal

Table 2.2.3. Associations of maternal milk consumption with the risks of neonatal complications.

Milk consumption (glasses/d)	Neonatal complications											
	Preterm birth (*n*=160)				Small for gestational age (*n*=169)				Large for gestational age (*n*=185)			
	n	cases	OR	95% CI	*n*	cases	OR	95% CI	*n*	cases	OR	95% CI
0-1	943	47	Ref		938	52	Ref		951	40	Ref	
>1-2	761	43	1.12	0.68, 1.83	765	41	0.81	0.49, 1.34	762	43	1.21	0.73, 2.01
>2-3	903	37	0.82	0.49, 1.38	899	41	0.79	0.28, 2.19	879	61	1.56	0.97, 2.49
>3	636	33	1.15	0.66, 2.00	635	35	0.84	0.49, 1.43	629	41	1.59	0.94, 2.70
P for trend			*0.68*				*0.25*				*0.17*	

Values are based on multiple logistic regression models and reflect the odds ratio and 95% confidence interval for neonatal complications for each level of daily milk consumption compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex and gestational age at measurement.

total intake of animal protein was not associated with birth weight. Since removing intake of milk protein from total animal protein turned the direction of the association, the association of milk protein intake is unlikely to be driven by a general animal protein effect. (Results are presented in Supplement 2.2.8 and Supplement 2.2.9)

DISCUSSION

Main findings

In this prospective cohort study of pregnant women in the Netherlands, we found that maternal milk consumption during pregnancy was associated with a greater fetal weight gain, resulting in a higher birth weight. It seems that the growth promoting effect of milk may have impact on fetal growth throughout pregnancy, but tends to have the greatest impact on fetal growth in the third trimester of pregnancy. Furthermore, we found that specifically higher intake of protein from milk, but not intake of fats or carbohydrates from milk, was associated with a higher birth weight.

Methodological considerations

To our knowledge, this is the first study that assessed the associations of first trimester maternal milk consumption with fetal growth characteristics measured in second and third trimester. The strength of this study is that we assessed fetal growth characteristics by actually measuring fetal growth instead of using birth outcomes as a proxy for fetal growth. In total, 88% of all eligible mothers fully completed the food frequency questionnaire. Mean maternal age was 31.4 years which is slightly higher than the mean maternal age of 29.4 years in the study area.[34] Our study population was relatively high educated;

59% finished higher education, as compared to 31% in the study area.[35] This difference may be largely due to restriction of the study population to Caucasian women. Selective participation may thereby have led to biased estimates. However, it has been shown that selection bias in cohort studies primarily arise from loss to follow up, rather than non-response at baseline.[36] We prospectively collected detailed information on milk and dairy consumption which enables separate analyses. Also, we extensively collected information on many potential confounding variables. However, as in any observational study, residual confounding might still be an issue. Nonetheless, adjustment for confounders only marginally influenced the studied associations. Another limitation of the study is that the FFQ that was used was validated in an older Caucasian population. Also, we only assessed maternal milk consumption once during pregnancy. Therefore we cannot infer whether the associations are primarily due to milk consumption in first trimester or later in pregnancy.

Interpretation of main results

Our results suggest that the growth promoting effect of maternal milk consumption mainly affects fetal weight gain in the third trimester of pregnancy. The effect of milk consumption on head growth remains uncertain. Several studies assessed the associations of maternal milk consumption with birth outcomes, however, not with fetal growth characteristics. In a large cohort in Denmark, higher maternal milk consumption was associated with higher neonatal weight, length, abdominal and head circumference.[5] Likewise, we observed an association of milk consumption with offspring birth weight and a tendency toward an association of milk consumption with head circumference at birth. However, we did not observe an association of maternal milk consumption with length. This difference in results may be due to the smaller size of our study population, or, less likely, a slightly higher intake of milk in the Danish population as compared with the Dutch population. A retrospective cohort in Sweden, reported an birth weight increase of 75 g and 134 g in offspring of mothers who consumed respectively ≤2 dl and >1L milk daily.[11] In a prospective study in India, milk consumption at 28 weeks was positively associated with offspring birth weight, birth length, head circumference in rural mothers, and offspring triceps skin fold thickness in urban mothers.[8] In a prospective study in Canada, maternal daily consumption of one additional cup of milk was associated with a 41 g increase of offspring birth weight.[12] A prospective Australian study among 557 mothers, found that protein intake from dairy products was associated with higher offspring birth weight.[10] In a small randomized controlled trial of 72 adolescent pregnant mothers, 25 mothers were counseled to consume >4 servings of dairy products a day, which resulted in a 240 g higher birth weight in this group as compared with controls.[37] In a case control study of 844 small for gestational age cases and 870 normal weight controls in New

Zealand, no significant association of dairy consumption and a lower risk of being small for gestational age was observed.[9]

Our findings on macronutrient intakes from milk suggest that the growth promoting effect was specifically driven by protein intake from milk, not by protein intake from cheese or other foods. In line with our findings, protein intake, but not fat intake, from dairy products was associated with higher birth weight in the Danish study.[5] High intake of protein from dairy resulted in a 65 g higher birth weight, which is comparable to our findings. Likewise, intake of protein from non-dairy products and cheese were not associated with birth weight in the Danish study. The explanation for the difference between the effects of protein from milk and cheese on fetal growth is unknown. A methodological explanation may be that reported milk consumption is a better predictor for intake of beneficial constituents in dairy and thus leads to higher effect estimates. Alternatively, from a biological perspective, alteration of the structure of casein proteins or loss of whey proteins during the process of cheese production may lead to a different biological activity of its constituents.[38]

Potential underlying mechanisms

Milk contains various nutrients potentially beneficial for fetal growth. Milk consumption is known to increase IGF-I blood concentration in both adults and children.[17-18,39-41] Some of the previous studies that reported an association of milk protein intake with IGF-I also reported an association calcium intake with IGF-I concentration.[14-15,21,39] Since we did not measure IGF-I or calcium blood concentrations, further studies are needed to explore whether IGF-I or calcium levels are involved in the underlying mechanism for the associations between maternal milk consumption and fetal growth. Vitamin D has also been suggested to contribute to the association between milk consumption and fetal growth.[12] However, vitamin D is unlikely to explain our findings because in the Netherlands milk is not enriched with vitamin D and naturally occurring concentrations of vitamin D in milk are negligible.[42]

Conclusion

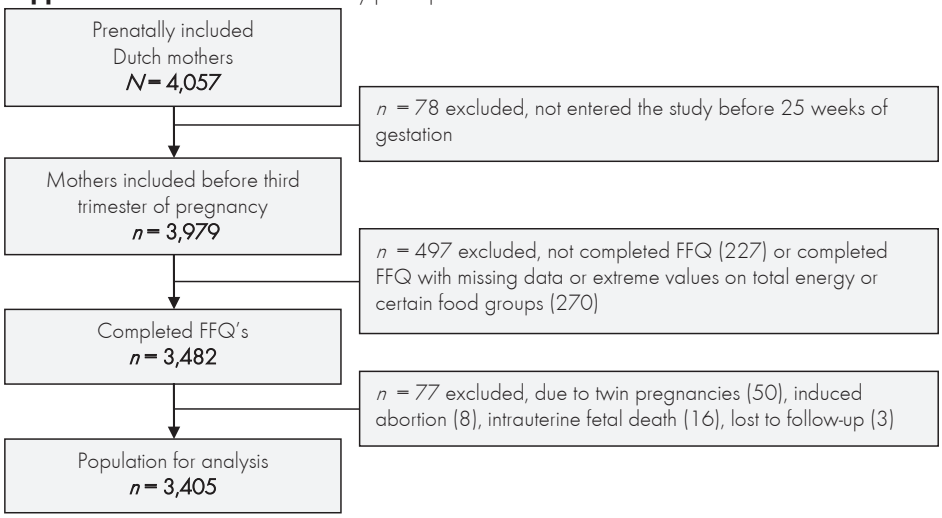
In a Dutch population-based cohort, we observed an association between higher milk consumption and greater fetal weight gain, particularly in the third trimester of pregnancy. Our results suggest that this association is mediated by the protein fraction and not the fat or carbohydrate fraction of milk. Further research is needed to clarify the mechanisms underlying these associations and the long term consequences.

REFERENCES

1. Godfrey KM, Barker DJ. Maternal nutrition in relation to fetal and placental growth. *Eur J Obstet Gynecol Reprod Biol* 1995;61:15-22.
2. Barker DJ. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* 1997;13:807-13.
3. Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop KA. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr* 2007;97:1036-46.
4. Chmurzynska A. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. *Nutr Rev* 2010;68:87-98.
5. Olsen SF, Halldorsson TI, Willett WC, *et al.* Milk consumption during pregnancy is associated with increased infant size at birth: prospective cohort study. *Am J Clin Nutr* 2007;86:1104-10.
6. Elwood PC, Haley TJ, Hughes SJ, Sweetnam PM, Gray OP, Davies DP. Child growth (0-5 years), and the effect of entitlement to a milk supplement. *Arch Dis Child* 1981;56:831-5.
7. Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *BMJ* 1996;312:410-4.
8. Rao S, Yajnik CS, Kanade A, *et al.* Intake of micronutrient-rich foods in rural Indian mothers is associated with the size of their babies at birth: Pune Maternal Nutrition Study. *J Nutr* 2001;131: 1217-24.
9. Mitchell EA, Robinson E, Clark PM, *et al.* Maternal nutritional risk factors for small for gestational age babies in a developed country: a case-control study. *Arch Dis Child* Fetal Neonatal Ed 2004;89:F431-5.
10. Moore VM, Davies MJ, Willson KJ, Worsley A, Robinson JS. Dietary composition of pregnant women is related to size of the baby at birth. *J Nutr* 2004;134:1820-6.
11. Ludvigsson JF, Ludvigsson J. Milk consumption during pregnancy and infant birthweight. *Acta Paediatr* 2004;93:1474-8.
12. Mannion CA, Gray-Donald K, Koski KG. Association of low intake of milk and vitamin D during pregnancy with decreased birth weight. *CMAJ* 2006;174:1273-7.
13. Netherlands-Nutrition-Center. Nevo: Dutch food composition database 2006 The Hague, The Netherlands., 2006.
14. Holmes MD, Pollak MN, Willett WC, Hankinson SE. Dietary correlates of plasma insulin-like growth factor I and insulin-like growth factor binding protein 3 concentrations. *Cancer Epidemiol Biomarkers Prev* 2002;11:852-61.
15. Ma J, Giovannucci E, Pollak M, *et al.* Milk intake, circulating levels of insulin-like growth factor-I, and risk of colorectal cancer in men. *J Natl Cancer Inst* 2001;93:1330-6.
16. Giovannucci E, Pollak M, Liu Y, *et al.* Nutritional predictors of insulin-like growth factor I and their relationships to cancer in men. *Cancer Epidemiol Biomarkers Prev* 2003;12:84-9.
17. Hoppe C, Udam TR, Lauritzen L, Molgaard C, Juul A, Michaelsen KF. Animal protein intake, serum insulin-like growth factor I, and growth in healthy 2.5-y-old Danish children. *Am J Clin Nutr* 2004; 80:447-52.
18. Larnkjaer A, Hoppe C, Molgaard C, Michaelsen KF. The effects of whole milk and infant formula on growth and IGF-I in late infancy. *Eur J Clin Nutr* 2009;63:956-63.
19. Gunnell D. Can adult anthropometry be used as a 'biomarker' for prenatal and childhood exposures? *Int J Epidemiol* 2002;31:390-4.
20. Juul A, Dalgaard P, Blum WF, *et al.* Serum levels of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1, IGFBP-2, age, sex, body mass index, and pubertal maturation. *J Clin Endocrinol Metab* 1995; 80:2534-42.

21. Hoppe C, Molgaard C, Dalum C, Vaag A, Michaelsen KF. Differential effects of casein versus whey on fasting plasma levels of insulin, IGF-1 and IGF-1/IGFBP-3: results from a randomized 7-day supplementation study in prepubertal boys. *Eur J Clin Nutr* 2009;63:1076-83.
22. Jaddoe VW, van Duijn CM, van der Heijden AJ, *et al*. The Generation R Study: design and cohort update 2010. *Eur J Epidemiol* 2010;25:823-41.
23. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, *et al*. Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. *Eur J Clin Nutr* 1998;52:588-96.
24. Donders-Engelen M vdHL, Hulshof K. Tabel Maten en gewichten Second ed. Zeist, the Netherlands: University of Wageningen, 2003.
25. Verburg BO, Steegers EA, De Ridder M, *et al*. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol* 2008;31:388-96.
26. Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements–a prospective study. *Am J Obstet Gynecol* 1985;151: 333-7.
27. Devlin TF, Weeks BJ. Spline functions for logistic regression modeling. Proceedings of the 11th Annual SAS Users Group International Conference: SAS Institute Inc., Cary, NC, 1986:646–651.
28. Jaddoe VW, Verburg BO, de Ridder MA, *et al*. Maternal smoking and fetal growth characteristics in different periods of pregnancy: the Generation R Study. *Am J Epidemiol* 2007;165:1207-15.
29. Bakker R, Steegers EA, Obradov A, Raat H, Hofman A, Jaddoe VW. Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. *Am J Clin Nutr* 2010;91:1691-8.
30. Timmermans S, Jaddoe VW, Hofman A, Steegers-Theunissen RP, Steegers EA. Periconception folic acid supplementation, fetal growth and the risks of low birth weight and preterm birth: the Generation R Study. *Br J Nutr* 2009;102:777-85.
31. Silva L, Coolman M, Steegers E, *et al*. Maternal educational level and risk of gestational hypertension: the Generation R Study. *J Hum Hypertens* 2008;22:483-92.
32. Ay L, Kruithof CJ, Bakker R, *et al*. Maternal anthropometrics are associated with fetal size in different periods of pregnancy and at birth. The Generation R Study. *BJOG* 2009;116:953-63.
33. Sterne JA, White IR, Carlin JB, *et al*. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ* 2009;338:b2393.
34. Jaddoe VW, Mackenbach JP, Moll HA, *et al*. The Generation R Study: Design and cohort profile. *Eur J Epidemiol* 2006;21:475-84.
35. GGD-Rotterdam-Rijmond. Gezondheidsenquête 2008-2009 (Health survey 2008-2009). 2008.
36. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology* 2006;17:413-8.
37. Chan GM, McElligott K, McNaught T, Gill G. Effects of dietary calcium intervention on adolescent mothers and newborns: A randomized controlled trial. *Obstet Gynecol* 2006;108:565-71.
38. Carlson A, Hill CG, Jr., Olson NF. Kinetics of milk coagulation: II. Kinetics of the secondary phase: micelle flocculation. *Biotechnol Bioeng* 1987;29:590-600.
39. Norat T, Dossus L, Rinaldi S, *et al*. Diet, serum insulin-like growth factor-I and IGF-binding protein-3 in European women. *Eur J Clin Nutr* 2007;61:91-8.
40. Rogers I, Emmett P, Gunnell D, Dunger D, Holly J, Tteam AS. Milk as a food for growth? The insulin-like growth factors link. *Public Health Nutr* 2006;9:359-68.
41. Hoppe C, Kristensen M, Boiesen M, Kudsk J, Fleischer Michaelsen K, Molgaard C. Short-term effects of replacing milk with cola beverages on insulin-like growth factor-I and insulin-glucose metabolism: a 10 d interventional study in young men. *Br J Nutr* 2009;102:1047-51.
42. Voedingscentrum. Zo eet Nederland 1998. Resultaten van de voedselconsumptiepeiling 1997–1998. Dutch. (Results of the Dutch National Food Consumption Survey 1997–1998). Den Haag, The Netherlands, 1998.

Supplement 2.2.1 Flowchart of study participants



Supplement 2.2.2 Associations of maternal milk consumption with longitudinally measured fetal growth

Milk consumption (glasses/d)	Head circumference (SDS) *n*=8,542		Length (SDS) *n*=8,947		Weight (SDS) *n*=10,025	
	ß	95% CI	ß	95% CI	ß	95% CI
0-1	Ref		Ref		Ref	
>1-2	0.07	-0.07, 0.20	0.10	-0.03, 0.23	**0.11**	**0.02, 0.20***
>2-3	0.08	-0.05, 0.22	0.04	-0.08, 0.16	**0.11**	**0.02, 0.20***
>3	0.12	-0.03, 0.27	0.04	-0.10, 0.17	**0.14**	**0.04, 0.24***

Values are based on spline regression models and reflect the absolute growth difference at 40 weeks of pregnancy and 95% confidence interval for each level of daily milk consumption compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height and fetal sex.***P*-value <0.05**

Supplement 2.2.3 Associations of maternal milk consumption with fetal growth characteristics

Milk consumption (glasses/d)	Trimester 2			Trimester 3			Birth		
	n	ß	95% CI	*n*	ß	95% CI	*n*	ß	95% CI
	Head circumference (mm) *n*=3331			Head circumference (mm) *n*=3301			Head circumference (cm) *n*=1936		
0-1	959	Ref		969	Ref		564	Ref	
>1-2	784	**0.7**	**0.1, 1.3***	773	0.8	-0.0, 1.7	455	1.1	-0.8, 3.1
>2-3	926	0.3	-0.2, 0.9	**911**	**0.9**	**0.1, 1.7***	551	1.3	-0.6, 3.1
>3	662	0.3	-0.3, 0.9	648	-0.1	-1.0, 0.8	366	1.6	-0.4, 3.7
P for trend		*0.76*			*0.96*			*0.07*	
	Femur length (mm) *n*=3330			Femur length (mm) *n*=3334			Birth length (cm) *n*=2321		
0-1	965	Ref		974	Ref		663	Ref	
>1-2	783	0.1	-0.0, 0.3	784	0.0	-0.2, 0.3	545	**2.2**	**0.1, 4.3***
>2-3	922	0.1	-0.1, 0.3	919	0.0	-0.2, 0.2	653	1.1	-0.9, 3.1
>3	660	-0.0	-0.2, 0.1	657	-0.1	-0.4, 0.1	460	1.4	-0.8, 3.6
P for trend		*0.86*			*0.35*			*0.52*	
	Estimated fetal weight (g) *n*=3314			Estimated fetal weight (g) *n*=3322			Birth weight (g) *n*=3392		
0-1	961	Ref		969	Ref		987	Ref	
>1-2	779	**5.3**	**1.3, 9.4***	780	15.1	-2.7, 32.8	800	**58.1**	**16.3, 100***
>2-3	921	**4.3**	**0.4, 8.1***	917	17.5	0.5, 34.6	939	**63.5**	**23.4, 104***
>3	653	2.0	-2.3, 6.2	656	-2.9	-21.6, 15.8	666	**74.4**	**30.3, 119***
P for trend		*0.37*			*0.81*			*<0.01**	

Values are based on multivariate linear regression models and reflect the difference and 95% confidence interval for each level of milk consumption compared to the lowest reference group. **Models are only adjusted for gestational age at measurement and fetal sex**. * ***P* value <0.05**

Supplement 2.2.4 Associations of maternal milk consumption with the risks of neonatal complications

Milk consumption (glasses/d)	Neonatal complications											
	Preterm birth (*n*=160)				Small for gestational age (*n*=169)				Large for gestational age (*n*=185)			
	n	cases	OR	95% CI	*n*	cases	OR	95% CI	*n*	cases	OR	95% CI
0-1	943	47	Ref		938	52	Ref		951	40	Ref	
>1-2	761	43	1.13	0.74, 1.73	765	41	0.57	0.32, 1.04	762	43	0.97	0.64, 1.47
>2-3	903	37	0.82	0.53, 1.27	899	41	0.44	0.24, 0.83	879	61	0.82	0.54, 1.25
>3	636	33	1.04	0.66, 1.64	635	35	0.75	0.41, 1.37	629	41	0.99	0.64, 1.54
P for trend			*0.76*				*0.53*				*0.40*	

Values are based on multivariate logistic regression models and reflect the odds ratio and 95% CI for neonatal complications for each level of daily milk consumption compared to the lowest reference group. **Models are only adjusted for gestational age at birth and fetal sex.**

Supplement 2.2.5 Associations of maternal macronutrient intake from dairy products with birth weight

Macronutrients from dairy products (*n*=3,405)								
	Birth weight (g)			Birth weight (g)			Birth weight (g)	
Fat intake	ß	95% CI	Carbohydrate intake	ß	95% CI	Protein intake	ß	95% CI
Q1	Ref		Q1	Ref		Q1	Ref	
Q2	37	-14, 87	Q2	13	-38, 64	Q2	36	-16, 89
Q3	-20	-73, 34	Q3	-3	-56, 51	Q3	52	-4, 108
Q4	-13	-72, 45	Q4	-14	-71, 44	Q4	71	10, 132*
Q5	-53	-119, 12	Q5	-50	-117, 18	Q5	65	-7, 138
P for trend	*0.04*		*P for trend*	*0.06*		*P for trend*	***0.01***	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily macronutrient intake from dairy products compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. ***P*-value <0.05**

Supplement 2.2.6 Associations of maternal protein intake from total diet, non-dairy, and dairy products with birth weight

Total diet (*n*=3,405)			Non-dairy products (*n*=3,405)			Dairy products (*n*=3,405)		
Protein intake	Birth weight (g)		Protein intake	Birth weight (g)		Protein intake	Birth weight (g)	
	ß	95% CI		ß	95% CI		ß	95% CI
Q1	Ref		Q1	Ref		Q1	Ref	
Q2	39	-24, 103	Q2	-20	-75, 35	Q2	36	-16, 89
Q3	35	-45, 115	Q3	-29	-93, 34	Q3	52	-4, 108
Q4	55	-44, 155	Q4	-19	-92, 55	Q4	71	10, 132*
Q5	5	-130, 140	Q5	-75	-167, 18	Q5	65	-7, 138
P for trend	*0.15*		*P for trend*	*0.93*		*P for trend*	***0.01***	

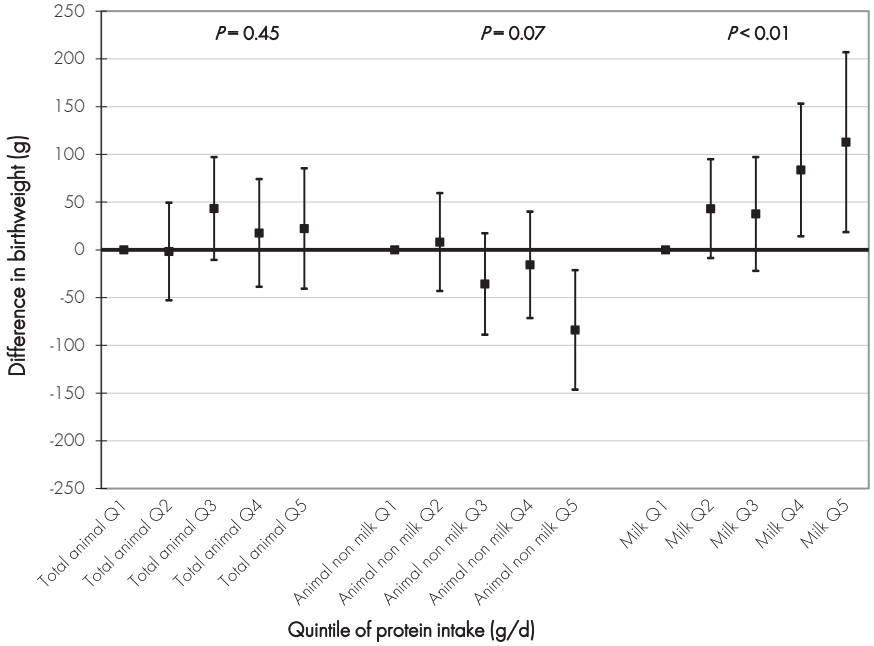
Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily protein intake from the total diet, non-dairy products or dairy products compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. ***P*-value <0.05**

Supplement 2.2.7 Associations of maternal protein intake from dairy products, milk, and cheese with birth weight

Dairy products (*n*=3,405)			Milk (*n*=3,405)			Cheese (*n*=3,405)		
Protein intake	Birth weight (g)		Protein intake	Birth weight (g)		Protein intake	Birth weight (g)	
	ß	95% CI		ß	95% CI		ß	95% CI
Q1	Ref		Q1	Ref		Q1	Ref	
Q2	36	-16, 89	Q2	44	-8, 96	Q2	-41	-93, 11
Q3	52	-4, 108	Q3	39	-21, 98	Q3	15	-43, 72
Q4	**71**	**10, 132***	Q4	**85**	**16, 155***	Q4	-61	-133, 11
Q5	65	-7, 138	Q5	**115**	**20, 209***	Q5	**-102**	**-203, -2***
P for trend	***0.01****		*P for trend*	***<0.01****		*P for trend*	*0.24*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily protein intake from dairy products, milk, or cheese consumption compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables, meat, fish and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. ***P-value* <0.05**

Supplement 2.2.8 Associations of maternal animal protein intake, animal protein intake without milk proteins or protein intake from milk with birth weight



Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of protein intake compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, vomiting, nausea, daily energy intake, consumption of fruits, vegetables and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. Corresponding effect estimates are presented in **Supplement 2.2.9**

Supplement 2.2.9 Associations of maternal intake of total animal protein, of animal protein without milk protein and of protein from milk, with birth weight

Total animal protein (*n*=3,405)			Animal protein without milk (*n*=3,405)			Milk protein (*n*=3,405)		
Protein intake	Birth weight (g)		Protein intake	Birth weight (g)		Protein intake	Birth weight (g)	
	ß	95% CI		ß	95% CI		ß	95% CI
Q1	Ref		Q1	Ref		Q1	Ref	
Q2	-2	-53, 49	Q2	8	-43, 59	Q2	43	-9, 95
Q3	43	-11, 97	Q3	-36	-89, 17	Q3	37	-22, 97
Q4	18	-39, 74	Q4	-16	-72, 40	Q4	**84**	**14, 153***
Q5	22	-41, 85	Q5	**-84**	**-147, -22***	Q5	**113**	**18, 207***
P for trend	*0.45*		*P for trend*	*0.07*		*P for trend*	***<0.01****	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily maternal animal protein intake, animal protein intake without milk proteins or protein intake from milk compared to the lowest reference group. Models are adjusted for maternal age, height, body mass index, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, vomiting, nausea, daily energy intake, consumption of fruits, vegetables and coffee, paternal height, fetal sex, gestational age at measurement and mutually for the other macronutrients. ***P-value* <0.05**

CHAPTER 3

PARENTAL, FETAL AND INFANT DETERMINANTS OF CHILDHOOD ADIPOSITY

CHAPTER 3.1

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Pediatr Res. 2013 Jan;73(1):120-7.

PARENTAL, FETAL AND INFANT RISK FACTORS FOR PRESCHOOL OVERWEIGHT

ABSTRACT

Background Overweight has its origins largely in early life. We aimed to identify the most important parental, fetal and infant risk factors of preschool overweight.

Methods In a prospective cohort study, among 3,610 Caucasian preschool children, we assessed the associations of 34 putative parental, fetal and infant factors with overweight risk.

Results Higher maternal BMI, paternal BMI and birth weight were associated with higher risk of preschool overweight (OR 1.23, 95% CI 1.10, 1.39, OR 1.35, 95% CI 1.19, 1.53 and OR 2.71, 95% CI 2.27, 3.25 respectively per SD increase). The same model identified low household income (OR 1.74, 95% CI 1.24, 2.45), being female (OR 1.55, 95% CI 1.20, 2.01), experiencing third trimester accelerated growth (OR 1.73, 95% CI 1.24, 2.40) or postnatal accelerated growth (OR 6.39, 95% CI 4.54, 8.99) as risk factors of preschool overweight. Higher poly unsaturated fat intake at 14 months was associated with lower risk of preschool overweight (OR 0.77, 95% CI 0.62, 0.96 per SD).

Conclusion Parental anthropometrics and household income, fetal and infant accelerated growth, and infant dietary fat intake are the major risk factors for development of preschool overweight. Further studies need to explore whether these risk factors could be potential targets for preventive interventions.

INTRODUCTION

Overweight is a major public health problem.[1] Childhood overweight is a well-documented risk factor for various adverse health outcomes in childhood and adulthood.[2] Since childhood overweight tends to track into adulthood, prevention should focus on risk factors for overweight in early life.[3] Numerous studies have identified associations of individual risk factors with childhood overweight.[4] However, the development of childhood overweight is not due to one individual risk factor, but results from interacting genetic, behavioral and environmental risk factors. Few studies have considered the relative impact of these individual risk factors by simultaneous assessment.[5-14] These studies suggested that infant growth rates are the strongest risk factor for overweight.[8,11,12] Infant and fetal growth rates are highly inversely correlated.[15] Decelerated fetal growth in second or third trimester and accelerated fetal growth in the third trimester have been associated with a higher fat mass percentage at 6 months of age.[16] However, not much is known about the relative effect of fetal growth on the risk of overweight, since none of the earlier studies that simultaneously assessed risk factors for overweight considered fetal growth. Simultaneous assessment of risk factors, including fetal growth, is needed to clarify which determinants are the most important in overweight development. Identification of independent risk factors in infancy and especially in fetal life may help to design childhood overweight prevention strategies focused on the earliest phase of life. Therefore, in a population-based prospective cohort study of 3,610 mothers, fathers and their children we assessed the independent associations of parental, fetal and infant risk factors with the risk of preschool-age overweight.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[26] The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all mothers. Of all eligible children in the study area, 61% participated at birth in the study. Flowchart is given in Figure 3.1.1.

Maternal risk factors

Information about maternal age, parity, education, marital status and family household income was obtained by questionnaire at enrolment in the study.[26] We categorized highest level of achieved education in: "low education", "mid-low/mid-high education"

or "higher education" following to the Dutch standard classification.[22] Marital status was dichotomized into "single motherhood" or "no single motherhood". Household income was dichotomized into "< € 2,200 per month" or "≥ € 2,200 per month", which is the average income per household in the study area.[17] We measured maternal anthropometrics, without shoes and heavy clothing, in each trimester at the research center. Information about maternal weight before pregnancy was obtained by questionnaire. BMI was calculated in kg/m². We defined weight gain by increase in weight per week from pre-pregnancy until the third trimester. We assessed maternal smoking and alcohol use in the first, second, and third trimester of pregnancy by questionnaires.[27] Based on answers from the repeated questionnaires, we categorized smoking and alcohol use during pregnancy into "no" and "yes". Maternal use of folic acid supplements was assessed by questionnaire at enrolment in the study and categorized into "no" and "yes".[28] We assessed maternal dietary intake at enrolment in the study using a modified version of a validated semi-quantitative food frequency questionnaire (FFQ).[29] This FFQ consisted of 293 food items and considered dietary intakes over the prior three months, thereby generally covering the first trimester of pregnancy.

Paternal risk factors

Paternal anthropometrics were measured, without shoes and heavy clothing, at the research center at intake. BMI (kg/m²) was calculated. Paternal smoking was assessed by questionnaire which was filled in by the mother at enrolment in the study.[27]

Fetal risk factors

We measured head circumference (HC), abdominal circumference (AC) and femur length (FL) in the second trimester (median 20.5 weeks of gestation, IQR 1.3 weeks) and third trimester (median 30.4 weeks of gestation, IQR 1.1 weeks) of pregnancy using ultrasound.[30] Estimated fetal weight (EFW) was calculated by the formula of Hadlock.[31] SD scores adjusted for gestational age at measurement were calculated using reference growth curves from the whole study population.[30] We calculated second and third trimester fetal growth rate by the change in SD scores between second and third trimester or between third trimester and birth. In accordance with an earlier study within The Generation R Study, a decrease or increase in EFW greater than 0.67 SD was considered decelerated or accelerated growth respectively.[32]

Infant risk factors

Information about offspring sex, gestational age and weight at birth was obtained from medical records and hospital registries. Postnatal growth was routinely measured at the Community Health Centers. Sex- and age-adjusted SD scores were calculated using Growth Analyzer (Growth Analyser 3.5. www.growthanalyser.org. Dutch Growth

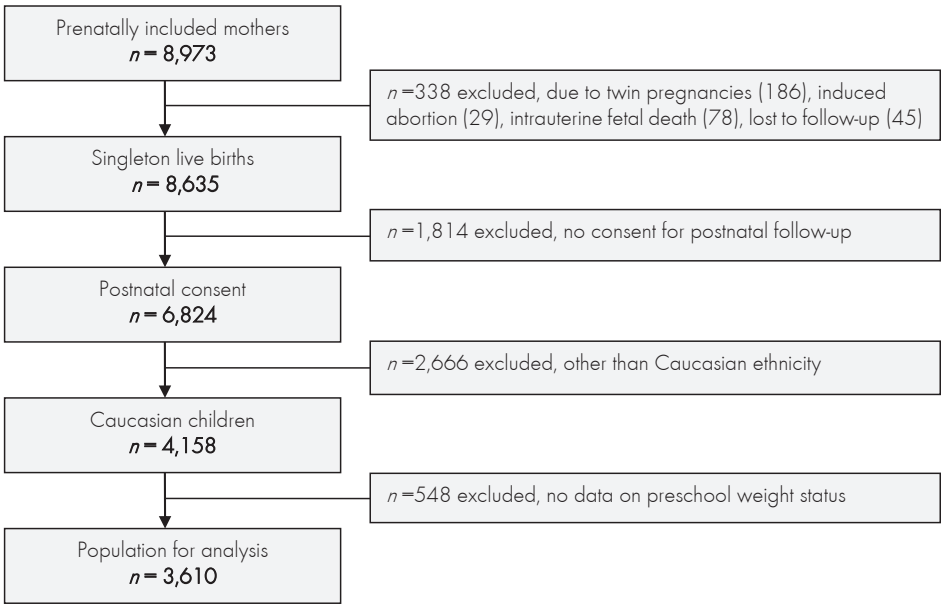

Figure 3.1.1 Flowchart of study participants

Research Foundation).[33] In accordance with earlier studies, a decrease or increase in weight greater than 0.67 SD birth and the age of 2 years was considered postnatal decelerated or accelerated growth.[8,11,12] Information about breast feeding was obtained from questionnaires at 2, 6 and 12 months and about introduction of solid foods at 6 and 12 months. We assessed the child's nutrition around the age of 14 months using a modified version of a validated semi quantitative FFQ[34] consisting of 211 food items. The FFQ has been validated against 3day-24hour recalls in Dutch children aged 14 months with the following intra-class correlation coefficients for macronutrients: total energy: 0.4, total protein: 0.7, total fat: 0.4, and carbohydrates: 0.4. This FFQ was implemented in a later stage in the study and was therefore available in a subgroup of participants (71%). Questionnaires were filled in by the primary caregiver. Information about night time sleep duration (hours/night), attending day-care (never, <8, 8-16, 16-24, 14-32 or >32 hours/week) and television watching during the week (never, <0.5, 0.5-1 or >1 hour/day) and weekend (never, <1, 1-2 or >2 hours/day) was collected by questionnaires at the age of 2 years. Questionnaires were filled in by the primary caregiver. Night time sleep duration was dichotomized into "<11.5 hours/night" and "≥11.5 hours/night" since according to the American Academy of Pediatrics (AAP) 11.5 hours is the mean night time sleep duration at age 2.[35] We summed TV watching during the week and weekend. According to the AAP no TV watching until the age of 2 is recommended. Therefore we categorized TV watching into "never" and "yes" [36].

Preschool children in the Netherlands attending day-care, on average spend 25 hours/week in day-care.[37] Therefore we categorized day-care use into "≤24 hour/week" and ">24 hour/week".

Overweight

Height and weight were routinely measured by well-trained staff in Community Health Centers. We aimed to measure all children before, but close to the age of 4 years. This is the age at which children enter primary school in the Netherlands. The median age of the measurements was 3.8 years (IQR 0.8 years). BMI (kg/m^2) was calculated. Overweight (including obesity) was defined based on the international growth charts presented by the International Obesity Taskforce.[38]

Statistical analysis

We used t-tests and chi-square tests to compare maternal, paternal, fetal and infant characteristics between non-overweight and overweight participants. We performed univariate logistic regression models to assess the associations of the putative risk factors with preschool overweight. We explored interactions between parental, fetal and infant risk factors that were biologically plausible by adding a multiplicative term to the univariate logistic regression models. Subsequently, we included all putative risk factors and significant interactions (P<0.10) simultaneously in a multiple logistic regression model. To take the time difference between fetal growth measurements into account, we additionally included the time difference between the time points of the measurements as a variable to the model. In order to identify the most significant independent risk factors, we performed logistic regression using backward selection (P<0.10). To explore the effect of the interactions that remained in the model, we stratified the analysis by these risk factors. Since total daily energy intake is strongly correlated with daily macronutrient intake, we used the energy partition method to adjust macronutrient intake for total energy intake.[39] We explored the effect of multiple testing by Bonferroni correction. To prevent bias associated with missing data, risk factors with missing values were multiple imputed (5 imputations) based on the correlation of the missing variables with other patient characteristics, according to the Markov Chain Monte Carlo method.[40] The amount of missing values ranged from 1-23%, with exception of the dietary intakes at the age of 14 months (40%) which is due to start of this data collection at a later stage in the study (response was 85%). Results are reported as Odds Ratios and 95% Confidence Intervals. P-values are two-sided. Analyses were performed using the Predictive Analytic Software version 17.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

Subject characteristics

The prevalence of overweight, including obesity, in boys and girls was 7.1% and 11.4% respectively. Obesity was prevalent in 1.6% and 1.3% of the boys and girls. As compared to normal weight children, overweight children had fathers who were heavier. Also, their mothers were heavier and gained more weight during pregnancy, were younger and lower educated , more often smoked, and less often used alcohol or folic acid supplements and had a lower household income (Table 3.1.1). As compared to normal weight children, overweight children more frequently had experienced fetal or infant accelerated growth and had a higher birth weight. Also, overweight children were more often introduced to solid foods before the age of 6 months.

Risk factors of preschool overweight

Children living in a family with a household income < € 2,200 (*US$ 2,855*) per month were more likely to be overweight at preschool age, as compared to children with family with a household income ≥ € 2200 per month (OR 1.74, 95% CI 1.24, 2.45) (Table 3.1.2). Higher maternal pre-pregnancy BMI and paternal BMI were associated with an increased risk of overweight (OR 1.23, 95% CI 1.10, 1.39 and OR 1.35, 95% CI 1.19, 1.53 per 1 SD increase, respectively). Maternal smoking during pregnancy and low maternal education showed a borderline significant association with higher risk of preschool overweight (*P*=0.10 and *P*=0.09). Paternal smoking and maternal intakes of proteins and carbohydrates, parity, alcohol or folic acid use were not independently associated with the risk of preschool overweight.

Higher birth weight and female gender were associated with an increased risk of preschool overweight (OR 2.71, 95% CI 2.27, 3.25 per 1 SD increase and OR 1.55, 95% CI 1.20, 2.01 respectively). As compared to normal growth, both third trimester fetal accelerated growth and infant accelerated growth were associated with an increased risk of preschool overweight (OR 1.73, 95% CI 1.24, 2.40 and OR 6.39, 95% CI 4.54, 8.99 respectively). Third trimester fetal decelerated growth showed a borderline significant association with higher risk of preschool overweight (*P*=0.08). Introduction of solid foods after the age of 6 months, as compared to before the age of 6 months, was associated with a lower risk of preschool overweight (OR 0.45, 95% 0.24, 0.84). A higher fat intake at the age of 14 months was associated with a lower risk of preschool overweight (OR 0.88, 95% CI 0.78, 0.99 per 100 kcal increase of energy intake from fat). We further explored this association by separately analyzing intakes of saturated, mono- and poly- unsaturated fats (Table 3.1.3), which revealed that only poly-unsaturated fat (PUFA) intake, but not saturated fat or mono-unsaturated fat intake, was inversely associated with the risk of preschool overweight (*P*=0.03). Infant breast

Table 3.1.1 Characteristics of the normal weight and overweight study population

Risk factors	Unit	Preschool weight status		*P*-value
		Normal *n*=3,275	Overweight *n*=335	
Maternal risk factors				
Socio-demographic factors				
Age	year	31.4 (4.4)	30.6 (5.2)	0.001
Parity ≥1	No	1961 (60)	203 (61)	0.81
	Yes	1311 (40)	132 (39)	
Single motherhood	No	2963 (91)	298 (89)	0.05
	Yes	225 (7)	33 (10)	
	Missing	87 (3)	4 (1)	
Educational status	Primary	87 (3)	24 (7)	<0.001
	Secondary	1177 (36)	134 (40)	
	Higher	1979 (60)	176 (53)	
	Missing	32 (1)	1 (0)	
Household income < € 2,200 / month	No	2245 (69)	193 (58)	0.003
	Yes	744 (23)	95 (28)	
	Missing	286 (9)	47 (14)	
Anthropometrics				
Pre-pregnancy body mass index	kg/m²	22.9 (3.7)	24.4 (4.3)	<0.001
Gestational weight gain	kg/week	0.28 (0.11)	0.30 (0.13)	0.02
Lifestyle-related factors				
Smoking during pregnancy	Never	2281 (70)	220 (66)	0.01
	Yes	735 (22)	94 (28)	
	Missing	259 (8)	21 (6)	
Alcohol use during pregnancy	No	961 (29)	120 (36)	0.03
	Yes	2031 (62)	195 (58)	
	Missing	283 (9)	20 (6)	
Folic acid supplement use	No	298 (9)	50 (15)	0.001
	Yes	2364 (72)	228 (68)	
	Missing	613 (19)	57 (17)	
Nutrition				
Energy intake	kcal/day	2121 (478)	2115 (476)	0.62
Protein intake	g/ day	75.5 (19.1)	79.0 (18.7)	0.67
Fat intake	g/ day	85.7 (24.1)	84.6 (25.3)	0.46
Carbohydrate intake	g/ day	257 (74)	258 (73)	0.89
Paternal risk factors				
Body mass index	kg/m²	25.0 (3.3)	26.4 (1.4)	<0.001
Paternal smoking	No	1773 (54)	173 (52)	0.41

Table 3.1.1 (continued)

Risk factors	Unit	Preschool weight status		*P*-value
		Normal *n*=3,275	Overweight *n*=335	
	Yes	1227 (38)	144 (43)	
	Missing	275 (8)	18 (5)	
Fetal and infant risk factors				
Growth				
Estimated fetal weight trimester 2	g	377 (86)	388 (91)	0.12
Estimated fetal weight trimester 3	g	1631 (250)	1650 (245)	<0.001
Growth velocity trimester 2	Decelerated	565 (17)	52 (16)	0.60
	Constant	1570 (48)	160 (48)	
	Accelerated	935 (29)	103 (31)	
	Missing	205 (6)	20 (6)	
Growth velocity trimester 3	Decelerated	807 (25)	89 (27)	<0.001
	Constant	1808 (55)	153 (46)	
	Accelerated	591 (18)	89 (27)	
	Missing	69 (2)	4 (1)	
Gestational age at birth	weeks	40.0 (1.7)	40.1 (1.4)	0.12
Birth weight	g	3481 (546)	3632 (538)	<0.001
Gender	Male	1672 (51)	128 (38)	<0.001
	Female	1603 (49)	207 (62)	
Growth velocity birth to 2 years	Decelerated	813 (25)	146 (9)	<0.001
	Constant	1350 (41)	115 (34)	
	Accelerated	589 (18)	137 (41)	
	Missing	523 (16)	52 (16)	
Nutrition				
Breast feeding	No	302 (9)	27 (8)	0.61
	Yes	2823 (86)	281 (84)	
	Missing	150 (5)	27 (8)	
Introduction of fruits & vegetables	<6 months	2253 (69)	222 (63)	0.02
	≥6 months	303 (9)	16 (5)	
	Missing	719 (22)	97 (29)	
Energy intake at 14 months	kcal/ day	1309 (283)	1300 (273)	0.19
Protein intake at 14 months	g/ day	41.7 (11.6)	42.0 (12.1)	0.72
Fat intake at 14 months	g/ day	41.7 (15.9)	39.4 (15.7)	0.06
Carbohydrate intake at 14 months	g/ day	191.5 (50.8)	192.4 (51.9)	0.82
Activity & leisure time				
Sleep duration at age 2	≥11.5 hours/night	1602 (70)	159 (67)	0.35
	<11.5 hours/night	1186 (15)	104 (12)	

Table 3.1.1 (continued)

Risk factors	Unit	Preschool weight status		*P*-value
		Normal *n*=3,275	Overweight *n*=335	
	Missing	487 (15)	72 (22)	
Day-care at age 2	≤3 days /week	2033 (62)	191 (57)	0.35
	>3 days /week	514 (16)	51 (15)	
	Missing	728 (22)	93 (28)	
TV-watching at age 2	Yes	2588 (79)	246 (73)	0.93
	Never	175 (5)	17 (5)	
	Missing	512 (16)	72 (22)	

Values reflect the mean (standard deviation) for continuous variables or absolute numbers (%) for categorical variables. *P*-values are obtained by Students *t*-tests for continuous variables and chi-square tests for categorical variables. Overweight is defined as overweight and obese weight status according to the definition of the International Obesity Task Force.[38]

feeding, TV-watching, attending day-care or sleep duration were not independently associated with preschool overweight. Time difference between fetal growth measurements did not significantly contribute to the explained variance of the final model.

In single mothers, older maternal age was found to lower the risk of preschool overweight (*P*=0.01), but not in mothers who were not single (*P*=0.77) (Table 3.1.4). In mothers who were in the highest tertile of daily fat intake, gestational weight gain tended to be a stronger risk factor for preschool overweight as compared to gestational weight gain in mothers with lower daily fat intakes.

However, in stratified analysis this association was not significant (*P*=0.12). After Bonferroni correction all findings from the backward model remained significant with exception of the association of infant fat intake at age 14 months with a lower risk of overweight.

DISCUSSION

Main findings

In this prospective cohort study in the Netherlands, we found that low family household income, high maternal and paternal BMI, female gender, higher birth weight and both fetal and infant accelerated growth were associated with increased risk of preschool overweight. Late introduction of solid foods and infant intake of PUFA were associated with a reduced risk of preschool overweight. In single mothers, older age was found to reduce the risk of preschool overweight.

Table 3.1.2 Associations of maternal, paternal, fetal and childhood risk factors with the risk of preschool overweight

	Unadjusted model		Adjusted model		Final model	
Risk factors	OR	95% CI	OR	95% CI	OR	95% CI
Maternal risk factors						
Socio-demographic factors						
Age – SD	**0.83**	**0.75, 0.93**	1.04	0.51, 2.11	-	-
Parity ≥ 1	0.97	0.77, 1.22	1.05	0.78, 1.40	-	-
Single motherhood – Yes	**1.48**	**1.01, 2.17**	0.74	0.35, 1.54	-	-
Educational status						
Primary school	**3.07**	**1.91, 4.94**	1.44	0.56, 3.72	1.70	0.91, 3.17
Secondary school	**1.28**	**1.01, 1.62**	0.84	0.59, 1.22	0.84	0.62, 1.15
Higher education	Ref	-	Ref	-	-	-
Household income < € 2,200 / month – Yes	**1.55**	**1.23, 2.04**	2.55	0.58, 11.1	**1.74**	**1.24, 2.45**
Anthropometrics						
Pre-pregnancy body mass index – SD	**1.38**	**1.25, 1.52**	**1.22**	**1.08, 1.39**	**1.23**	**1.10, 1.39**
Gestational weight gain– SD	**1.12**	**1.01, 1.26**	0.78	0.46, 1.31	-	-
Lifestyle-related factors						
Smoking during pregnancy – Yes	**1.35**	**1.04, 1.74**	1.33	0.96, 1.84	1.28	0.96, 1.73
Alcohol use during pregnancy – Yes	**0.77**	**0.60, 0.99**	0.91	0.66, 1.25	-	-
Folic acid supplement use – No	**1.67**	**1.17. 2.38**	1.35	0.87, 2.10	-	-
Nutrition						
Energy from protein intake – /100 kcal	1.05	0.89, 1.22	1.32	0.98, 1.78	-	-
Energy from fat intake – /100 kcal	0.98	0.92, 1.03	0.93	0.86, 1.02	-	-
Energy from carbohydrate intake – /100 kcal	1.01	0.96, 1.05	1.00	0.94, 1.06	-	-
Paternal risk factors						
Body mass index – SD	**1.47**	**1.32, 1.63**	**1.35**	**1.19, 1.53**	**1.35**	**1.19, 1.53**
Paternal smoking – Yes	1.20	0.94, 1.52	1.03	0.77, 1.38	-	-
Fetal and infant risk factors						
Growth-related factors						
Estimated fetal weight trimester 2 – SD	**1.17**	**1.04, 1.31**	1.17	0.88, 1.56	-	-
Estimated fetal weight trimester 3 – SD	**1.24**	**1.11, 1.38**	0.79	0.53, 1.19	-	-
Decelerated growth trimester 2 to 3 – Yes	0.91	0.65, 1.28	0.78	0.47, 1.29	-	-
Accelerated growth trimester 2 to 3 – Yes	1.07	0.83, 1.40	1.08	0.68, 1.73	-	-
Decelerated growth trimester 3 to birth – Yes	1.29	0.98, 1.69	1.47	0.89, 2.43	1.32	0.96, 1.80
Accelerated growth trimester 3 to birth – Yes	**1.75**	**1.33, 2.31**	1.47	0.89, 2.43	**1.73**	**1.24, 2.40**
Gestational age at birth – SD	1.10	0.97, 1.24	0.97	0.72, 1.30	-	-
Birth weight – SD	**1.37**	**1.22, 1.53**	**4.39**	**2.36, 8.16**	**2.71**	**2.27, 3.25**
Gender – Female	**1.69**	**1.34, 2.13**	**1.56**	**1.19, 2.05**	**1.55**	**1.20, 2.01**
Decelerated growth 0-2 years – Yes	**0.43**	**0.28, 0.63**	**0.13**	**0.07, 0.25**	**0.17**	**0.11, 0.27**

Table 3.1.2 (continued)

Risk factors	Unadjusted model		Adjusted model		Final model	
	OR	95% CI	OR	95% CI	OR	95% CI
Accelerated growth 0-2 years – Yes	**2.78**	**2.15, 3.60**	**6.30**	**4.36, 9.10**	**6.39**	**4.54, 8.99**
Nutrition						
No breast feeding	0.95	0.63, 1.42	0.77	0.47, 1.28	-	-
Introduction of fruits & vegetables ≥ 6 months – Yes	0.55	0.30, 1.01	**0.44**	**0.23, 0.84**	**0.45**	**0.24, 0.84**
Energy from protein intake at 14 months– /100 kcal	1.03	0.72, 1.48	0.90	0.46, 1.76	-	-
Energy from fat intake at 14 months – /100 kcal	0.92	0.81, 1.05	0.84	0.67, 1.06	**0.88**	**0.78, 0.99**
Energy from carbohydrate intake at 14 months – /100kcal	1.02	0.93, 1.12	1.07	0.95, 1.21	-	-
Activity & leisure time						
Sleep duration <11.5 hours/night at age 2 – Yes	0.88	0.56, 1.40	0.83	0.46, 1.48	-	-
>3 days/week day-care at age 2 – Yes	1.06	0.77, 1.47	1.14	0.79, 1.64	-	-
Never watching TV at age 2	0.97	0.58, 1.62	1.19	0.64, 2.19	-	-

Values reflect the odds ratio and 95% confidence interval. **Unadjusted model** is based on univariate logistic regression models for each risk factor. **Adjusted model** is based on a logistic regression model including all risk factors. The model was additionally adjusted for the interaction of single motherhood with age, of maternal education with age, of family income with age, of gestational weight gain with age, of maternal fat intake with gestational weight gain, of maternal education with family income, of maternal smoking with birth weight, and of birth weight with postnatal catch-down/up growth to the model, and for the time difference between the ultrasound measurements in trimester 2 and 3, and between trimester 3 and birth. **Final model** is based on a logistic regression model using stepwise backward selection and shows the risk factors that remained in the model ($P<0.10$). The interactions of single motherhood with age and of maternal fat intake with gestational weight gain remained in the model as well. **Bold font** denotes a ***P*-value <0.05**

Strengths and limitations

To our knowledge, this is the first study that simultaneously assessed the influence of maternal, paternal, fetal and infant risk factors on preschool overweight. The strength of this study is that we prospectively collected detailed information on many potential risk factors. Mothers in our study population were relatively high educated; 60% finished higher education, as compared to 31% in the study area.[17] This difference may be largely due to restriction of the study population to Caucasian participants. Also, mothers of children that lacked data on preschool weight status were more often low educated. In these children, exposure to risk factors for overweight was higher and birth weight lower. If selective participation has influenced our results, it might more likely have weakened than strengthened our results, however, this cannot be excluded. Our results should therefore be carefully generalized to other populations. Nevertheless, the overweight prevalence in our study population was comparable to the study area; 7.1% and 11.4% in boys and girls in our study population as compared to 6.4% and 11.9% in 3-6 year old Dutch boys and girls in the study area.[18] Our results showed that both higher fetal and infant growth

Table 3.1.3 Association of child fat intake at 14 months of age with the risk of preschool overweight

	Unadjusted model		Final model	
Risk factors	OR	95% CI	OR	95% CI
Fat intake child at 14 months				
Total fats – SD	0.88	0.75, 1.03	**0.83**	**0.70, 0.99**
Saturated fats – SD	0.95	0.81, 1.12	1.04	0.78, 1.37
Mono-unsaturated fats – SD	0.90	0.77, 1.06	0.95	0.65, 1.38
Poly-unsaturated fats – SD	0.86	0.73, 1.01	**0.77**	**0.62, 0.96**

Values reflect the odds ratio and 95% confidence interval. **Unadjusted model** is based on univariate logistic regression models. **Final model** is based on a logistic regression model using stepwise backward selection and is adjusted for the risk factors that remained in het model: household income, maternal BMI, educational level and smoking, paternal BMI, fetal decelerated and accelerated growth in 3rd trimester, infant birth weight, gender, postnatal decelerated and accelerated growth, age at introduction of solids, the interaction of single motherhood with age, and of maternal fat intake with gestational weight gain, and the other fat intakes. **Bold font** denotes a ***P*-value <0.05**

Table 3.1.4 Interactions between maternal and infant risk factors for preschool overweight

		Unadjusted model		Final model	
Interactions		OR	95% CI	OR	95% CI
Maternal age	Single motherhood				
Age – SD	No	0.91	0.80, 1.03	1.02	0.88, 1.19
	Yes	**0.64**	**0.47, 0.85**	**0.56**	**0.36, 0.88**
Gestational weight gain	Maternal fat intake (g/day)				
Weight gain – SD	Tertile 1	1.02	0.84, 1.23	1.00	0.79, 1.26
	Tertile 2	1.13	0.90, 1.42	1.11	0.89, 1.39
	Tertile 3	1.25	0.94, 1.67	1.27	0.93, 1.72

Values reflect the odds ratio and 95% confidence interval. **Unadjusted model** is based on univariate logistic regression models. **Final model** is based on a logistic regression model using stepwise backward selection and is adjusted for the risk factors that remained in het model: household income, maternal BMI, educational level and smoking, paternal BMI, fetal decelerated and accelerated growth in 3rd trimester, infant birth weight, gender, postnatal decelerated and accelerated growth, age at introduction of solids, fat intake at age 14 months and the other interaction. **Bold font** denotes a ***P*-value <0.05**

rates lead to preschool overweight. The independent effect of infant accelerated growth on overweight risk is well-established.[5,8,11,12] However, to our knowledge, the effect of fetal growth, independent of other risk factors, has not been studied yet. In an earlier study within a subgroup of our cohort, both third trimester decelerated and accelerated growth were associated with a higher fat mass at the age of 6 months.[16]

Comparison with other studies

In a small study of 58 twins, high fetal growth velocity was not associated with adiposity in adulthood.[19] The thrifty phenotype hypothesis proposed that fetal growth restriction in early pregnancy leads to metabolic adaptations.[20] which have beneficial effects in short

term, but predispose to adiposity and type 2 diabetes in long term. Accelerated fetal growth may be an early consequence of these adaptations, resulting in increased risk of adiposity in later life. More research is needed to further explore the independent consequences of decelerated or accelerated fetal growth in the development of overweight.

Our finding that maternal pre-pregnancy BMI is associated with overweight in preschoolers is in line with previous studies.[5-9,14] In two studies among preschoolers, that took paternal BMI into account, paternal BMI was associated with overweight, independent of maternal BMI.[8,9] Parental anthropometrics may affect childhood overweight by shared genetic, environmental and behavioral mechanisms.

In single mothers, older age decreased the risk of preschool overweight. This interaction was not reported in earlier studies. Single motherhood has been suggested to be a risk factor for childhood overweight in studies focused on family structure.[21] As a result of financial and time constraints, children of single mothers were suggested to be less physically active and more often fed a low quality diet.[21] Speculating, it might be that in most studies, including the current study, single motherhood in itself was not a significant risk factor, since these factors were accounted for in the analyses. Nevertheless, single mothers of older age might for example be less challenged by financial constraints as compared to younger single mothers.

Children of families having an income lower than the average household income in the study area were at increased risk of developing overweight. Dubois *et al.* and Brophy *et al.* describe a similar effect for preschool children being raised in middle-income or poor families.[8,14] Other studies focused on parental education as measure of socioeconomic status (SES). Low parental educational level was associated with a higher BMI.[5,9] In our study, after adding household income to the model, low maternal educational level was only a borderline significant risk factor. Low SES reduces financial possibilities and may precede overweight development by living in deprived areas, lower health-related knowledge and adverse overweight-related behaviour.[22]

In our population, preschool overweight risk was higher in females than in males. This is in agreement with the results in preschool children[7] and older children.[10,12] However, it was not reported by all studies.[6,11,13] Our results correspond to the higher overweight prevalence among Dutch females compared to Dutch males in the age range of 3 to 6 years.[18] Since this dissimilarity in overweight prevalence between boys and girls attenuates with age and differs between ethnic groups, a direct comparison of these results between countries is complicated.

We found that introduction of solid foods after the age of 6 months was associated with lower risk of preschool overweight. Likewise, Brophy *et al.* suggested that introduction of solids before the age of 3 months increased the risk of obesity.[14] In the study of Reilly *et al.* early introduction of solids seemed to increase the risk of obesity in univariable analysis, but not in simultaneous analysis.[11] A recent systematic review about

timing of introduction of solids and obesity risk in later life reported to not have found a clear association.[23] Studying this association is challenged by methodological issues, however, since promotion of delaying introduction of solids is a simple and low-cost act further study may be warranted.[24] Higher intake of poly-unsaturated fats at the age of 14 months was associated with a lower risk of preschool overweight. This specific risk factor was not studied in previous studies simultaneously assessing other risk factors for overweight. However, mounting evidence from studies assessing the influence of PUFA on lipid metabolism suggests a fat lowering effect of n-3 PUFA intake. The n-3 PUFA were found to exert their fat-lowering effect through regulation of lipid metabolism by promoting lipolysis and fatty acid oxidation, and inhibiting lipogenesis.[25] Research on the influence of n-3 PUFA in infant diet is lacking and is needed to confirm our findings.

In contrast to several other studies we did not find an association of infant lifestyle-related risk factors with preschool overweight, such as night time sleep duration[11] or TV-watching.[5,7,11-13] It might be that our study population is too young to adequately measure these risk factors or that these factors were dependent on other stronger risk factors of preschool overweight.

Conclusion

In a Dutch population-based cohort, several early life risk factors were independently associated with preschool overweight. Besides previously identified risk factors, we found evidence for an independent role of fetal growth rate and infant dietary PUFA intake. Further research is needed to develop prediction models on the basis of these determinants for risk stratification in overweight prevention at a very young age.

REFERENCES

1. James WP. WHO recognition of the global obesity epidemic. *Int J Obes (Lond).* 2008 Dec;32 Suppl 7:S120-6.
2. Reilly JJ, Kelly J. Long-term impact of overweight and obesity in childhood and adolescence on morbidity and premature mortality in adulthood: systematic review. *Int J Obes (Lond).* 2011 Jul; 35(7):891-8.
3. Bayer O, Kruger H, von Kries R, Toschke AM. Factors Associated With Tracking of BMI: A Meta-Regression Analysis on BMI Tracking. *Obesity.* 2010 Oct 14.
4. Monasta L, Batty GD, Cattaneo A, Lutje V, Ronfani L, Van Lenthe FJ, *et al.* Early-life determinants of overweight and obesity: a review of systematic reviews. *Obes Rev.* 2010 Oct;11(10):695-708.
5. Beyerlein A, Toschke AM, von Kries R. Risk factors for childhood overweight: shift of the mean body mass index and shift of the upper percentiles: results from a cross-sectional study. *Int J Obes (Lond).* 2010 Apr;34(4):642-8.
6. Kitsantas P, Gaffney KF. Risk profiles for overweight/obesity among preschoolers. *Early Hum Dev.* 2010 Sep;86(9):563-8.
7. Jouret B, Ahluwalia N, Cristini C, Dupuy M, Negre-Pages L, Grandjean H, *et al.* Factors associated with overweight in preschool-age children in southwestern France. *Am J Clin Nutr.* 2007 Jun; 85(6):1643-9.
8. Dubois L, Girard M. Early determinants of overweight at 4.5 years in a population-based longitudinal study. *Int J Obes (Lond).* 2006 Apr;30(4):610-7.
9. Huus K, Ludvigsson JF, Enskar K, Ludvigsson J. Risk factors in childhood obesity-findings from the All Babies In Southeast Sweden (ABIS) cohort. *Acta Paediatr.* 2007 Sep;96(9):1321-5.
10. Juliusson PB, Eide GE, Roelants M, Waaler PE, Hauspie R, Bjerknes R. Overweight and obesity in Norwegian children: prevalence and socio-demographic risk factors. *Acta Paediatr.* 2010 Jun; 99(6):900-5.
11. Reilly JJ, Armstrong J, Dorosty AR, Emmett PM, Ness A, Rogers I, *et al.* Early life risk factors for obesity in childhood: cohort study. *BMJ.* 2005 Jun 11;330(7504):1357.
12. Blair NJ, Thompson JM, Black PN, Becroft DM, Clark PM, Han DY, *et al.* Risk factors for obesity in 7-year-old European children: the Auckland Birthweight Collaborative Study. *Arch Dis Child.* 2007 Oct;92(10):866-71.
13. Kuhle S, Allen AC, Veugelers PJ. Perinatal and childhood risk factors for overweight in a provincial sample of Canadian Grade 5 students. *Int J Pediatr Obes.* 2010;5(1):88-96.
14. Brophy S, Cooksey R, Gravenor MB, Mistry R, Thomas N, Lyons RA, *et al.* Risk factors for childhood obesity at age 5: analysis of the millennium cohort study. *BMC Public Health.* 2009; 9:467.
15. Mook-Kanamori DO, Durmus B, Sovio U, Hofman A, Raat H, Steegers EA, *et al.* Fetal and infant growth and the risk of obesity during early childhood. The Generation R Study. *Eur J Endocrinol.* 2011 Jul 20;65:623-30.
16. Ay L, Van Houten VA, Steegers EA, Hofman A, Witteman JC, Jaddoe VW, *et al.* Fetal and postnatal growth and body composition at 6 months of age. *J Clin Endocrinol Metab.* 2009 Jun; 94(6):2023-30.
17. Goodfellow LR, Cooper C, Harvey NC. Regulation of placental calcium transport and offspring bone health. *Front Endocrinol.* 2011;2:3.
18. de Wilde JA, van Dommelen P, Middelkoop BJ, Verkerk PH. Trends in overweight and obesity prevalence in Dutch, Turkish, Moroccan and Surinamese South Asian children in the Netherlands. *Arch Dis Child.* 2009 Oct;94(10):795-800.
19. Pilgaard K, Hammershaimb Mosbech T, Grunnet L, Eiberg H, Van Hall G, Fallentin E, *et al.* Differential nongenetic impact of birth weight versus third-trimester growth velocity on glucose

metabolism and magnetic resonance imaging abdominal obesity in young healthy twins. *J Clin Endocrinol Metab*. 2011 Sep;96(9):2835-43.
20. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol*. 2010 Apr; 25(4):669-77.
21. Byrne LK, Cook KE, Skouteris H, Do M. Parental status and childhood obesity in Australia. *Int J Pediatr Obes*. 2011 Oct;6(5-6):415-8.
22. van Rossem L, Silva LM, Hokken-Koelega A, Arends LR, Moll HA, Jaddoe VW, *et al*. Socioeconomic status is not inversely associated with overweight in preschool children. *J Pediatr*. 2010 Dec;157(6):929-35 e1.
23. Moorcroft KE, Marshall JL, McCormick FM. Association between timing of introducing solid foods and obesity in infancy and childhood: a systematic review. *Matern Child Nutr*. [Review]. 2011 Jan;7(1):3-26.
24. Seach KA, Dharmage SC, Lowe AJ, Dixon JB. Delayed introduction of solid feeding reduces child overweight and obesity at 10 years. *Int J Obes (Lond)*. 2010 Oct;34(10):1475-9.
25. Tai CC, Ding ST. N-3 polyunsaturated fatty acids regulate lipid metabolism through several inflammation mediators: mechanisms and implications for obesity prevention. *J Nutr Biochem*. 2010 May;21(5):357-63.
26. Jaddoe VW, van Duijn CM, van der Heijden AJ, Mackenbach JP, Moll HA, Steegers EA, *et al*. The Generation R Study: design and cohort update 2010. *Eur J Epidemiol*. 2010 Nov;25(11): 823-41.
27. Durmus B, Kruithof CJ, Gillman MH, Willemsen SP, Hofman A, Raat H, *et al*. Parental smoking during pregnancy, early growth, and risk of obesity in preschool children: the Generation R Study. *Am J Clin Nutr*. 2011 Jul;94(1):164-71.
28. Timmermans S, Jaddoe VW, Hofman A, Steegers-Theunissen RP, Steegers EA. Periconception folic acid supplementation, fetal growth and the risks of low birth weight and preterm birth: the Generation R Study. *Br J Nutr*. 2009 Sep;102(5):777-85.
29. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, Geleijnse JM, Hofman A, Grobbee DE, *et al*. Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. *Eur J Clin Nutr*. 1998 Aug;52(8):588-96.
30. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, *et al*. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol*. 2008 Apr;31(4):388-96.
31. Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements–a prospective study. *Am J Obstet Gynecol*. 1985 Feb 1; 151(3):333-7.
32. Sonnenschein-van der Voort AM, Jaddoe VW, Raat H, Moll HA, Hofman A, de Jongste JC, *et al*. Fetal and infant growth and asthma symptoms in preschool children: the Generation R Study. *Am J Respir Crit Care Med*. 2012 Apr 1;185(7):731-7.
33. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). *Acta Paediatr Scand*. 1991 Aug-Sep;80(8-9):756-62.
34. Feunekes GI, Van Staveren WA, De Vries JH, Burema J, Hautvast JG. Relative and biomarker-based validity of a food-frequency questionnaire estimating intake of fats and cholesterol. *Am J Clin Nutr*. 1993 Oct;58(4):489-96.
35. Iglowstein I, Jenni OG, Molinari L, Largo RH. Sleep duration from infancy to adolescence: reference values and generational trends. *Pediatrics*. 2003 Feb;111(2):302-7.
36. American Academy of Pediatrics. Committee on Public E. American Academy of Pediatrics: Children, adolescents, and television. *Pediatrics*. 2001 Feb;107(2):423-6.

37. Statistics Netherlands (Centraal Bureau voor Statistiek). Labor force survey '09 (Enquête Beroepsbevolking '09). 2009.
38. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ*. 2000 May 6;320(7244):1240-3.
39. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr*. 1997 Apr;65(4 Suppl):1220S-8S; discussion 9S-31S.
40. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, *et al*. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ*. 2009;338: b2393.

CHAPTER 3.2

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Adapted from *Int J Obes* (Lond). 2014 Jul;38(7):966-72.

MATERNAL SMOKING DURING PREGNANCY AND CHILDHOOD ADIPOSITY

ABSTRACT

Background Fetal smoke exposure may influence growth and body composition in later life. We examined the associations of maternal and paternal smoking during pregnancy with total and abdominal fat distribution in school-age children.

Methods We performed a population-based prospective cohort study among 5,243 children followed from early pregnancy onwards in the Netherlands. Information about parental smoking was obtained by questionnaires during pregnancy. At the median age of 6.0 years (90% range 5.7–7.4), we measured anthropometrics, total fat and android/gynoid fat ratio by Dual-energy X-ray absorptiometry (DXA), and preperitoneal and subcutaneous abdominal fat by ultrasound.

Results The associations of maternal smoking during pregnancy with childhood fat distribution were only present among girls (*P*-value for sex interaction < 0.05). Compared with girls from mothers who did not smoke during pregnancy, those from mothers who smoked during the first trimester had a higher android/gynoid fat ratio (difference 0.23 (95% Confidence Interval (CI) 0.09-0.37) standard deviation scores (SDS)). Girls from mothers who continued smoking throughout pregnancy had a higher body mass index (difference 0.24 (95% CI 0.14-0.35) SDS), total fat mass (difference 0.23 (95% CI 0.14-0.33) SDS), android/gynoid fat ratio (difference 0.34 (95% CI 0.22-0.46) SDS), increased subcutaneous abdominal fat (difference 0.22 (95% CI 0.11-0.33) SDS), and preperitoneal abdominal fat (difference 0.20 (95% CI 0.08-0.31) SDS). Similar associations with child body fat distribution were observed for paternal smoking during pregnancy. Both continued maternal and paternal smoking during pregnancy were associated with an increased risk of childhood overweight; odds ratios 1.19 (95% CI 0.98 -1.46) and 1.32 (1.10-1.58), respectively.

Conclusions Maternal and paternal smoking during pregnancy are associated with an adverse body and abdominal fat distribution and increased risk of overweight in children. Similar effects of maternal and paternal smoking suggest that direct intrauterine mechanisms and common family based lifestyle-related factors explain the associations.

INTRODUCTION

Adverse exposures in fetal life may have lifelong consequences.[1] Maternal smoking during pregnancy is one of the most prevalent adverse fetal exposures.[2] Cigarette smoke contains many different substances including nicotine, carbon monoxide, cadmium and other toxic factors, that have direct toxic effects and might affect placental function and the fetal supply line.[3-6] Continued maternal smoking during pregnancy leads to lower birth weight and infant catch-up growth.[2,7] Infant catch-up growth may subsequently lead to higher risks of overweight and obesity in children of mothers who smoked during pregnancy.[7] Consistent associations have been reported of maternal smoking during pregnancy with higher risks of overweight and obesity in the offspring.[8-10] These studies were mainly focused on body mass index (BMI) as adiposity outcome. Not much is known about the effect of fetal smoke exposure on specific body and abdominal fat distribution outcomes. These outcomes might be more important for the prediction of cardiometabolic diseases in later life.[11-13] Also, it is not clear whether the associations of maternal smoking during pregnancy with childhood adiposity is explained by direct intrauterine mechanisms or reflect common family based lifestyle-related factors. Comparing the associations of maternal and paternal smoking during pregnancy with childhood adiposity may help to explore the underlying mechanisms. Stronger effect estimates for maternal smoking than paternal smoking would suggest direct intrauterine effects of fetal smoke exposure, whereas similar effect estimates indicate that the observed associations are driven by common family based lifestyle-related factors.[7,14-16]

We examined in 5,243 children participating in a population-based prospective cohort study, the associations of maternal and paternal smoking in different trimesters of pregnancy with BMI and detailed measures of total and abdominal body fat distribution in children at the age of 6 years.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[17] Enrolment in the study was aimed in first trimester but was allowed until the birth of the child. All children were born between April 2002 and January 2006. Of all eligible children in the study area, 61% of children were participating in the study at birth.[17] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents. Information about maternal smoking during pregnancy was available in 7,534 singleton live born children. Of these

children, 6,838 participated in the follow-up measurements at the age of 6 years, of whom 5,243 participated in the detailed measurements evaluated in the current study at the median age of 6.0 years (90% range 5.7 – 7.4) (Figure 3.2.1).

Maternal and paternal smoking status assessments

Information about maternal smoking during pregnancy was obtained by postal questionnaires sent in first, second and third trimester of pregnancy. Response rates for these questionnaires were 91%, 80%, 77%, respectively.[17] Active maternal smoking at enrollment was assessed in the first questionnaire by asking whether she smoked during her pregnancy. This questionnaire was sent to all mothers independent of the gestational age at enrollment. We grouped mothers into 3 categories as follows: 1) never smoked during pregnancy; 2) only smoked until their pregnancy was acknowledged (first trimester only); and 3) continued to smoke during pregnancy. In the second and third questionnaires, mothers were asked whether they had smoked during the past 2 months (yes, no). Mothers who reported in the first questionnaire not to have smoked or to have smoked until their pregnancy was acknowledged but reported to have smoked in the second or third questionnaire were reclassified as ''continued smoking''. Active paternal smoking was assessed in the first questionnaire by asking the mother whether the father smoked

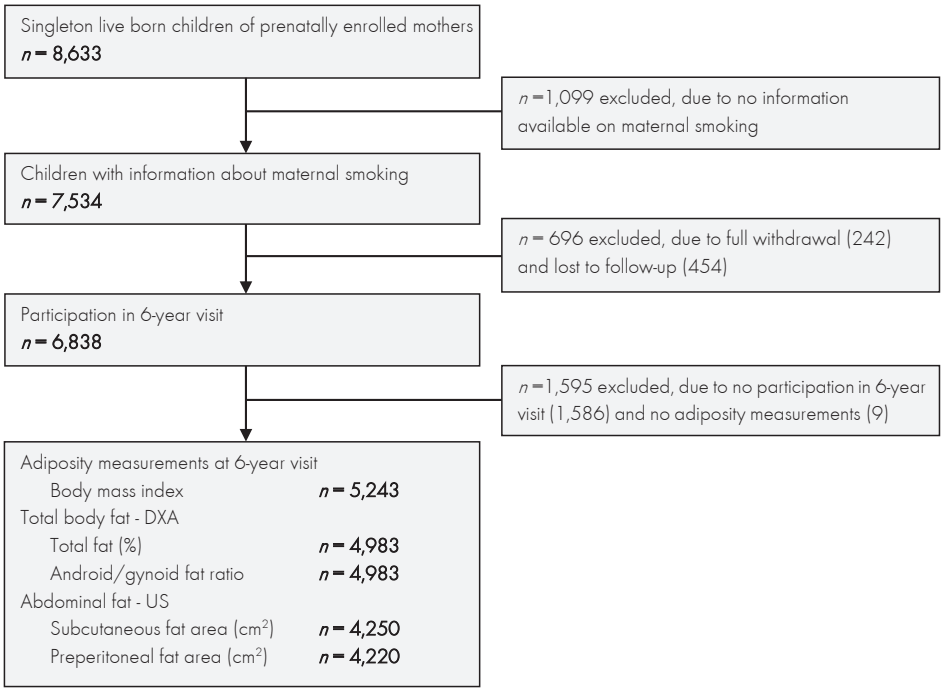

Figure 3.2.1 Flowchart of study participants

during pregnancy (yes, no, do not know). Similar information completed by the father was available in a subset of participants (*n* = 3,473). The inter-rater agreement between these two qualitative assessments was good (Cohen's kappa = 0.86). We used data collected from the mother's questionnaire because this information was almost available for all children (*n* = 5,096). When we used information completed by the father himself, the conclusion drawn from the results was not different. Mothers and fathers who smoked, were asked how many cigarettes per day they smoked. To perform dose-response analyses, we categorized cigarette dose into: 1) no smoking; 2) <5 cigarettes per day and 3) ≥5 cigarettes per day. Mothers included in these analyses were selected on the basis of complete information about the duration of smoking during pregnancy. The number of cigarettes smoked per day was therefore not known for all mothers (known for 87%, *n*=4,539).

Body and abdominal fat distribution assessments

Anthropometrics of the child were measured in a dedicated research center by well-trained staff. Height was determined in a standing position to the nearest millimeter without shoes by a Harpenden stadiometer (Holtain Limited, Dyfed, UK). Weight was measured with a mechanical personal scale (SECA, Almere, The Netherlands), and BMI (kg/m^2) was calculated. Age- and sex-adjusted standard deviation scores (SDS) for all childhood height, weight and BMI were obtained with Dutch reference growth charts (Growth Analyzer 3.5, Dutch Growth Research Foundation, Rotterdam, the Netherlands).[18] Subsequently, childhood overweight was based on the definition of Cole *et al.* who provided age- and sex-adjusted cut-off points from 2 to 18 years based on international criteria.[19]

Total body and regional fat mass was measured by a Dual-energy X-ray absorptiometry (DXA) scan (iDXA, GE-Lunar, 2008, Madison, WI, USA), which estimated the percentages of fat, lean and bone mineral masses for the whole body and specific regions with the enCORE software. [20,21] Previous studies have validated adiposity assessment by the DXA scanner against Computed Tomography (CT).[20-22] Well-trained research assistants obtained the DXA scans following standard manufacturer and positioning protocols. Quality assurance tests were run every day using a standard calibration block of tissue-equivalent material supplied by the manufacturer. Children were placed in supine position on the DXA table, without shoes, heavy clothing and metal objects, with their hands lying flat and pronated and were asked to remain motionless.[22] Total fat mass (kg) was assessed as a percentage of total body weight (kg) measured by DXA. Android and gynoid fat mass and their ratio were calculated as a percentage of total fat mass.[22] We used the android/gynoid fat mass ratio as a measure of body fat distribution, since waist/hip ratio was not measured. Higher waist/hip ratio and android/gynoid fat mass ratio reflect an adverse body fat distribution and are related with mortality in adults and

insulin resistance in children, respectively.[23,24] We measured abdominal fat mass as preperitoneal and subcutaneous fat thicknesses and areas by ultrasound (GE LOGIQ E9, Milwaukee, WI, USA) in supine position. Preperitoneal fat is a measure of abdominal visceral fat, whereas subcutaneous fat reflects the abdominal subcutaneous fat compartment. The methods have been described in detail and were validated previously.[25-27] Briefly, a Linear Array probe L12-5 (38 mm, 5-12 MHz) was placed at the median upper abdomen and images were taken when children were relaxed and showed no or little movements. All measurements were conducted by well-trained research nurses. Using a standard protocol, thicknesses and areas of subcutaneous and preperitoneal fat were measured offline to the nearest millimetre.[25-28]

Covariates

Information on maternal age, parity (nulliparity, multiparity), educational level (lower, higher) and ethnicity (European, Non-European) was obtained from the first questionnaire at enrollment in the study. Ethnicity and educational level were defined according to the classification of Statistics Netherlands.[29,30] Maternal and paternal anthropometrics were assessed at enrollment. Height and weight were measured in standing position without shoes and heavy clothing, and BMI was calculated (kg/m^2). Gestational weight gain in pregnancy was defined as the difference between weight before pregnancy and weight at gestational age of 30 weeks. Information on maternal intake of folic acid (no use, start within 10 weeks of pregnancy, periconception start) and alcohol consumption (no use, in first trimester only, continued) was assessed by questionnaire at enrollment. Information about gestational diabetes was available from records from midwives and obstetricians. Information on breastfeeding (ever, never) was obtained by postnatal questionnaires. The average child's television (TV) watching time (<2 hours/day, ≥2 hours/day) was assessed by questionnaire at the age of 6 years.[31]

Statistical analysis

First, we assessed differences in subject characteristics between the maternal smoking categories with the Student's t-tests and Mann-Whitney-U tests for continuous variables, and the Chi-square tests for categorical variables. We used the same tests for assessing differences in childhood adiposity between boys and girls. Second, we explored the associations of maternal and paternal smoking during pregnancy and the reported number of cigarettes smoked per day with childhood BMI, total and abdominal body fat distribution at the age of 6 years by using multiple linear regression models. These models were first adjusted for child's age at visit and sex (crude models). Height was included in all models focused on total and abdominal fat mass measures. Subsequently, the models were adjusted for parental covariates (ethnicity, education, BMI at enrollment, parity, age, folic acid use, gestational weight gain and alcohol consumption) and

infant covariates (breastfeeding, TV watching) (adjusted models). To explore whether any association was explained by differences in birth outcomes, we further adjusted these models for gestational age and weight at birth. Analyses of abdominal fat measures were additionally adjusted for performing sonographer. Covariates were included in the regression models based on their associations with body fat distribution and cardio-metabolic outcomes in previous studies, a significant association with the determinants and outcomes, or a change in effect estimates of >10%. On the basis of these criteria, gestational diabetes was not included in the models. Analyses on maternal smoking, but not on paternal smoking, were stratified for child's sex because we observed a significant interaction between maternal smoking category and child's sex in the analysis of body fat measures (*P*-value for interaction < 0.05). As the body fat measures had skewed distributions, we applied log transformation. To enable comparison of effect estimates, results are presented in SDS ((observed value-mean) / SD). Tests for trends for dose-response analyses were performed by treating the categorized variable as a continuous term and by entering the variable into the fully adjusted regression model. Third, multiple logistic regression models were used for analyzing the associations of maternal and paternal smoking during pregnancy with the risk of overweight at the age of 6 years. All analyses of paternal smoking were performed in children of mothers who did not smoke during pregnancy. We performed sensitivity analyses in which maternal smoking analyses were additionally adjusted for paternal smoking and *vice versa*. In order to reduce potential bias associated with missing data, we performed multiple imputations of missing covariates (<25% missing values) by generating 5 independent datasets using the Markov Chain Monte Carlo (MCMC) method after which the pooled effect estimates (95% Confidence Interval (CI)) of these 5 datasets are presented.[32] Imputations were based on the relationships between covariates, determinants and outcomes. Although small differences in some effect estimates in the adjusted models were observed between analyses with imputed missing data and complete cases only, the main conclusions of the results were similar. Statistical analyses were performed using the Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Of all mothers included in the analyses, 8.9% reported first trimester only smoking and 17.2% reported continued smoking during pregnancy (Table 3.2.1). Supplement 3.2.1 shows weak-to- moderate correlations between all adiposity measures. Table 3.2.2 shows that girls at the age of 6 years have higher total fat mass and higher subcutaneous and preperitoneal fat mass (*P*<0.01). The percentage of mothers who continued smoking during pregnancy was higher in those who were not included in the current analyses

Table 3.2.1 Characteristics of mothers, fathers and children according to category of maternal smoking during pregnancy

	Smoking during pregnancy (*n*=5,243)		
	No *n*=3,874 (74%)	First trimester only *n*=468 (9%)	Continued *n*=901 (17%)
Maternal characteristics			
Age - years	31.2 (21.8 – 38.3)	30.6 (20.6 – 38.3)*	29.3 (19.4 – 37.6)**
Height - cm	167.7 (7.4)	168.7 (6.9)**	166.9 (7.1)**
Weight -kg	67.0 (53.0 – 94.0)	67.5 (53.0 – 92.0)	68 .0 (53.0 – 100.0)
Body mass index - kg/m^2	23.7 (19.4 – 33.3)	23.4 (19.6 – 31.6)	24.2 (19.4 – 34.3)**
Parity - %			
0	56.7	71.1**	56.4
>=1	43.3	28.9**	43.6
Education - %			
Lower	49.3	54.7*	78.2**
Higher	50.7	45.3*	21.8**
Ethnicity - %			
European	69.3	73.2	73.4*
Non-European	30.7	26.8	26.6*
Folic acid supplement use - %			
No use	25.2	19.2**	37.8**
First 10 weeks of pregnancy	28.7	45.1**	38.2**
Preconception	46.1	35.7**	24.0**
Gestational diabetes - %	1.1	0.4	0.9
Paternal characteristics			
Age - years	33.2 (24.3 – 42.9)	32.2 (22.5 – 43.4)**	31.7 (21.6 – 41.2)**
Weight - kg	83.0 (58.0 – 118.0)	82.0 (55.0 – 111.6)	82.0 (55.0 – 125.0)
Height - cm	181.9 (7.1)	182.5 (7.5)	180.4 (6.9)**
Body mass index - kg/m2	24.9 (18.2 – 34.6)	24.6 (18.6 – 35.4)	25.2 (16.9 – 37.9)
Smoking - %			
Yes	35.4	65.0**	76.9**
Missing	1.1	1.1	11.1
Birth and infant characteristics			
Boys - %	49	47	55**
Gestational age - weeks	40.1 (37.1 – 42.1)	40.1 (37.0 – 42.0)	40.0 (36.4 – 42.1)**
Preterm birth - %	4.5	4.3	6.0
Weight - grams	3,459 (541)	3,440 (548)	3,284 (558)**
Low birth weight - %	3.9	4.3	6.8**
Small for gestational age - %	8.5	8.3	15.3**
Ever breastfeeding - %	93.6	92.3	84.6**

Values reflect means (standard deviation), percentages, or medians (90% range) for variables with skewed distribution. The values represent the pooled results after multiple imputation. Differences are tested by Student's T-tests or Mann-Whitney-U tests for continuous variables, and chi-squared tests for categorical variables. **P*-value < 0.05 and ***P*-value <0.01

Table 3.2.2 Characteristics of children according to sex

Child characteristics	Boys (*n*=2,610)	Girls (*n*=2,633)
Age - years	6.0 (5.7-7.5)	6.0 (5.7-7.3)
Height - cm	119.9 (6.0)	119.1 (6.1)**
Weight - kg	23.5 (4.1)	23.3 (4.6)
Body mass index - kg/m^2	15.9 (14.0 – 19.5)	15.9 (13.9 – 20.3)
Total fat mass - %	21.8 (16.5 – 33.2)	26.5 (19.9 – 37.8)**
Android/gynoid fat ratio	0.24 (0.17 – 0.36)	0.24 (0.17 – 0.40)
Subcutaneous fat area - mm^2	35.0 (17.0 – 35.6)	44.0 (21.0 -113.0)**
Preperitoneal fat area - mm^2	41.0 (19.0 – 129.7)	58.0 (26.0 – 174.0)**

Values reflect means (standard deviation) or medians (90% range) for variables with skewed distribution. Differences are tested by Students t- tests or Mann-Whitney-U tests for continuous variables. ** *P*-value <0.01

(22%) than in those who were included (17%). Also, mothers not included in the analyses were younger, of smaller statue and more likely to have a non-European ethnicity and lower education. Their children had a lower weight and gestational age at birth and were less likely to have been breastfed.

Table 3.2.3 shows that in the adjusted models, in the total group, compared with children of mothers who never smoked during pregnancy, children of mothers who continued smoking during pregnancy had a higher BMI, android/gynoid fat ratio and increased abdominal subcutaneous and preperitoneal fat (all *P*-values <0.01). Stratified analyses for child's sex showed that these associations were only statistically significant among girls (*P*-value for sex interaction <0.05 for all body fat measures). Compared with girls from mothers who did not smoke during pregnancy, those from mothers who smoked during the first trimester only had a higher android/gynoid fat ratio (difference 0.23 (95% Confidence Interval (CI) 0.09-0.37) SDS). Girls from mothers who continued smoking throughout pregnancy had a higher BMI (difference 0.24 (95% CI 0.14-0.35) SDS), total fat mass (difference 0.23 (95% CI 0.14-0.33) SDS), android/gynoid fat ratio (difference 0.34 (95% CI 0.22-0.46) SDS), subcutaneous abdominal fat (difference 0.22 (95% CI 0.11-0.33) SDS), and preperitoneal abdominal fat (difference 0.20 (95% CI 0.08-0.31) SDS). Similar associations were observed for the dose-response analyses (*P* for trends < 0.05). No associations were observed in boys. The crude models only adjusted for child's age at visit, sex and height are presented in Supplement 3.2.2 and showed that boys of mothers who continued smoking during pregnancy also had a higher BMI, android/gynoid fat ratio, and increased abdominal preperitoneal and subcutaneous fat.

Table 3.2.4 shows that in the fully adjusted models, paternal smoking during pregnancy was associated with a higher BMI (difference 0.09 (95% CI 0.03-0.15) SDS), total fat (0.09 (95% CI 0.03-0.15) SDS), android/gynoid fat ratio (0.12 (95% CI 0.06-0.19)

Table 3.2.3 Associations of maternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years

		Body Mass Index *n*=5,243		Total fat mass *n*=4,983		Android/gynoid fat ratio *n*=4,983		Subcutaneous fat area *n*=4,250		Preperitoneal fat area *n*=4,220	
Maternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Never	3874	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	468	0.01	-0.09, 0.10	0.06	-0.03, 0.16	**0.16**	**0.06, 0.25****	0.05	-0.04, 0.15	**0.11**	**0.01, 0.21***
Continued	901	**0.11**	**0.04, 0.18****	0.03	-0.05, 0.10	**0.20**	**0.12, 0.28****	**0.12**	**0.05, 0.19****	**0.11**	**0.03, 0.18****
<5 cigarettes/day	345	0.02	-0.08, 0.12	-0.04	-0.14, 0.05	0.08	-0.04, 0.19	-0.02	-0.13, 0.08	0.02	-0.09, 0.13
≥5 cigarettes/day	320	**0.14**	**0.03, 0.25***	**0.15**	**0.05, 0.25****	**0.24**	**0.12, 0.35****	**0.18**	**0.07, 0.28****	0.08	-0.03, 0.19
P for trend		***0.02***		***0.03***		***<0.01***		***<0.01***		*0.17*	
Boys *n*=2,610	*n*	**Body Mass Index** *n*=2,610		**Total fat mass** *n*=2,465		**Android/gynoid fat ratio** *n*=2,465		**Subcutaneous fat area** *n*=2,145		**Preperitoneal fat area** *n*=2,128	
Never	1879	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	221	0.03	-0.10, 0.15	0.05	-0.08, 0.17	0.09	-0.04, 0.23	0.04	-0.10, 0.18	0.12	-0.02, 0.26
Continued	492	0.00	-0.10, 0.09	-0.07	-0.17, 0.03	0.09	-0.01, 0.19	0.03	-0.07, 0.13	0.04	-0.07, 0.14
<5 cigarettes/day	191	-0.12	-0.25, 0.01	**-0.17**	**-0.31, -0.04***	-0.01	-0.15, 0.14	-0.05	-0.20, 0.10	-0.05	-0.20, 0.10
≥5 cigarettes/day	182	0.04	-0.10, 0.18	-0.01	-0.15, 0.13	0.09	-0.06, 0.24	0.06	-0.09, 0.21	0.03	-0.12, 0.18
P for trend		*0.89*		*0.28*		*0.31*		*0.66*		*0.94*	
Girls *n*=2,633	*n*	**Body Mass Index** *n*=2,633		**Total fat mass** *n*=2,518		**Android/gynoid fat ratio** *n*=2,518		**Subcutaneous fat area** *n*=2,105		**Preperitoneal fat area** *n*=2,092	
Never	1977	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	247	-0.01	-0.14, 0.12	0.04	-0.07, 0.15	**0.23**	**0.09, 0.37****	0.06	-0.07, 0.19	0.11	-0.03, 0.25
Continued	409	**0.24**	**0.14, 0.35****	**0.23**	**0.14, 0.33****	**0.34**	**0.22, 0.46****	**0.22**	**0.11, 0.33****	0.20	**0.08, 0.31****
<5 cigarettes/day	154	**0.20**	**0.04, 0.35***	0.12	-0.02, 0.25	**0.19**	**0.02, 0.36***	0.01	-0.15, 0.16	0.12	-0.05, 0.28
≥5 cigarettes/day	138	**0.27**	**0.10, 0.44****	**0.33**	**0.19, 0.47****	**0.43**	**0.25, 0.62****	**0.32**	**0.15, 0.48****	0.15	-0.02, 0.33
P for trend		***<0.01***		***<0.01***		***<0.01***		***<0.01***		***0.04***	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence interval. Trend tests are based on dose-response analyses in continued smoking mothers. Models are adjusted for maternal ethnicity, education, BMI at enrollment, age, parity, folic acid supplement use, gestational weight gain and alcohol use; and sex, breastfeeding, TV watching, current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. BMI models are not adjusted for height. *P*-value for interaction of child's sex with maternal smoking categories < 0.05. ***P*-value <0.05** and ****P*-value <0.01**

Table 3.2.4 Associations of paternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years

		Body Mass Index *n*=5,382		Total fat mass *n*=3,636		Android/gynoid fat ratio *n*=3,636		Subcutaneous fat area *n*=3,100		Preperitoneal fat area *n*=3,075	
Paternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
No	2461	Ref		Ref		Ref		Ref		Ref	
Yes	1355	0.09	0.03, 0.15**	0.09	0.03, 0.15**	0.12	0.06, 0.19**	0.10	0.04, 0.17**	0.08	0.01, 0.14*
<5 cigarettes/day	593	0.09	0, 0.17*	0.05	-0.03, 0.13	0.09	-0.01, 0.18	0.06	-0.03, 0.15	0.05	-0.04, 0.14
≥5 cigarettes/day	732	0.10	0.02, 0.18*	0.13	0.06, 0.20**	0.15	0.07, 0.23**	0.16	0.08, 0.24**	0.11	0.03, 0.20**
P for trend		*<0.01*		*<0.01*		*<0.01*		*<0.01*		*<0.01*	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence intervals. Analyses are performed in nonsmoking mothers. Models are adjusted for paternal ethnicity, education, BMI and age; maternal parity, folic acid use, gestational weight gain and alcohol use; and sex, breastfeeding, TV watching, current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. ***P*-value <0.05 and ****P*-value <0.01

SDS), and increased abdominal preperitoneal fat (0.08 (95% CI 0.01-0.14) SDS) and abdominal subcutaneous fat (0.10 (95% CI 0.04-0.17) SDS) in children at the age of 6 years. Dose-response analyses showed that children of fathers who smoked 5 cigarettes or more per day had the highest BMI, total fat, android/gynoid fat ratio and the most abdominal preperitoneal and subcutaneous fat (*P* for trends < 0.01). We did not observe sex-specific effects (*P*-value for sex interaction > 0.05). The crude models adjusted for child's age at visit, sex and height only are presented in Supplement 3.2.3. Additional adjustment of paternal smoking analyses for maternal smoking did not materially change the results. When maternal smoking analyses were adjusted for paternal smoking, the associations with childhood BMI and android/gynoid fat ratio remained statistically significant (data not shown).

Table 3.2.5 shows that children of mothers who continued smoking during pregnancy had a borderline increased risk of overweight at the age of 6 years (OR 1.19 (95% CI 0.98-1.46)). Paternal smoking during pregnancy was also associated with an increased risk of childhood overweight (OR 1.32 (95% CI 1.10-1.58)). We did not observe sex-specific effects (*P*-value for sex interaction > 0.05).

Results that are additionally adjusted for gestational age and weight at birth are given in Supplement 3.2.4, 3.2.5 and 3.2.6. These tables show that additional adjustment for birth outcomes only slightly changed the effect estimates.

Table 3.2.5 Associations of parental smoking during pregnancy with childhood overweight risk

	Overweight risk			
	n	cases	OR	95% CI
Maternal smoking				
Never	3874	658	Ref	
First trimester only	468	77	1.02	0.77, 1.36
Continued	901	215	1.19	0.98, 1.46
Total	*5243*	*950*		
Paternal smoking				
No	2461	368	Ref	
Yes	1355	279	1.32	1.10, 1.58**
Total	*3874*	*647*		

Values reflect odds ratios and 95% confidence intervals obtained by multiple logistics regression models. Models are adjusted for parental ethnicity, education, BMI, age, parity, folic acid supplement use, gestational weight gain and alcohol use; and sex, breastfeeding, TV watching, current age and height of the child. ***P*-value <0.01

DISCUSSION

We observed that both maternal and paternal smoking during pregnancy were associated with a higher BMI, a higher android/gynoid fat ratio, increased abdominal fat and increased overweight risk in school-age offspring. Associations of maternal smoking during pregnancy with school-age body fat distribution were only observed in girls.

Adverse exposures *in utero* may lead to early developmental adaptations of growth and body composition.[1] An accumulating body of evidence suggests that maternal smoking during pregnancy is a prevalent adverse fetal exposure, which also programs later body fat distribution.[6,33] A study among 8,815 adults aged 45 years showed higher BMIs and waist circumferences in those from mothers who smoked during their pregnancy.[13] Another recent study among 33,000 adults suggested that both maternal and paternal smoking during pregnancy are associated with the risk of adverse body weight throughout life.[16] Similarly, a study in Australia among 2,038 21-year old adolescents suggested that maternal smoking during pregnancy was associated with a higher BMI, waist circumference and pulse rate.[34] Studies focused on the associations of maternal smoking during pregnancy with fat distribution in childhood are important. The effect of lifestyle-related factors on the associations of interest are probably smaller in children than in adults. Observational studies have demonstrated associations of maternal smoking during pregnancy with higher body fat in the children and adolescents.[11-13,33] These associations might be explained by increased infant weight gain or may result from a long term-effect of *in utero* exposure to nicotine, which may alter neurotransmitter levels and hypothalamic function, influencing appetite control.[33] Previously, in the same population

as in the current study, we observed that maternal smoking during pregnancy led to different growth patterns for early height and weight with, subsequently, an increased overweight risk at preschool age.[7] Like our previous study, most studies used BMI as outcome measure.[12] Not much is known about the effects of trimester-specific fetal smoke exposure on body fat in later life. It has been suggested that that maternal smoking during pregnancy leads to increased intra-abdominal adiposity, measured by Magnetic Resonance Imaging (MRI), through accelerated weight gain in late puberty.[12] Similarly, Leary *et al.,* in a prospective cohort study among 5,689 children, observed that maternal smoking during pregnancy was associated with higher DXA-derived total fat mass at the age of 10 years. [11] They observed stronger effect estimates in girls than in boys. The effect estimates for paternal smoking were somewhat smaller than those for maternal smoking. In line with results of this study, we observed that in the fully adjusted models maternal smoking during pregnancy was associated with a higher childhood BMI, total fat, android/gynoid fat ratio and increased abdominal fat, only in girls. Sex differences in the associations of maternal smoking during pregnancy with body fat distribution in the offspring have only been assessed in a few studies. Previous studies suggested that body fat development, including the timing of the adiposity rebound, might differ between boys and girls.[34,35] It might be that in girls the effects of early adverse exposures on body fat can be observed at younger age than in boys. Another explanation might be that girls are more susceptible to the adverse effects of fetal smoke exposure. Further studies are needed to explore the mechanisms underlying these sex differences.

We observed that effect estimates for the associations of smoking with childhood body fat distribution were similar for mothers and fathers. From these results, we can conclude that the associations of maternal smoking during pregnancy with increased body fat are at least partly explained by unmeasured family-based or lifestyle-related factors, and not fully by direct intrauterine effects.[12,36,37] Alternatively, the associations of paternal smoking during pregnancy with childhood body fat distribution may reflect detrimental effects of passive smoke exposure in early childhood.[4,37] However, thus far, there is no consistent evidence that passive smoke exposure causes childhood adiposity.[38] It is more likely that passive smoke exposure act as a marker for other obesogenic lifestyle habits as paternal smoking is stronger associated with total and abdominal body fat than maternal smoking.

Some methodological considerations should be discussed. We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. Of the total group of singleton live born children information about maternal smoking during pregnancy was missing in 22%. This was mainly owing to mothers who did not participate in questionnaire data collection during pregnancy. Selection bias in cohort studies mainly arises from loss to follow-up rather than from non-response at baseline.[39] Of all children with information about maternal smoking

during pregnancy, 70% did participate in the follow-up measurements at the age of 6 years. This loss to follow-up would lead to selection bias if the associations of maternal smoking during pregnancy with body fat distribution would be different between those included and not included in the final analyses. The percentage of mothers who continued smoking during pregnancy was higher in those who were not included in the current analyses than in those who were included. Also, the reported number of cigarettes smoked per day was higher in those who were not included. This difference may have led to loss of statistical power and underestimation of estimated effects. We assessed active maternal and paternal smoking habits during pregnancy by questionnaire instead of cotinine levels in biological fluids. Assessing smoking by questionnaire is accepted in epidemiological studies, but may still have led to misclassification by underreporting.[40,41] We performed detailed measurements of childhood body fat distribution. DXA quantifies fat content with high precision and has the capacity for regional analysis but cannot differentiate the amount of the two abdominal fat compartments.[20,22,42] Ultrasound is a reliable method to differentiate between the abdominal visceral and subcutaneous fat compartments.[42] Both DXA and abdominal ultrasound have been validated against computed tomography, which is assumed to be the reference standard for measuring fat distribution.[42] Although we performed a meticulous adjustment for a large number of potential confounders, residual confounding in the observed associations might still be present, as in any observational study. We were unable to take account of detailed measures of childhood diet and postnatal tobacco smoke exposure, because this information was only available in a small subgroup. Importantly, by comparing the associations between maternal smoking and paternal smoking with childhood outcomes, we attempted to identify direct intrauterine effects of fetal smoke exposure. In conclusion, our study suggests that continued maternal and paternal smoking during pregnancy are associated with an adverse body fat distribution and increased risk of overweight in school-age girls. Both direct intrauterine mechanisms and common family based lifestyle-related factors may explain the associations.

REFERENCES

1. Bateson P, Barker D, Clutton- Brock T, Deb D, D'Udine B, Foley RA, *et al*. Developmental plasticity and human health. *Nature* 2004; 430: 419-21.
2. Jaddoe VW, Troe EJ, Hofman A, Mackenbach JP, Moll HA, Steegers EA, *et al*. Active and passive maternal smoking during pregnancy and the risks of low birthweight and preterm birth: the Generation R Study. *Paediatr Perinatal Epidemiol* 2008; 22: 162-71.
3. Ng SP, Conklin DJ, Bhatnagar A, Bolanowski DD, Lyon J, Zelikoff JT. Prenatal exposure to cigarette smoke induces diet- and sex-dependent dyslipidemia and weight gain in adult murine offspring. *Environ Health Perspect* 2009; 117: 1042-8.
4. Thiering E, Brüske I, Kratzsch J, Thiery J, Sausenthaler S, Meisinger C, *et al*. Prenatal and postnatal tobacco smoke exposure and development of insulin resistance in 10 year old children. *Int J Hyg Environ Health* 2011; 214: 361-8.
5. Yuan H, Shyy JY, Martins-Green M. Second-hand smoke stimulates lipid accumulation in the liver by modulating AMPK and SREBP-1. *J Hepatol* 2009; 51: 535-47.
6. Bakker H, Jaddoe VW. Cardiovascular and metabolic influences of fetal smoke exposure. *Eur J Epidemiol* 2011; 26: 763-70.
7. Durmuş B, Kruithof CJ, Gillman MH, Willemsen SP, Hofman A, Raat H, *et al*. Parental smoking during pregnancy, early growth, and risk of obesity in preschool children: the Generation R Study. *Am J Clin Nutr* 2011; 94: 164-71.
8. Ino T, Shibuya T, Saito K, Inaba Y. Relationship between body mass index of offspring and maternal smoking during pregnancy. *Int J Obes* 2012; 36: 554-8.
9. Ino T. Maternal smoking during pregnancy and offspring obesity: meta-analysis. *Pediatr Int* 2010; 52: 94-9.
10. Oken E, Levitan EB, Gillman MW. Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J of Obesity* 2008; 32: 201-10.
11. Leary SD, Smith GD, Rogers IS, Reilly JJ, Wells JC, Ness AR. Smoking during pregnancy and offspring fat and lean mass in childhood. *Obesity* 2006; 14: 2284-93.
12. Syme C, Abrahamowicz M, Mahboubi A, Leonard GT, Perron M, Richer L, *et al*. Prenatal exposure to maternal cigarette smoking and accumulation of intra-abdominal fat during adolescence. *Obesity* 2010; 18: 1021-5.
13. Power C, Atherton K, Thomas C. Maternal smoking in pregnancy, adult adiposity and other risk factors for cardiovascular disease. *Atherosclerosis* 2010; 211: 643-8.
14. Brion MJ, Leary SD, Smith GD, Ness AR. Similar associations of parental prenatal smoking suggest child blood pressure is not influenced by intrauterine effects. *Hypertension* 2007; 49: 1422-8.
15. Howe LD, Matijasevich A, Tilling K, Brion MJ, Leary SD, Smith GD, *et al*. Maternal smoking during pregnancy and offspring trajectories of height and adiposity: comparing maternal and paternal associations. *Int J Epidemiol* 2012; 41: 722-32
16. Harris HR, Willett WC, Michels KB. Parental smoking during pregnancy and risk of overweight and obesity in the daughter. *Int J Obes (Lond)*. 2013. doi: 10.1038/ijo.2013.101.
17. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, *et al*. The Generation R Study: design and cohort update 2012. *Eur J Epidemiol* 2012; 27: 739-56.
18. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, *et al*. Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res* 2000; 47: 316-23.
19. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 2000; 7244: 1240-3.
20. Kaul S, Rothney MP, Peters DM, Wacker WK, Davis CE, Shapiro MD, *et al*. Dual-energy X-ray absorptiometry for quantification of visceral fat. *Obesity* 2012; 20: 1313-8.

21. Shuster A, Patlas M, Pinthus JH, Mourtzakis M. The clinical importance of visceral adiposity: a critical review of methods for visceral adipose tissue analysis. *Br J Radiol* 2012; 85: 1-10.
22. Helba M, Binkovitz LA. Pediatic body composition analysis with dual-energy X-ray absorptiometry. *Pediatr Radiol* 2009; 39: 647-656.
23. Pischon T, Boeing H, Hoffmann K, Bergmann M, Schulze MB, Overvad K, *et al*. General and abdominal adiposity and risk of death in Europe. *N Engl J Med*. 2008;359:2105-20.
24. Aucouturier J, Meyer M, Thivel D, Taillardat M, Duché P. Effect of android to gynoid fat ratio on insulin resistance in obese youth. *Arch Pediatr Adolesc Med*. 2009;163:826-31.
25. Suzuki R, Watanabe S, Hirai Y, Akiyama K, Nishida T, Matsushima Y, *et al*. Abdominal wall fat index, estimated by ultrasonography, for assessment of the ratio of visceral fat to subcutaneous fat in the abdomen. *Am J Med* 1993; 95: 309-14.
26. Mook-Kanamori DO, Holzhauer S, Hollestein LM, Durmuş B, Manniesing R, Koek M, *et al*. Abdominal fat in children measured by ultrasound and computed tomography. *Ultrasound Med Biol* 2009; 35: 1938-46.
27. Holzhauer S, Zwijsen RML, Jaddoe VWV, Boehm G, Moll HA, Mulder PG, *et al*. Sonographic assessment of abdominal fat distribution in infancy. *Eur J Epidemiol* 2009; 24: 521-9.
28. Zheng X, Tang Q, Tao Y, Lu W, Cai W. Methods in Analyzing Abdominal Fat of Obese Children and Adolescents. *J Clin Pediatr* 2009; 27: 1-6.
29. Allochtonen in Nederland 2004. Voorburg/Heerlen: Statistics Netherlands; 2004.
30. Standaard onderwijsindeling 2003. Voorburg/ Heerlen: Statistics Netherlands; 2004.
31. American Academy of Pediatrics. Children, adolescents, and television. *Pediatrics* 2001; 107: 423-426.
32. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, *et al*. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ* 2009; 338: b2393.
33. Mamun AA, O'Callaghan MJ, Williams GM, Najman JM. Maternal smoking during pregnancy predicts adult offspring cardiovascular risk factors - evidence from a community-based large birth cohort study. *PLoS One* 2012; 7: e41106.
34. Ay L, Van Houten VA, Steegers EA, Hofman A, Witteman JC, Jaddoe VW, *et al*. Fetal and postnatal growth and body composition at 6 months of age. *J Clin Endocrinol Metab* 2009; 94: 2023-30.
35. Staiano AE, Katzmarzyk PT. Ethnic and sex differences in body fat and visceral and subcutaneous adiposity in children and adolescents. *Int J Obes* 2012; 36: 1261-9.
36. Kwok MK, Schooling CM, Lam TH, Leung GM. Paternal smoking and childhood overweight: evidence from the Hong Kong "Children of 1997". *Pediatrics* 2010; 126: e46-56.
37. Raupach T, Schäfer K, Konstantinides S, Andreas S. Secondhand smoke as an acute threat for the cardiovascular system: a change in paradigm. *Eur Heart J* 2006; 27: 386-92.
38. von Kries R, Bolte G, Baghi L, Toschke AM; GME Study Group. Parental smoking and childhood obesity–is maternal smoking in pregnancy the critical exposure? *Int J Epidemiol* 2008; 37: 210-6.
39. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology* 2006; 17: 413-18.
40. Gorber SC, Schofield-Hurwitz S, Hardt J, Levasseur G, Tremblay M. The accuracy of self-reported smoking: a systematic review of the relationship between self-reported and cotinine-assessed smoking status. *Nicotine Tob Res* 2009; 11: 12-24.
41. Gorog K, Pattenden S, Antova T, Niciu E, Rudnai P, Scholtens S, *et al*. Maternal smoking during pregnancy and childhood obesity: results from the CESAR Study. *Matern Child Health J* 2011; 15: 985-92.
42. Bazzocchi A, Filonzi G, Ponti F, Sassi C, Salizzoni E, Battista G, *et al*. Accuracy, reproducibility and repeatability of ultrasonography in the assessment of abdominal adiposity. *Acad Radiol* 2011; 18: 1133-43.

Supplement 3.2.1 Correlation coefficients between body fat measures (*n*=5,243)

	BMI	Total fat	Android/gynoid fat ratio	Preperitoneal fat area	Subcutaneous fat area
BMI	1	0.57	0.48	0.43	0.61
Total fat	0.57	1	0.56	0.55	0.82
Android/gynoid fat ratio	0.48	0.56	1	0.40	0.57
Preperitoneal fat area	0.43	0.55	0.40	1	0.66
Subcutaneous fat area	0.61	0.82	0.57	0.66	1

Correlation coefficients are based on Spearman's rho test. *P*<0.01 for all correlation coefficients.

Supplement 3.2.2 Associations of maternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years (crude models)

		Body Mass Index *n*=5,243		Total fat mass *n*=4,983		Android/gynoid fat ratio *n*=4,983		Subcutaneous fat area *n*=4,250		Preperitoneal fat area *n*=4,220	
Maternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Never	3874	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	468	-0.01	-0.11, 0.08	0.03	-0.07, 0.13	0.13	0.03, 0.23**	0.07	-0.03, 0.16	0.02	-0.07, 0.12
Continued	901	0.20	0.13, 0.27**	0.14	0.07, 0.21**	0.27	0.20, 0.35**	0.17	0.09, 0.24**	0.23	0.16, 0.30**
<5 cigarettes/day	345	0.11	0.00, 0.21*	0.05	-0.05, 0.15	0.14	0.03, 0.25*	0.08	-0.03, 0.19	0.08	-0.03, 0.19
≥5 cigarettes/day	320	0.22	0.11, 0.33**	0.27	0.16, 0.37**	0.31	0.19, 0.42**	0.13	0.02, 0.24*	0.27	0.15, 0.38**
P for trend		*<0.01*		*<0.01*		*<0.01*		*0.01*		*<0.01*	
Boys *n*=2,610	*n*	Body Mass Index *n*=2,610		Total fat mass *n*=2,465		Android/gynoid fat ratio *n*=2,465		Subcutaneous fat area *n*=2,145		Preperitoneal fat area *n*=2,128	
Never	1879	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	221	0.01	-0.12, 0.14	0.03	-0.10, 0.16	0.07	-0.07, 0.20	0.07	-0.07, 0.21	0.01	-0.13, 0.15
Continued	492	0.10	0.01, 0.19*	0.09	-0.01, 0.18	0.16	0.06, 0.25**	0.11	0.01, 0.20*	0.17	0.07, 0.27**
<5 cigarettes/day	191	-0.04	-0.17, 0.10	-0.06	-0.20, 0.08	0.04	-0.10, 0.18	-0.00	-0.15, 0.15	0.06	-0.09, 0.21
≥5 cigarettes/day	182	0.15	0.01, 0.28*	0.17	0.02, 0.31*	0.17	0.03, 0.32*	0.10	-0.04, 0.25	0.19	0.04, 0.34*
P for trend		*0.11*		*0.10*		*0.02*		*0.22*		*0.01*	
Girls *n*=2,633	*n*	Body Mass Index *n*=2,633		Total fat mass *n*=2,518		Android/gynoid fat ratio *n*=2,518		Subcutaneous fat area *n*=2,105		Preperitoneal fat area *n*=2,092	
Never	1977	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	247	-0.03	-0.17, 0.11	-0.00	-0.12, 0.11	0.19	0.05, 0.34**	0.07	-0.08, 0.21	0.04	-0.10, 0.17
Continued	409	0.33	0.22, 0.44**	0.30	0.21, 0.39**	0.40	0.29, 0.52**	0.24	0.13, 0.35**	0.30	0.19, 0.41**
<5 cigarettes/day	154	0.29	0.12, 0.46**	0.19	0.05, 0.33**	0.27	0.10, 0.44**	0.19	0.02, 0.35*	0.10	-0.06, 0.26
≥5 cigarettes/day	138	0.32	0.14, 0.50**	0.39	0.24, 0.54**	0.48	0.30, 0.67**	0.17	-0.01, 0.34	0.36	0.19, 0.52**
P for trend		*<0.01*		*<0.01*		*<0.01*		*0.01*		*<0.01*	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence interval. Trend tests are based on dose-response analyses in continued smoking mothers. Models are only adjusted for sex and current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. BMI models are not adjusted for height. *P*-value for interaction of child's sex with maternal smoking categories < 0.05. ***P*-value <0.05 and ****P*-value <0.01

Supplement 3.2.3 Associations of paternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years (crude models)

		Body Mass Index *n*=5,382		Total fat mass *n*=3,636		Android/gynoid fat ratio *n*=3,636		Subcutaneous fat area *n*=3,100		Preperitoneal fat area *n*=3,075	
Paternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
No	2461	Ref		Ref		Ref		Ref		Ref	
Yes	1355	0.13	0.07, 0.19**	0.15	0.09, 0.21**	0.16	0.09, 0.22**	0.12	0.05, 0.18**	0.16	0.10, 0.23**
<5 cigarettes/day	593	0.09	0.01, 0.17*	0.06	-0.02, 0.14	0.08	-0.01, 0.17	0.05	-0.04, 0.14	0.08	-0.01, 0.17
≥5 cigarettes/day	732	0.16	0.08, 0.24**	0.22	0.15, 0.29**	0.21	0.13, 0.29**	0.17	0.09, 0.25**	0.24	0.16, 0.32**
P for trend		*<0.01*		*<0.01*		*<0.01*		*<0.01*		*<0.01*	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence intervals. Analyses are performed in nonsmoking mothers. Models are only adjusted for sex and current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. ***P*-value <0.05 and ****P*-value <0.01

Supplement 3.2.4 Associations of paternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years, adjusted for birth weight and gestational age at birth

		Body Mass Index *n*=5,382		Total fat mass *n*=3,636		Android/gynoid fat ratio *n*=3,636		Subcutaneous fat area *n*=3,100		Preperitoneal fat area *n*= 3,075	
Paternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
No	2461	Ref		Ref		Ref		Ref		Ref	
Yes	1355	0.09	0.04, 0.15**	0.09	0.04, 0.15**	0.13	0.06, 0.19**	0.11	0.04, 0.17**	0.08	0.01, 0.14*
<5 cigarettes/day	593	0.08	-0.01, 0.16	0.04	-0.04, 0.11	0.08	-0.01, 0.17	0.05	-0.04, 0.14	0.03	-0.06, 0.12
≥5 cigarettes/day	732	0.11	0.04, 0.19**	0.15	0.08, 0.22**	0.16	0.08, 0.25**	0.17	0.09, 0.25**	0.12	0.04, 0.20**
P for trend		*<0.01*		*<0.01*		*<0.01*		*<0.01*		*<0.01*	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence intervals. Analyses are performed in nonsmoking mothers. Models are adjusted for paternal ethnicity, education, BMI and age; maternal parity, folic acid use, gestational weight gain and alcohol use; and sex, gestational age at birth, birth weight, breastfeeding, TV watching, current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. ***P*-value <0.05 and ****P*-value <0.01

Supplement 3.2.5 Associations of maternal smoking during pregnancy with body fat measures (SDS) at the age of 6 years, adjusted for birth weight and gestational age at birth

		Body Mass Index *n*=5,243		Total fat mass *n*=4,983		Android/gynoid fat ratio *n*=4,983		Subcutaneous fat area *n*=4,250		Preperitoneal fat area *n*=4,220	
Maternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Never	3874	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	468	-0.02	-0.11, 0.07	0.01	-0.08, 0.11	**0.12**	**0.02, 0.21***	0.01	-0.08, 0.11	0.06	-0.04, 0.16
Continued	901	**0.15**	**0.08, 0.22****	-0.03	-0.10, 0.05	**0.17**	**0.10, 0.25****	**0.09**	**0.01, 0.16***	0.07	-0.01, 0.14
<5 cigarettes/day	345	0.05	-0.05, 0.15	-0.08	-0.17, 0.02	0.05	-0.06, 0.16	-0.05	-0.16, 0.05	-0.02	-0.13, 0.09
≥5 cigarettes/day	320	**0.21**	**0.10, 0.32****	**0.12**	**0.02, 0.22***	**0.22**	**0.10, 0.34****	**0.15**	**0.04, 0.26****	0.06	-0.06, 0.17
P for trend		***<0.01***		***0.17***		***<0.01***		*0.05*		*0.50*	
Boys *n*=2,610	*n*	Body Mass Index *n*=2,610		Total fat mass *n*=2,465		Android/gynoid fat ratio *n*=2,465		Subcutaneous fat area *n*=2,145		Preperitoneal fat area *n*=2,128	
Never	1879	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	221	0.00	-0.12, 0.12	-0.00	-0.13, 0.12	0.05	-0.08, 0.19	-0.01	-0.14, 0.13	0.06	-0.08, 0.20
Continued	492	0.02	-0.07, 0.11	**-0.11**	**-0.20, -0.01***	0.07	-0.03, 0.17	-0.00	-0.11, 0.10	0.00	-0.10, 0.10
<5 cigarettes/day	191	-0.11	-0.23, 0.02	**-0.22**	**-0.35, -0.09****	-0.03	-0.17, 0.11	-0.09	-0.24, 0.05	-0.10	-0.25, 0.05
≥5 cigarettes/day	182	0.09	-0.05, 0.23	-0.04	-0.18, 0.11	0.08	-0.07, 0.23	0.04	-0.11, 0.19	0.01	-0.14, 0.16
P for trend		*0.60*		*0.09*		*0.51*		*0.99*		*0.72*	
Girls *n*=2,633	*n*	Body Mass Index *n*=2,633		Total fat mass *n*=2,518		Android/gynoid fat ratio *n*=2,518		Subcutaneous fat area *n*=2,105		Preperitoneal fat area *n*=2,092	
Never	1977	Ref		Ref		Ref		Ref		Ref	
Until pregnancy was known	247	-0.03	-0.16, 0.09	-0.01	-0.12, 0.10	**0.19**	**0.05, 0.33****	0.02	-0.11, 0.15	0.06	-0.08, 0.19
Continued	409	**0.31**	**0.20, 0.42****	**0.20**	**0.10, 0.29****	**0.30**	**0.18, 0.42****	**0.19**	**0.08, 0.29****	**0.15**	**0.04, 0.26****
<5 cigarettes/day	154	**0.24**	**0.09, 0.40****	0.08	-0.05, 0.22	0.16	-0.01, 0.33	-0.01	-0.17, 0.14	0.08	-0.08, 0.25
≥5 cigarettes/day	138	**0.37**	**0.20, 0.54****	**0.30**	**0.16, 0.45****	**0.41**	**0.22, 0.59****	**0.29**	**0.12, 0.45****	0.12	-0.06, 0.30
P for trend		***<0.01***		***<0.01***		***<0.01***		***<0.01***		*0.12*	

Values are based on multiple linear regression models and reflect standardized regression coefficients and 95% confidence interval. Trend tests are based on dose-response analyses in continued smoking mothers. Models are adjusted for maternal ethnicity, education, BMI at enrollment, age, parity, folic acid supplement use, gestational weight gain and alcohol use; and sex, gestational age at birth, birth weight, breastfeeding, TV watching, current age and height of the child. Abdominal fat measures are additionally adjusted for performing sonographer. BMI models are not adjusted for height. *P*-value for interaction of child's sex with maternal smoking categories < 0.05. ***P*-value <0.05** and ****P*-value <0.01**

Supplement 3.2.6 Associations of parental smoking during pregnancy with childhood overweight risk, adjusted for gestational age and weight at birth

	Overweight risk			
	n	cases	OR	95% CI
Maternal smoking				
Never	3874	658	Ref	
First trimester only	468	77	0.94	0.71, 1.24
Continued	901	215	1.20	0.99, 1.46
Total	*5243*	*950*		
Paternal smoking				
No	2461	368	Ref	
Yes	1355	279	1.32	1.11, 1.58**
Total	*3874*	*647*		

Values reflect odds ratios and 95% confidence intervals obtained by multiple logistics regression models. Models are adjusted for parental ethnicity, education, BMI, age, parity, folic acid supplement use, gestational weight gain and alcohol use; and sex, gestational age at birth, birth weight, breastfeeding, TV watching, current age and height of the child. ***P*-value <0.01

CHAPTER 4

PARENTAL, FETAL AND INFANT DETERMINANTS OF CHILDHOOD BONE MASS

CHAPTER 4.1

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J Bone Miner Res. 2014 Dec;29(12):2584-93.

FETAL AND CHILDHOOD GROWTH PATTERNS AND CHILDHOOD BONE MASS

ABSTRACT

Low birth weight is associated with lower bone accrual in children and peak bone mass in adults. We assessed how different patterns of longitudinal fetal and early childhood growth influence bone properties at school age. In 5,431 children participating in a population-based prospective cohort study, we measured fetal growth by ultrasound at 20 and 30 weeks gestation, and childhood growth at birth, 1, 2, 3 and 4 years of age. We analyzed these growth measurements in relation to total body (less head) BMD measured by DXA at age 6. We used conditional growth modelling; a technique which takes into account correlation between repeatedly measured growth measures. Our results showed that estimated fetal weight gain, femur length growth between 20 and 30 weeks gestation, femur length growth between 30 weeks and birth, as well as all height and weight growth measurements from birth to 4 years of age were all positively associated with BMC, BA and BMD (all $P<0.01$). Fetal femur length growth between 30 weeks and birth was positively associated with BMC and BA (both $P<0.001$), but not with BMD. Overall, childhood growth measurements exerted a larger influence on bone measures than fetal growth measures. The strongest effect estimate was observed during the first year of life. Children born small (<10th percentile) for gestational age (SGA) had lower BMC and BA, but not BMD, than children born appropriate for gestational age (AGA),whereas children born large (>90th percentile) for gestational age (LGA) had higher BMC and BA (all $P<0.001$). These differences were no longer present in children showing subsequent accelerated and decelerated infant growth, respectively. We conclude that both fetal and childhood growth patterns are associated with bone mineral accrual, showing the strongest effect estimates in infancy. Compensatory infant growth counteracts the adverse consequences of fetal growth restriction on bone development.

INTRODUCTION

Early life factors influence the development of bone health and osteoporosis during the life-course.[1] Several studies have consistently shown that low birth weight leads to lower bone accrual in children and peak bone mass acquisition in adults.[2,3] However, birth weight is an inappropriate measure of fetal growth, as different adverse fetal growth patterns may still result in the same birth weight.[4] Also, birth weight is strongly correlated with infant growth. A low or high birth weight is frequently compensated for by catch-up growth or catch-down growth during the first two years of life.[5] Studies assessing the effects of directly measured fetal growth in different trimesters along with early post-natal growth on bone mineral accrual in later life are scarce. Nevertheless, these studies are important to identify specific early critical periods for bone development. A previous study among 380 children suggested that fetal growth from 19 to 34 weeks of gestation already affected childhood bone development at age 4 years.[6] In another study, among the same population, including 628 children, fetal as well as early postnatal growth contributed to bone development at age 4 years.[7] On the other hand, a study among 123 adolescents, found fetal growth and early postnatal growth to be a less crucial determinant of adolescent bone development than pre-pubertal growth.[8] These findings suggest that bone accrual is influenced by different critical periods, though diverse methodological challenges interfere with identifying effects across time in a conclusive manner; when growth measurements are widely separated in time, pinpointing the most influential period of growth is very difficult.[8] Furthermore, the identification of a critical period of growth on subsequent bone development is challenged by the correlation existing between repeatedly collected growth measures;[9] and the unknown influence of growth realignment following an earlier period of growth deviation.

We investigated the independent associations of repeatedly measured fetal and childhood growth characteristics bone mineral density measured by DXA at age 6 years in 5,431 children participating in a population-based birth cohort. We applied conditional growth modelling,[10] which enables the simultaneous assessment of correlated growth measures to identify independent critical periods, to further elucidate the independent role of fetal and childhood growth on bone development.

METHODS

Study design

This study is embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[11] All mothers who were resident in the study area and had an expected delivery date between April 2002

and January 2006 were eligible. The study aimed enrolment in first trimester but allowed enrolment until delivery of the child. In total, 75% of all mothers enrolled before 18 weeks of gestation. Of all eligible children in the study area, 61% participated at birth in the study. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31), and conducted according to the guidelines of the Helsinki Declaration. Written informed consent was obtained from all participants.

In total, 7,893 children of mothers who gave consent for follow-up in the preschool phase (0-4 years) were eligible for this study. Of the 7,696 singleton born children, growth was measured at least once in 7,683 children. Of these children, 5,602 visited our research center around the age of 6 years. DXA-scanning was successfully performed in 5,431 children (69% of the eligible population). A flowchart of included participants is shown in Figure 4.1.1.

Fetal and childhood growth characteristics

Fetal ultrasound examinations were performed in each trimester of pregnancy. Medians (IQR range, interquartile range) of these visits were 13.1 (2.4), 20.5 (1.3) and 30.4 (1.1) weeks of gestation for the first, second and third trimester, respectively. In total, 88% of the examinations took place at either of the two research centers of the study. The remaining examinations were carried out in one of five hospitals in the vicinity under

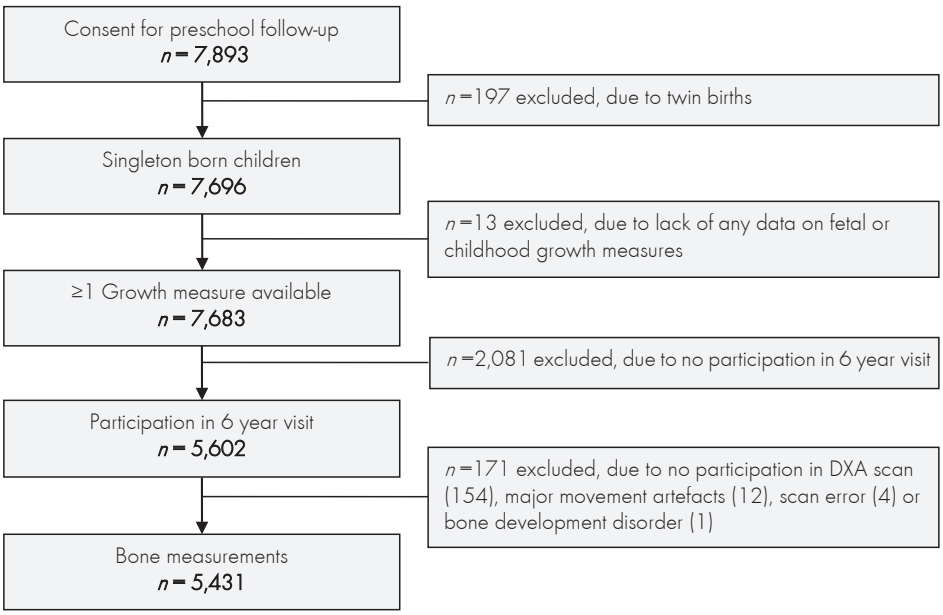


Figure 4.1.1 Flowchart of study participants

guidance of our research staff. In order to achieve optimal reproducibility all sonographers were experienced and underwent additional training according to guidelines from The Fetal Medicine Foundation.[12] Gestational age was determined at the first fetal ultrasound examination, since 39% of pregnant women had unknown or irregular last menstrual periods and because using last menstrual period for this purpose has several described limitations.[12] In the second and third trimesters, fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) were measured to the nearest millimeter using standardized ultrasound procedures.[13-16] A brief description of the applied techniques is given in **Supplement 4.1.1**. Fetal weight (EFW) was estimated using head circumference, abdominal circumference and femur length in the formula from Hadlock: ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[17] In a previous study within the Generation R Study reference curves were developed based on fetal growth characteristics of the whole study population.[12] In the current study we used these reference curves to calculate gestational age adjusted SD scores.

Information about offspring sex, gestational age and weight at birth was obtained from medical records and hospital registries. Very preterm birth was defined as birth occurring before 32.0 weeks of gestation, and preterm birth as birth between 32.0 and 37.0 weeks of gestation. Small for gestational age (SGA) and large for gestational age (LGA) was defined as sex- and gestational age adjusted birth weight below the 10^{th} percentile and above the 90^{th} percentile, respectively. Childhood growth was routinely measured at the Community Health Centers at the median ages of 6.2 (IQR 0.4), 11.1 (IQR 0.7), 24.8 (IQR 1.6), 36.7 (IQR 1.4) and 45.8 (IQR 1.3) months following standardized protocols. Sex- and age-adjusted SD scores were calculated using Growth Analyzer 3.5 (www.growthanalyser.org, Dutch Growth Research Foundation, Rotterdam, the Netherlands).[18,19] In accordance with earlier studies, we defined an increase or decrease in weight greater than 0.67 SD from birth to the age of 24 months as accelerated or decelerated growth, respectively.[20,21] At the age of 6 years, we measured weight in our research center using an electronic personal scale (Seca, Almere, The Netherlands) and height using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K) following standardized protocols.

Bone mineral density measurements

Total body bone mineral density (BMD; g/cm^2), bone mineral content (BMC; g) and bone area (BA; cm^2) were measured at a median age of 6.0 (IQR 0.37) years using a DXA scanner (iDXA, General Electrics –Lunar, 2008, Madison, WI, USA). As described in detail earlier,[22] well-trained research assistants obtained the DXA scans using the same device and software (enCORE) following standard manufacturer protocols. In our analyses, we used areal total body less head (TBLH) BMD, BMC and BA as recommended

by the International Society for Clinical Densitometry for pediatric evaluations of bone health.[23] All measures were adjusted for skeletal size by using body height or weight as covariate in the models to correct for artefacts arising from periods of rapid growth;[24] this is needed as areal BMD measured on larger bones overestimates true (volumetric) BMD, while on smaller bones it can underestimate BMD across individuals.[25,26] In subsequent comparative analyses, we, in addition to the other covariates, corrected BMC for BA to further adjust for size effects.[25]

Covariates

We registered maternal age at enrolment and collected information about maternal education, marital status, parity and country of birth, and country of birth of the father and grandparents by questionnaire at enrolment in the study. Maternal smoking and alcohol habits were assessed in each trimester. We measured parental height and weight at the research center and obtained information about maternal weight before pregnancy by questionnaire. As the enrolment in our study was during pregnancy, we were not able to measure maternal weight before pregnancy. Yet, correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was high (ρ=0.95; P<0.01). We categorized ethnicity into three main groups: Western (Dutch, Turkish, other European, American, and Oceanic), African (Moroccan, other African, Antillean, Surinamese-Creole and Cape Verdean) and Asian (Indonesian, other Asian and Surinamese-Hindu) descent according to the three largest transcontinental ancestral groups. Information about breast feeding[27] and participation in sports was obtained from postnatal questionnaires.

Statistical analysis

We used t-tests and chi-square tests to compare differences in subject characteristics between boys and girls. We calculated age adjusted standard deviation (SD) scores for all bone measures based on their distribution in the whole study population and analyzed them following four strategies. First, we performed multiple linear regression analyses to assess the individual associations of fetal and childhood growth measures with bone measures at the age of 6 years. Second, we assessed the associations of these growth measures with bone measures using conditional change modelling.[10] In conditional growth modelling, a growth measure at a specific time point is adjusted for growth predicted by prior growth measures. Accordingly, we calculated standardized residuals by regression of the growth measure of interest on prior growth measures,[10] obtaining growth measures independent of prior growth measures and statistically independent of each other across time. This approach enabled a simultaneous analysis of all growth measures with bone measures in order to identify the period of growth most critical to bone development. In an attempt to eliminate potential artefacts caused by bone size

and to further distinguish potential effects on bone size from bone mineral accrual, we additionally corrected BMC for BA in a sensitivity analysis. Third, we assessed associations of birth outcomes (gestational age, birth weight, gestational age adjusted birth weight) with bone measures at the age of 6 years. Fourth, we explored the associations of gestational age adjusted birth weight with bone measures stratified for the postnatal growth pattern. Based on previous literature, all models were adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, paternal weight and height, and child's sex, ethnicity, breastfeeding duration and participation in sports.[28-31] Models concerning weight measures were additionally adjusted for current height, whereas models concerning height were adjusted for current soft tissue weight calculated as "lean + fat mass" (thereby excluding the contribution of bone mass to the child's weight). As missing values add up in conditional modelling and to prevent bias associated with missing data, we used multiple imputations (5 imputations) to impute missing values in growth measures and covariates. Missing values for growth measures and covariates were imputed based on the correlation of the missing variables with other participant characteristics and other available growth measures, according to the Markov Chain Monte Carlo method [32]. The percentage of missing values for any fetal growth measure was lower than16%, and for any childhood growth measure lower than 38%. Of all children, 6% did not have any data on growth from 1 to 4 years of age. Results from the complete case analyses were similar to results from the imputed analyses. We only present results for the imputed analyses. Comparing infants born SGA and not born SGA, we would be able to detect statistically significant difference in childhood BMD of 0.13 SD (type I error of 5% and a type II error of 20% (power 80%)).[33] Analyses were performed using the SPSS Predictive Analytic Software version 17.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

Characteristics of study population

Subject characteristics for boys and girls separately are shown in **Table 4.1.1**. At 20 and 30 weeks gestation estimated fetal weight was higher in boys than in girls (**Table 4.1.2**). At 30 weeks gestation femur length was longer in girls than in boys. From birth onwards, boys were heavier and taller. At 6 years of age, boys had a higher BMC than girls, whereas no differences in BMD or BA were observed.

Fetal growth, childhood growth and bone measures

The associations of individually modelled growth measures with bone parameters at age 6 are shown in **Supplement 4.1.2**. In short, all fetal length and weight measures were

Table 4.1.1 Parental, fetal and child characteristics

Characteristic	Unit	Boys *n*=2,718	Girls *n*=2,732	*P*-value
Maternal characteristics				
Age	year	30.9 (5.1)	30.8 (5.0)	0.38
Height	cm	167.9 (7.2)	167.8 (7.5)	0.62
Pre-pregnancy weight	kg	66.2 (12.1)	66.8 (12.5)	0.19
Pre-pregnancy BMI	kg/m²	23.4 (4.1)	23.6 (4.2)	0.13
Parity ≥1	No	1466 (54)	1499 (55)	0.39
	Yes	1150 (42)	1129 (42)	
	Missing	106 (4)	90 (3)	
Single motherhood	No	2192 (81)	2203 (81)	0.80
	Yes	285 (11)	284 (10)	
	Missing	245 (9)	231 (9)	
Educational status	Primary	199 (7)	214 (8)	0.76
	Secondary	1021 (38)	1038 (38)	
	Higher	1259 (46)	1234 (45)	
	Missing	243 (9)	232 (9)	
Smoking during pregnancy	Never	1766 (65)	1797 (66)	0.08
	Until pregnancy was known	199 (7)	233 (9)	
	Continued	382 (14)	333 (12)	
	Missing	375 (14)	355 (13)	
Alcohol use during pregnancy	No	1200 (44)	1226 (45)	
	Yes	930 (34)	952 (35)	0.23
	Missing	592 (22)	1045 (36)	
Start folic acid supplement use	Preconception	812 (30)	886 (33)	0.18
	First 10 weeks	588 (22)	571 (21)	
	No	439 (16)	418 (15)	
	Missing	883 (32)	843 (31)	
Paternal characteristics				
Age	age	33.4 (5.4)	33.5 (5.5)	0.44
Height	cm	182.4 (7.9)	182.4 (7.8)	0.39
Weight	kg	83.8 (12.8)	84.2 (12.9)	0.95
Body mass index	kg/m²	25.2 (3.3)	25.3 (3.4)	0.26
Child characteristics				
Gestational age at birth	weeks	39.9 (1.7)	39.8 (1.7)	0.17
Ethnicity	Caucasian	2026 (74)	2022 (74)	0.89
	African	413 (15)	403 (15)	
	Asian	146 (5)	144 (5)	
	Missing	137 (5)	149 (6)	

Table 4.1.1 (continued)

Characteristic	Unit	Boys *n*=2,718	Girls *n*=2,732	*P*-value
Breast feeding	Never	186 (7)	188 (7)	0.64
	>0-3 months	655 (23)	656 (23)	
	>3 months	1021 (38)	1055 (39)	
	Missing	819 (30)	777 (29)	
Participation in sports at age 6	Never	1291 (47)	1226 (45)	<0.001
	1/week	739 (27)	976 (36)	
	≥2/week	289 (11)	88 (3)	
	Missing	403 (15)	428 (16)	

Values reflect the mean (standard deviation) for continuous variables or absolute numbers (percentage) for categorical variables. *P*-values are obtained by Students *t*-tests for continuous variables and chi-square tests for categorical variables.

Table 4.1.2 Fetal growth, childhood growth and bone measures until the age of 6 years

				Boys		Girls		
Period	(Gestational) age median (IQR)	Growth characteristic	Unit	*n*	mean (SD)	*n*	mean (SD)	*P*-value
Trimester 2	20.5 (1.3) weeks	Femur length	mm	2292	33.4 (3.5)	2316	33.4 (3.4)	0.72
		Estimated fetal weight	g	2281	385 (94)	2309	376 (87)	<0.001
Trimester 3	30.4 (1.1) weeks	Femur length	mm	2368	57.3 (3.0)	2394	57.6 (3.0)	0.007
		Estimated fetal weight	g	2364	1635 (258)	2383	1619 (260)	0.03
Birth	40.1 (1.9) weeks	Birth length	cm	1698	50.6 (2.4)	1703	49.9 (2.2)	<0.001
		Birth weight	g	2719	3503 (562)	2716	3374 (526)	<0.001
1 year	11.1 (0.7) months	Height	cm	2141	75.1 (2.5)	2116	73.5 (2.5)	<0.001
		Weight	kg	2143	10.0 (1.1)	2123	9.3 (1.0)	<0.001
2 years	24.8 (1.6) months	Height	cm	1987	88.9 (3.4)	1984	87.7 (3.4)	<0.001
		Weight	kg	2023	13.2 (1.5)	2006	12.7 (1.5)	<0.001
3 years	36.7 (1.4) months	Height	cm	1884	97.9 (3.8)	1911	96.8 (3.8)	<0.001
		Weight	kg	1908	15.5 (1.8)	1929	15.0 (1.9)	<0.001
4 years	45.8 (1.3) months	Height	cm	1687	103.7 (4.1)	1666	102.8 (4.2)	<0.001
		Weight	kg	1697	17.1 (2.2)	1669	16.8 (2.3)	<0.001
6 years	72.2 (4.1) months	Height	cm	2719	119.5 (5.7)	2713	118.7 (5.7)	<0.001
		Weight	kg	2719	23.2 (3.8)	2713	22.9 (4.1)	0.008
		BMC (total body less head)	g	2722	523 (100)	2718	519 (98)	0.01
		BA (total body less head)	cm^2	2722	942 (115)	2718	941 (109)	0.17
		BMD (total body less head)	g/cm^2	2722	0.552 (0.051)	2718	0.549 (0.052)	0.66

P-values are obtained by Student's *t*-tests for continuous variables and chi-square tests for categorical variables. Bone measures are shown for the total body less head. *SD* denotes standard deviation, *BMC* bone mineral content, *BA* bone area, *BMD* bone mineral density.

positively associated with BMC and BA (all $P<0.05$), whereas all childhood growth height and weight measures were positively associated with BMC, BA and BMD (all $P<0.01$). The magnitude of the effect estimates increased with advancing age. When applying a conditional model, estimated fetal weight gain between 20 and 30 weeks gestation, 30 weeks and birth, as well as childhood weight gain from birth to 4 years of age were all positively associated with BMC, BA and BMD (all $P<0.001$) (**Figure 4.1.2.A-F**). Fetal femur length growth between 20 and 30 weeks gestation and height growth from birth to 4 years were all positively associated with BMC, BA and BMD (all $P<0.01$). Fetal femur length growth between 30 weeks gestation and birth was positively associated with BMC and BA (both $P<0.001$), but not with BMD. Effect estimates (in SD) for childhood height and weight growth measures were larger than for fetal growth. The largest effect estimates were found for the associations of height growth during the first year with BMC and BA and weight gain during the first year with BMC, BA and BMD. The size of the effect estimates decreased after the first year of age, except for the association of height growth with BMD, which peaked at 2-3 years of age. The corresponding effect estimates are shown in **Supplement 4.1.3**.

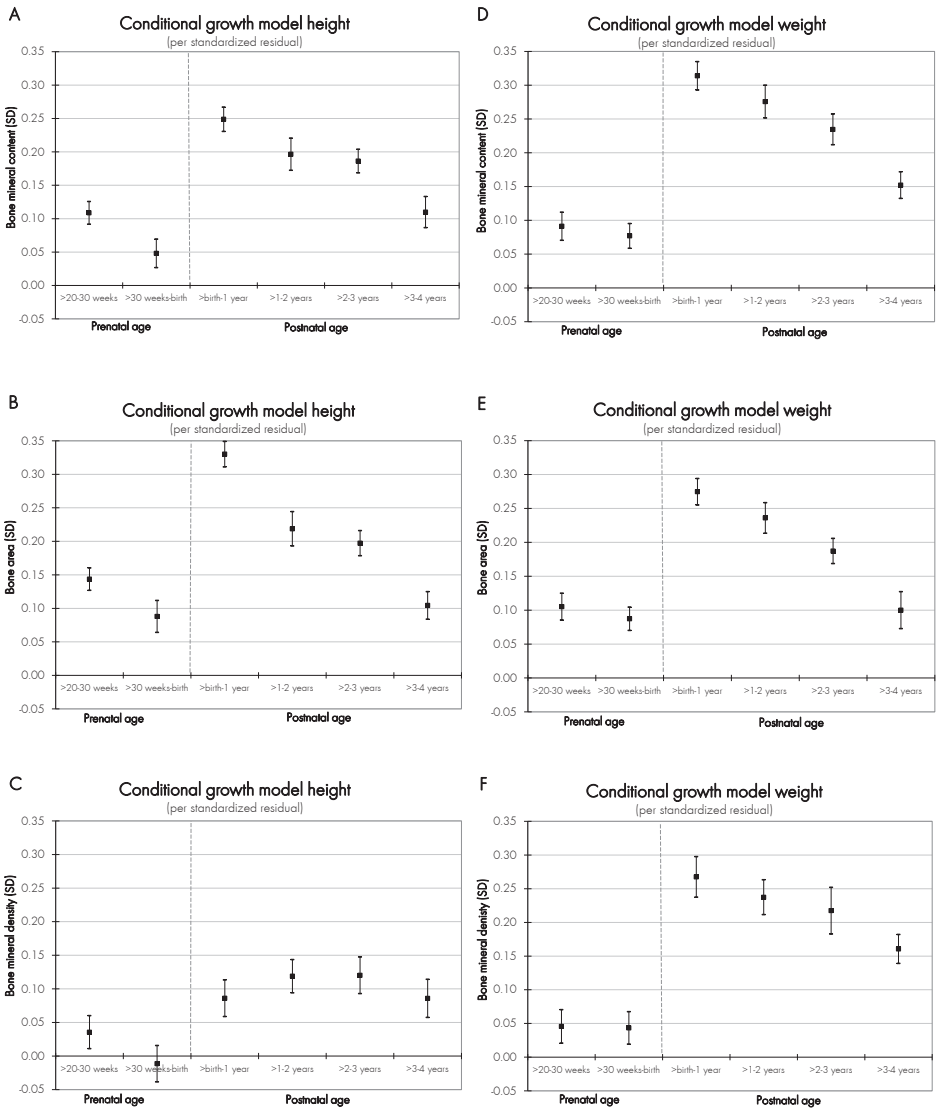
We further explored whether the associations of height and weight growth during the first year with bone measures were driven by growth during the first 6 months by replacing growth from birth to age 1 year by two separate measures for growth from birth to 6 months and from 6 to 12 months in our models. Height and weight growth during the first 6 months showed stronger associations with bone measures than growth from 6 to 12 months, yet effect estimates were not larger than those observed for growth during the first year as a whole (data not shown).

Size is a major determinant of bone mass. To demonstrate its impact on the conditional growth analysis, **Supplement 4.1.4** shows the results from the analyses not adjusted for size. Size adjustment reduced effect sizes approximately by one third. In a second sensitivity analysis, to further distinguish an increase in bone mineral accrual from bone size, we additionally adjusted BMC models for BA. As a result, fetal femur growth measures were no longer associated with BMC. However, estimated fetal weight measures, and postnatal height and weight growth measures remained positively associated with BMC, although effect estimates were less than half the size of the effect estimates for BMC not adjusted for BA (shown in **Supplement 4.1.5**).

Birth outcomes and bone measures

Gestational age at birth showed a weak positive association with BMC (*P* for trend 0.05) and BA (*P* for trend 0.02) at 6 years of age, not with BMD (**Table 4.1.3**). Children born pre-term had a -0.09 SD lower BMC (95% CI -0.19, 0.00) and a -0.08 SD lower BA (95% CI -0.18, 0.01) at school age. Birth weight showed a stronger positive association with both BMC and BA (both *P* for trend <0.001) and a weak positive association with

Figure 4.1.2.A-F Associations of conditionally modelled fetal and childhood growth with bone measures at age 6



Values are based on multiple linear regression models and reflect the coefficients and 95% confidence interval per standardized residual of conditionally modelled growth. Conditional growth variables are independent of prior growth. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, daily protein intake, folic acid supplements use, paternal weight and height, and sex, ethnicity, breastfeeding duration, participation in sports, and for current height (weight models) or weight measured as "lean + fat mass" (height models) of the child and mutually for the other growth measures.

Table 4.1.3 Associations of birth outcomes with bone measures at age 6

		Bone mineral content (SD)		Bone area (SD)		Bone mineral density (SD)	
Birth outcome	*n*	ß	95% CI	ß	95% CI	ß	95% CI
Gestational age (weeks) - *adjusted for birth weight*							
<32	28	-0.09	-0.35, 0.17	-0.06	-0.31, 0.19	-0.08	-0.40, 0.23
≥32-37	225	-0.09	-0.19, 0.00	-0.08	-0.18, 0.01	-0.06	-0.18, 0.05
≥37-42	4761	Ref		Ref		Ref	
≥42	380	0.01	-0.06, 0.09	0.01	-0.06, 0.08	0.01	-0.08, 0.10
P for trend		*0.05*		*0.03*		*0.35*	
Birth weight (g)							
<2000	64	-0.11	-0.29, 0.07	-0.06	-0.23, 0.11	-0.12	-0.34, 0.09
≥2000-2500	170	-0.10	-0.21, 0.01	**-0.15**	**-0.26, -0.05***	-0.01	-0.15, 0.12
≥2500-3000	808	**-0.08**	**-0.14, -0.02****	**-0.07**	**-0.13, -0.01****	**-0.07**	**-0.15, 0.00***
≥3000-3500	1912	Ref		Ref		Ref	
≥3500-4000	1755	0.04	-0.01, 0.09	**0.10**	**0.05, 0.14*****	-0.03	-0.09, 0.02
≥4000-4500	602	**0.11**	**0.04, 0.18****	**0.15**	**0.08, 0.21*****	0.05	-0.04, 0.13
≥4500	124	**0.19**	**0.06, 0.32****	**0.29**	**0.16, 0.41*****	-0.02	-0.18, 0.14
P for trend		***<0.001***		***<0.001***		*0.06*	
Birth weight (SD) - *adjusted for gestational age*							
SGA	501	**-0.07**	**-0.14, 0.00***	**-0.11**	**-0.18, -0.05*****	-0.01	-0.09, 0.07
AGA	4311	Ref		Ref		Ref	
LGA	576	**0.12**	**0.06, 0.18*****	**0.16**	**0.10, 0.23*****	0.02	-0.05, 0.10
P for trend		***<0.001***		***<0.001***		*0.15*	

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval for each category. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports and current height of the child. *SGA* denotes small for gestational age, *AGA* denotes appropriate for gestational age, *LGA* denotes large for gestational age. ***P*-value <0.05**, ****P*-value <0.01** and *****P*-value <0.001**

BMD (*P* for trend 0.06). When birth weight was adjusted for gestational age at birth, it was still positively associated with BMC and BA, but not with BMD. As compared to children born AGA, children born SGA had a -0.07 SD (95% CI -0.14, 0.00) lower BMC and a -0.11 SD (95% CI -0.18, -0.05) lower BA, whereas children born LGA had a 0.12 SD (95% CI 0.06, 0.18) higher BMC and a 0.16 SD (95% CI 0.10, 0.23) higher BA.

Birth weight, infant growth and bone measures

As compared to children born AGA with normal infant growth, children born SGA without growth realignment between 0 and 2 years of age had a -0.30 SD (95% CI -0.42, -0.18) lower BMC, a -0.35 SD (95%CI -0.47, -0.24) lower BA and a -0.21 SD (95%CI -0.36, -0.06) lower BMD at age 6 (**Figure 4.1.3.A-C**). Children born LGA

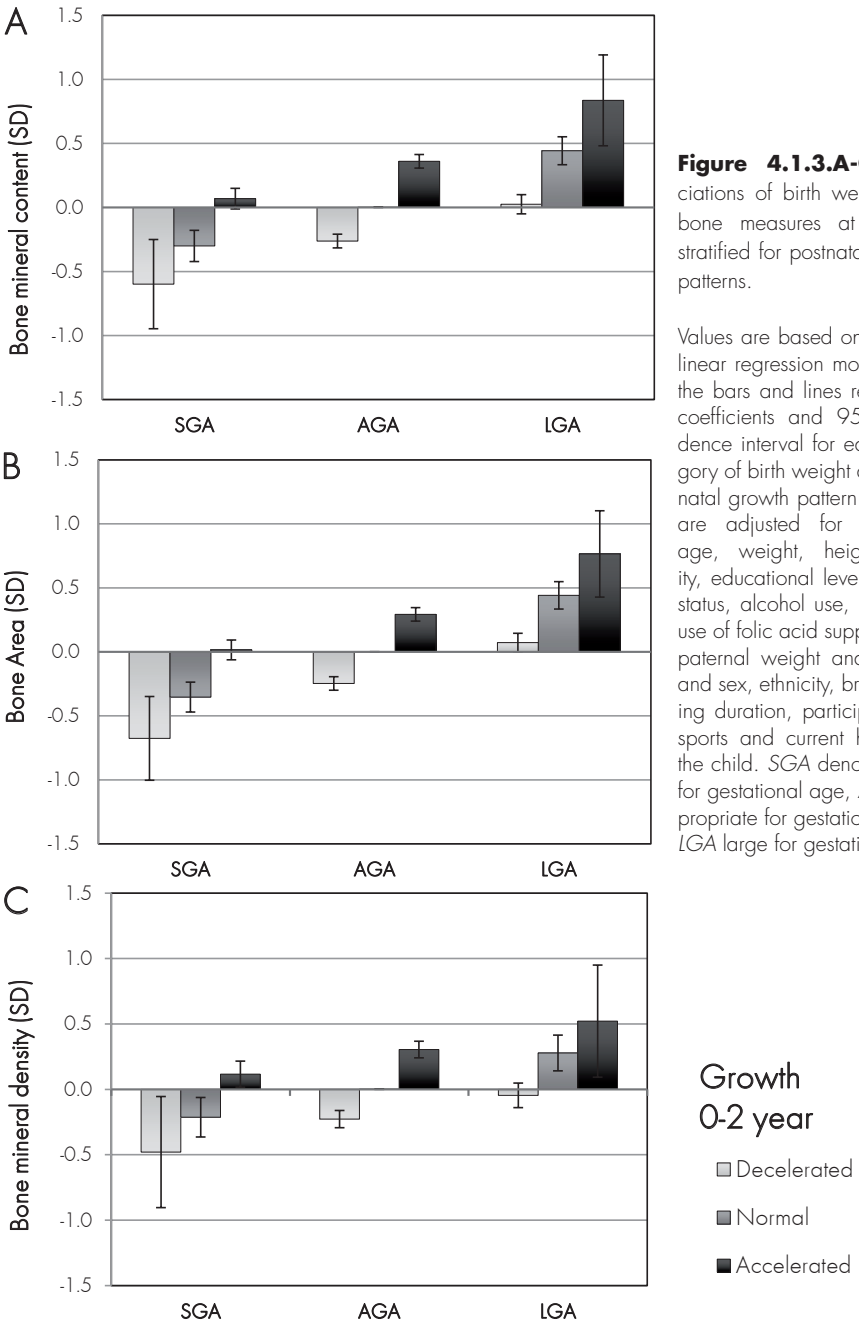


Figure 4.1.3.A-C Associations of birth weight with bone measures at age 6 stratified for postnatal growth patterns.

Values are based on multiple linear regression models and the bars and lines reflect the coefficients and 95% confidence interval for each category of birth weight and postnatal growth pattern. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, use of folic acid supplements, paternal weight and height, and sex, ethnicity, breastfeeding duration, participation in sports and current height of the child. *SGA* denotes small for gestational age, *AGA* appropriate for gestational age, *LGA* large for gestation age.

without growth realignment during infancy had a 0.44 SD (95% CI 0.33, 0.55) higher BMC, a 0.44 SD (95%CI 0.34, 0.55) higher BA and a 0.28 SD (95%CI 0.14, 0.41) higher BMD at age 6 than children born AGA with normal infant growth. Children born SGA and LGA who did show growth realignment during infancy had a similar BMD, BMC and BA to that of children born AGA with normal growth. The corresponding effect estimates are shown in Supplement 4.1.6.

DISCUSSION

Main findings

In this large population-based prospective cohort study of pregnant women and their children in the Netherlands, we found that both fetal and childhood growth, as reflected by height and weight gain, were positively associated with bone accrual at school age. Childhood growth showed larger effect estimates than fetal growth, whereas growth occurring in the first year of life showed the strongest positive association with bone mass accrual in later childhood. Gestational duration and birth weight were positively associated with bone parameters at 6 years of age. However, growth realignment between birth and 2 years of age in children born SGA or LGA led to similar bone measures at the age of 6 years to children born AGA who showed normal postnatal growth.

Methodological considerations

One of the major strengths of the study is that it is a large-scale, population-based prospective cohort study rich in assessments of prenatal and early childhood growth. This unique setting of repeated measures of fetal growth, childhood anthropometrics and bone measures enabled us to evaluate the independent associations of early growth with bone health. We used DXA, a well-validated technique to assess bone mass accrual in children. Nevertheless, our study is not free of limitations. Of the eligible children, 69% participated in the 6-year visit at our research center. Children who did not participate grew slower during fetal life and, accordingly, had lower birth weight and length than those participating in the study. They more often showed accelerated growth in the first 2 years of life, were of non-European descent and had mothers with a lower educational level (all $P<0.05$). Further, as in the Netherlands it is not obligatory to attend the routine Community Health Center visits, only 40% of the children had complete data on growth from 1 to 4 years of age and 6% did not have data from these routine visits at all. Children with incomplete data on average had a higher weight and BMC, and larger BA at the age of 6 years (all $P<0.05$), but overall similar BMD levels. Although we used multiple imputation, we cannot exclude that missing information may still have led to loss of power or biased estimates. Nevertheless, this will only be the case if effect estimates

would have differed systematically between those children included and not included in the analyses. This is unlikely, as loss to follow-up in our cohort is not expected to be related to the studied research question,[34] but cannot be fully excluded.

Establishing gestational age by ultrasound is considered superior to the use of the last menstrual period (LMP)[35] as almost 40% of pregnant women have unknown or irregular last menstrual periods.[12] However, use of first trimester ultrasounds assumes the variation in fetal growth before that ultrasound to be zero, possibly leading to underestimated effect estimates in early pregnancy. We minimized this unwanted side effect by using crown-rump length and biparietal diameter for pregnancy dating,[36,37] but not for assessing fetal growth. Yet, as a result of the correlation between fetal growth measures, underestimation of effect estimates may still have occurred. Nonetheless, since we studied relative change in size within time periods by conditional modeling, we do not expect our pregnancy dating strategy to have substantially influenced our results. The validity of ultrasound estimation of fetal weight has often been debated. A systematic review assessed the validity of estimated fetal weight measurements by reviewing measurement errors across 42 studies.[38] In this review, the authors found that all methods used to estimate fetal weight, including the method of Hadlock, have insignificant systematic error. Random error, on the other hand, averaged 10%. Another limitation possibly leading to random error may be the fact that growth measures from age 1 to 4 years were acquired from routine Community Health Centers. Nevertheless, measurements within these clinics were performed using standardized protocols. Even though routinely collected measurements of this type have previously been shown to have good accuracy by lacking systematic error,[39] random error may still have been introduced. Random error may reduce power and lead to underestimation of effects. Furthermore, the 0.67 SD cut-off that we used to define "accelerated growth" has been internationally recognized to represent clinically significant catch-up growth, but does not necessarily represent, and should not be interpreted as a biological phenomenon. Lastly, although we collected detailed information on many potential confounding variables, residual confounding due to unmeasured socio-demographic and lifestyle factors could still be influencing the results.

Interpretation of main results

Our results confirmed that early growth is associated with both bone size (BA) and bone mineral (BMC) accrual at school age. Overall, effect estimates for models including BMD were consistent in direction, yet smaller in magnitude. This attenuation of the effect on BMD is likely consequence of the influence of changes in bone area on BMD (e.g. larger bone areas result in comparatively lower BMD). Further, as DXA is a two-dimensional assessment of a three-dimensional structure, the overestimation of areal BMD in larger bones compared to volumetric BMD may also, although to a lesser extent, reduce the effect estimates. However, the prominent, yet incomplete, attenuation of effect estimates

by additional correction of BMC for BA supports the idea that early growth is associated with both increased bone size as well as mineral accrual. The attenuation of effect estimates by size correction seemed to be larger for height than for weight measures. Possibly, height growth, and in particular increase in fetal femur length, are more closely related to the actual skeletal frame size, while weight gain is only indicative of the loading effects on the skeleton during postnatal life.

Evidence supporting an influence of early growth on adult peak bone mass acquisition is increasing.[2] Recently, fast weight and height gain during childhood and adolescence were positively associated with bone strength among 1,658 60-64 year old adults.[40] Only few studies have assessed the association of fetal growth with bone development by actually measuring fetal growth instead of using birth weight as a proxy for fetal growth.[6-8,41] In line with our results, Beltrand *et al.* found that fetal growth restriction (≥20 percentiles reduction in estimated fetal weight between 22 weeks of gestation and birth) led to lower BMC in 185 newborns, independent of birth weight.[41] Among 380 British children, fetal femur length and abdominal circumference growth during 19 to 34 weeks of gestation were positively associated with BMC and BMD at age 6 years. The effect of fetal abdominal growth was independent of current height, weight or bone size.[6] Among 119 adolescents living in Denmark third trimester fetal growth velocity, birth weight and growth-in-the-first-year were positively associated with BMC.[8] However, these associations fully disappeared when adjusted for current height and weight. For that reason, the authors concluded that growth in later life, rather than early growth may be crucial to bone health in adolescence. However, pinpointing the most influential period of growth is less precise when assessments are so widely separated in time.

Assessment of repeatedly measured growth is challenged by some methodological issues. In fact, "early size" adjusted for "later size" in regression analysis is a measure of change in size between the earlier and later measurement, rather than a measure of absolute growth.[9] To overcome these issues, only one previous study used conditional modelling[10] to study the associations of linear and abdominal growth measured at 11, 19 and 34 weeks of gestation, birth and 1, 2, 3 and 4 years with bone measures among 628 4-year-olds.[7] The results were consistent with our observations, showing both fetal and childhood growth are positively associated with bone development at age 4, whereas growth in the first two postnatal years contributed most strongly. These results are also in line with our results indicating that children born with a low birth weight who showed growth realignment in the first 2 years had similar bone mass to children with a normal birth weight and normal postnatal growth. Our findings highlight the importance of early growth patterns determining bone health later in childhood.

Potential underlying mechanisms

Bone growth during fetal development and post-natal life involves complex regulatory processes mediated by growth factors, cytokines, hormones, mechanical stimuli and diverse environmental influences. These processes are largely controlled by genetic, epigenetic regulation, and availability of nutrients and diverse exposures during fetal life, childhood and adolescence.[42] By adaptation to environmental queues, early growth even from fetal life may already program later bone development.[43] Among others, hormones like leptin, growth hormone (GH) and cortisol have been suggested to play a prominent role in this "programming" of bone mineral accrual.[44,45] However, the exact mechanisms underlying the process remain unclear. Altered leptin levels, resulting from low or high nutrient availability, are proposed to program bone development by stimulating differentiation of mesenchymal stem cells into bone (osteoblasts) precursors, over adipogenic lineages, as well as stimulating cortical bone over trabecular bone formation.[44,46] Further, leptin levels are negatively correlated with fetal growth retardation[47] and positively with postnatal catch-up growth and neonatal BA and BMC.[48,49] Similarly, the GH/ IGF-I axis has long been considered a major determinant of bone mass acquisition. The axis is negatively affected by fetal growth restriction[42] and essential to achieve catch-up growth in fetal growth retarded infants.[50] IGF-I levels in neonates as well as in children are positively correlated with bone mass.[51,52] On the other hand, endogenous cortisol inhibits osteoblast function.[53] Serum cortisol levels are higher in infants born SGA[54], especially in those who do not achieve catch-up growth.[55] High normal endogenous cortisol levels have been negatively associated with bone mass, predominantly in boys.[56,57] Nutritional aspects like breast feeding, calcium and vitamin D intake, and environmental exposures like sunlight and physical activity may also exert an effect on these associations. Nevertheless, the fact that effect estimates remained essentially unchanged upon correction for a large number of nutritional and environmental factors does not seem to corroborate this contention.

Conclusion

Both fetal and childhood growth predict bone development at 6 years of age. Weight and height growth in the first year of life appeared to have the largest impact on bone mineral accrual. Compensatory growth in the first two postnatal years reduced the adverse consequences of slower growth velocity in fetal life on childhood bone mass. As childhood bone mass tends to track into adulthood, fetal life and infancy may be critical periods to attain optimal bone health and possibly reduce the risk of osteoporosis in later life. The mechanisms underlying these findings are largely unknown and warrant further study.

REFERENCES

1. Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Review: developmental origins of osteoporotic fracture. *Osteoporos Int*. 2006;17(3):337-47.
2. Baird J, Kurshid MA, Kim M, Harvey N, Dennison E, Cooper C. Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis. *Osteoporos Int*. 2011 May;22(5): 1323-34.
3. Martinez-Mesa J, Restrepo-Mendez MC, Gonzalez DA, Wehrmeister FC, Horta BL, Domingues MR, *et al*. Life-course evidence of birth weight effects on bone mass: systematic review and meta-analysis. *Osteoporos Int*. 2012 Aug 29.
4. Wilcox AJ. Intrauterine growth retardation: beyond birthweight criteria. *Early Hum Dev*. 1983 Oct;8(3-4):189-93.
5. Taal HR, Vd Heijden AJ, Steegers EA, Hofman A, Jaddoe VW. Small and large size for gestational age at birth, infant growth, and childhood overweight. *Obesity*. 2013 Jun;21(6):1261-8.
6. Harvey N, Mahon P, Robinson S, Nisbet C, Javaid M, Crozier S, *et al*. Different Indices of Fetal Growth Predict Bone Size and Volumetric Density at 4 Years Old. *J Bone Miner Res*. 2009 Oct 19.
7. Harvey NC, Mahon PA, Kim M, Cole ZA, Robinson SM, Javaid K, *et al*. Intrauterine growth and postnatal skeletal development: findings from the Southampton Women's Survey. *Paediatr Perinat Epidemiol*. 2012 Jan;26(1):34-44.
8. Jensen RB, Vielwerth S, Frystyk J, Veldhuis J, Larsen T, Molgaard C, *et al*. Fetal growth velocity, size in early life and adolescence, and prediction of bone mass: association to the GH-IGF axis. *J Bone Miner Res*. 2008 Mar;23(3):439-46.
9. Lucas A, Fewtrell MS, Cole TJ. Fetal origins of adult disease-the hypothesis revisited. *BMJ*. 1999 Jul 24;319(7204):245-9.
10. Keijzer-Veen MG, Euser AM, van Montfoort N, Dekker FW, Vandenbroucke JP, Van Houwelingen HC. A regression model with unexplained residuals was preferred in the analysis of the fetal origins of adult diseases hypothesis. *J Clin Epidemiol*. 2005 Dec;58(12):1320-4.
11. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, *et al*. The Generation R Study: design and cohort update 2012. *Eur J Epidemiol*. 2012 Sep; 27(9):739-56.
12. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, *et al*. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol*. 2008 Apr;31(4):388-96.
13. Robinson HP, Fleming JE. A critical evaluation of sonar "crown-rump length" measurements. *British Journal of Obstetrics and Gynaecology*. 1975 Sep;82(9):702-10.
14. Hadlock FP, Harrist RB, Deter RL, Park SK. Fetal femur length as a predictor of menstrual age: sonographically measured. *AJR American Journal of Roentgenology*. 1982 May;138(5):875-8.
15. Hadlock FP, Deter RL, Harrist RB, Park SK. Fetal abdominal circumference as a predictor of menstrual age. *AJR American Journal of Roentgenology*. 1982 Aug;139(2):367-70.
16. Shepard M, Filly RA. A standardized plane for biparietal diameter measurement. *Journal of ultrasound in medicine : Official Journal of the American Institute of Ultrasound in Medicine*. 1982 May;1(4):145-50.
17. Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements–a prospective study. *Am J Obstet Gynecol*. 1985 Feb 1; 151(3):333-7.
18. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). *Acta Paediatr Scand*. 1991 Aug-Sep;80(8-9):756-62.

19. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, *et al*. Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res*. 2000 Mar;47(3): 316-23.
20. Heppe DH, Kiefte-de Jong JC, Durmus B, Moll HA, Raat H, Hofman A, *et al*. Parental, fetal, and infant risk factors for preschool overweight: the Generation R Study. *Pediatr Res*. 2013 Jan;73(1): 120-7.
21. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *BMJ*. 2000 Apr 8;320(7240): 967-71.
22. Heppe DH, Medina-Gomez C, Hofman A, Franco OH, Rivadeneira F, Jaddoe VW. Maternal first-trimester diet and childhood bone mass: the Generation R Study. *Am J Clin Nutr*. 2013 Jul; 98(1):224-32.
23. Lewiecki EM, Gordon CM, Baim S, Binkley N, Bilezikian JP, Kendler DL, *et al*. Special report on the 2007 adult and pediatric Position Development Conferences of the International Society for Clinical Densitometry. *Osteoporos Int*. 2008 Oct;19(10):1369-78.
24. Heaney RP. Bone mineral content, not bone mineral density, is the correct bone measure for growth studies. *Am J Clin Nutr*. 2003 Aug;78(2):350-1; author reply 1-2.
25. Prentice A, Parsons TJ, Cole TJ. Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. *Am J Clin Nutr*. 1994 Dec;60(6):837-42.
26. Warner JT, Cowan FJ, Dunstan FD, Evans WD, Webb DK, Gregory JW. Measured and predicted bone mineral content in healthy boys and girls aged 6-18 years: adjustment for body size and puberty. *Acta Paediatr*. 1998 Mar;87(3):244-9.
27. Durmus B, van Rossem L, Duijts L, Arends LR, Raat H, Moll HA, *et al*. Breast-feeding and growth in children until the age of 3 years: the Generation R Study. *Br J Nutr*. 2011 Jun;105(11):1704-11.
28. Cooper C, Harvey N, Cole Z, Hanson M, Dennison E. Developmental origins of osteoporosis: the role of maternal nutrition. *Adv Exp Med Biol*. 2009;646:31-9.
29. Cooper C, Cawley M, Bhalla A, Egger P, Ring F, Morton L, *et al*. Childhood growth, physical activity, and peak bone mass in women. *J Bone Miner Res*. 1995 Jun;10(6):940-7.
30. Rudang R, Mellstrom D, Clark E, Ohlsson C, Lorentzon M. Advancing maternal age is associated with lower bone mineral density in young adult male offspring. *Osteoporos Int*. 2012 Feb;23(2): 475-82.
31. Harvey NC, Javaid MK, Poole JR, Taylor P, Robinson SM, Inskip HM, *et al*. Paternal skeletal size predicts intrauterine bone mineral accrual. *J Clin Endocrinol Metab*. 2008 May;93(5):1676-81.
32. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, *et al*. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ*. 2009;338: b2393.
33. Lenth RV. Java Applets for Power and Sample Size [Computer software]. Retrieved May 15th, 2014, from http://www.stat.uiowa.edu/~rlenth/Power. (2006-9).
34. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology*. 2006 Jul;17(4):413-8.
35. Tunon K, Eik-Nes SH, Grottum P. A comparison between ultrasound and a reliable last menstrual period as predictors of the day of delivery in 15,000 examinations. *Ultrasound Obstet Gynecol*. 1996 Sep;8(3):178-85.
36. Altman DG, Chitty LS. New charts for ultrasound dating of pregnancy. *Ultrasound Obstet Gynecol*. 1997 Sep;10(3):174-91.
37. Robinson HP, Sweet EM, Adam AH. The accuracy of radiological estimates of gestational age using early fetal crown-rump length measurements by ultrasound as a basis for comparison. *British Journal of Obstetrics and Gynaecology*. 1979 Jul;86(7):525-8.

38. Dudley NJ. A systematic review of the ultrasound estimation of fetal weight. *Ultrasound Obstet Gynecol.* 2005 Jan;25(1):80-9.
39. Howe LD, Tilling K, Lawlor DA. Accuracy of height and weight data from child health records. *Arch Dis Child.* 2009 Dec;94(12):950-4.
40. Kuh D, Wills AK, Shah I, Prentice A, Hardy R, Adams JE, *et al.* Growth from birth to adulthood and bone phenotype in early old age: a british birth cohort study. *J Bone Miner Res.* 2014 Jan; 29(1):123-33.
41. Beltrand J, Alison M, Nicolescu R, Verkauskiene R, Deghmoun S, Sibony O, *et al.* Bone mineral content at birth is determined both by birth weight and fetal growth pattern. *Pediatr Res.* 2008 Jul; 64(1):86-90.
42. Setia S, Sridhar MG. Changes in GH/IGF-1 axis in intrauterine growth retardation: consequences of fetal programming? *Horm Metab Res.* 2009 Nov;41(11):791-8.
43. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med.* 2008 Jul 3;359(1):61-73.
44. Devlin MJ, Bouxsein ML. Influence of pre- and peri-natal nutrition on skeletal acquisition and maintenance. *Bone.* 2012 Feb;50(2):444-51.
45. Goodfellow LR, Cooper C, Harvey NC. Regulation of placental calcium transport and offspring bone health. *Front Endocrinol.* 2011;2:3.
46. Wong IP, Nguyen AD, Khor EC, Enriquez RF, Eisman JA, Sainsbury A, *et al.* Neuropeptide Y is a critical modulator of leptin's regulation of cortical bone. *J Bone Miner Res.* 2012 Oct 8.
47. Jaquet D, Leger J, Levy-Marchal C, Oury JF, Czernichow P. Ontogeny of leptin in human fetuses and newborns: effect of intrauterine growth retardation on serum leptin concentrations. *J Clin Endocrinol Metab.* 1998 Apr;83(4):1243-6.
48. Javaid MK, Godfrey KM, Taylor P, Robinson SM, Crozier SR, Dennison EM, *et al.* Umbilical cord leptin predicts neonatal bone mass. *Calcif Tissue Int.* 2005 May;76(5):341-7.
49. Jaquet D, Leger J, Tabone MD, Czernichow P, Levy-Marchal C. High serum leptin concentrations during catch-up growth of children born with intrauterine growth retardation. *J Clin Endocrinol Metab.* 1999 Jun;84(6):1949-53.
50. Cianfarani S, Ladaki C, Geremia C. Hormonal regulation of postnatal growth in children born small for gestational age. *Hormone research.* 2006;65 Suppl 3:70-4.
51. Javaid MK, Godfrey KM, Taylor P, Shore SR, Breier B, Arden NK, *et al.* Umbilical venous IGF-1 concentration, neonatal bone mass, and body composition. *J Bone Miner Res.* 2004 Jan;19(1): 56-63.
52. van Coeverden SC, Netelenbos JC, de Ridder CM, Roos JC, Popp-Snijders C, Delemarre-van de Waal HA. Bone metabolism markers and bone mass in healthy pubertal boys and girls. *Clin Endocrinol (Oxf).* 2002 Jul;57(1):107-16.
53. Lillycrop KA, Slater-Jefferies JL, Hanson MA, Godfrey KM, Jackson AA, Burdge GC. Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr.* 2007 Jun;97(6):1064-73.
54. Verkauskiene R, Jaquet D, Deghmoun S, Chevenne D, Czernichow P, Levy-Marchal C. Smallness for gestational age is associated with persistent change in insulin-like growth factor I (IGF-I) and the ratio of IGF-I/IGF-binding protein-3 in adulthood. *The Journal of clinical endocrinology and metabolism.* 2005 Oct;90(10):5672-6.
55. Cianfarani S, Geremia C, Scott CD, Germani D. Growth, IGF system, and cortisol in children with intrauterine growth retardation: is catch-up growth affected by reprogramming of the hypothalamic-pituitary-adrenal axis? *Pediatr Res.* 2002 Jan;51(1):94-9.
56. Remer T, Boye KR, Hartmann MF, Neu C, Schoenau E, Manz F, *et al.* Adrenal steroid hormones and metaphyseal bone in children. *Hormone research.* 2004;62(5):221-6.

57. Remer T, Boye KR, Hartmann M, Neu CM, Schoenau E, Manz F, *et al.* Adrenarche and bone modeling and remodeling at the proximal radius: weak androgens make stronger cortical bone in healthy children. *J Bone Miner Res.* 2003 Aug;18(8):1539-46.

Supplement 4.1.1 Description of techniques applied to measure fetal growth characteristics[12]

CRL was measured in a true mid-sagittal plane with the genital tubercle and the fetal spine longitudinally in view. The maximum length from cranium to the caudal rump was measured as a straight line. HC was measured in a transverse section of the head with a central midline echo, interrupted in the anterior third by the cavity of the septum pellucidum with the anterior and posterior horns of the lateral ventricles in view. For HC an ellipse was drawn around the outline of the skull. AC was measured in a symmetrical, transverse, round section through the abdomen, with visualization of the vertebrae on a lateral position in alignment with the ribs. The measurement was taken in a plane with the stomach and the bifurcation of the umbilical and hepatic veins using an ellipse around the abdomen. FL was measured with the full length of the bone in view perpendicular to the ultrasound beam. Trans-vaginal scanning was performed in case of limited visibility by trans-abdominal scanning in early pregnancy.

Supplement 4.1.2 Associations of individually modelled fetal and childhood growth with bone measures at age 6

Growth characteristics (*n*=5,431)	Bone mineral content (SD)		Bone area (SD)		Bone mineral density (SD)	
	ß	95% CI	ß	95% CI	ß	95% CI
Femur length (SD)						
20 weeks of gestation	0.05	0.03, 0.06***	0.07	0.05, 0.09***	0.01	-0.02, 0.03
30 weeks of gestation	0.08	0.06, 0.10***	0.12	0.10, 0.13***	0.01	-0.01, 0.03
Length (SD)						
Birth length	0.02	0.00, 0.04	0.05	0.03, 0.08***	0.02	-0.01, 0.04*
1 year	0.22	0.20, 0.24***	0.32	0.29, 0.34***	0.21	0.18, 0.24**
2 years	0.25	0.23, 0.28***	0.29	0.27, 0.32***	0.31	0.28, 0.34***
3 years	0.37	0.35, 0.39***	0.46	0.44, 0.48***	0.40	0.36, 0.43***
4 years	0.40	0.37, 0.42***	0.48	0.45, 0.50***	0.43	0.40, 0.47***
6 years	0.54	0.52, 0.56***	0.63	0.61, 0.65***	0.63	0.60, 0.66***
Estimated fetal weight (SD)						
20 weeks of gestation	0.02	0.00, 0.04	0.02	0.00, 0.04*	0.01	-0.02, 0.03
30 weeks of gestation	0.05	0.03, 0.07***	0.07	0.05, 0.09***	0.02	-0.01, 0.04
Weight (SD)						
Birth weight	0.07	0.05, 0.09***	0.09	0.07, 0.11***	-0.02	-0.05, 0.00
1 year	0.27	0.24, 0.29***	0.25	0.23, 0.27***	0.04	0.01, 0.07***
2 years	0.39	0.37, 0.41***	0.35	0.33, 0.37***	0.11	0.09, 0.14***
3 years	0.47	0.45, 0.50***	0.42	0.40, 0.44***	0.16	0.13, 0.19***
4 years	0.49	0.47, 0.52***	0.42	0.39, 0.44***	0.19	0.16, 0.22***
6 years	0.68	0.66, 0.70***	0.55	0.53, 0.57***	0.29	0.26, 0.32***

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports, and for current height (weight models) or weight measured as "lean + fat mass" (height models) of the child. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

Supplement 4.1.3 Associations of conditionally modelled fetal and childhood growth with bone measures at age 6

(Gestational) age (*n*=5,431)	Bone mineral content (SD)		Bone area (SD)		Bone mineral density (SD)	
	ß	95% CI	ß	95% CI	ß	95% CI
(Femur) length						
>20 - 30 weeks	0.11	0.09, 0.13***	0.14	0.13, 0.16***	0.04	0.01, 0.06**
>30 weeks - birth	0.05	0.03, 0.07***	0.09	0.06, 0.11***	-0.01	-0.04, 0.02
>birth - 1 year	0.25	0.23, 0.27***	0.33	0.31, 0.35***	0.09	0.06, 0.11***
>1 - 2 years	0.20	0.17, 0.22***	0.22	0.19, 0.24***	0.12	0.09, 0.14***
>2 - 3 years	0.19	0.17, 0.20***	0.20	0.18, 0.22***	0.12	0.09, 0.15***
>3 - 4 years	0.11	0.09, 0.13***	0.11	0.09, 0.13***	0.09	0.06, 0.11***
(Estimated fetal) weight						
>20 - 30 weeks	0.09	0.07, 0.11***	0.11	0.09, 0.12***	0.05	0.02, 0.07***
>30 weeks - birth	0.08	0.06, 0.10***	0.09	0.07, 0.10***	0.04	0.02, 0.07***
>birth - 1 year	0.31	0.29, 0.33***	0.27	0.26, 0.29***	0.27	0.24, 0.30***
>1 - 2 years	0.28	0.25, 0.30***	0.24	0.21, 0.26***	0.24	0.21, 0.26***
>2 - 3 years	0.23	0.21, 0.26***	0.19	0.17, 0.21***	0.22	0.18, 0.25***
>3 - 4 years	0.15	0.13, 0.17***	0.10	0.07, 0.13***	0.16	0.14, 0.18***

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval per standardized residual of a conditionally modelled growth measure. Conditional growth measures are independent of prior growth. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports, and for current height (weight models) or weight measured as "lean + fat mass" (height models) of the child and mutually for the other growth measures. *P-value <0.05, **P-value <0.01 and ***P-value <0.001

Supplement 4.1.4 Associations of conditionally modelled fetal and childhood growth with bone measures at age 6 – *not adjusted for current size*

(Gestational) age (*n*=5,431)	Bone mineral content (SD)		Bone area (SD)		Bone mineral density (SD)	
	ß	95% CI	ß	95% CI	ß	95% CI
(Femur) length						
>20 - 30 weeks	0.17	0.15, 0.19***	0.19	0.17, 0.21***	0.06	0.03, 0.08***
>30 weeks - birth	0.13	0.10, 0.15***	0.15	0.12, 0.17***	0.09	0.07, 0.12***
>birth - 1 year	0.44	0.41, 0.46***	0.47	0.45, 0.49***	0.06	0.02, 0.09***
>1 - 2 years	0.31	0.27, 0.34***	0.30	0.27, 0.33***	0.25	0.22, 0.28***
>2 - 3 years	0.32	0.30, 0.34***	0.30	0.28, 0.32***	0.22	0.19, 0.25***
>3 - 4 years	0.19	0.16, 0.22***	0.17	0.14, 0.19***	0.24	0.21, 0.27***
(Estimated fetal) weight						
>20 - 30 weeks	0.13	0.11, 0.15***	0.11	0.09, 0.13***	0.06	0.04, 0.09***
>30 weeks - birth	0.11	0.09, 0.13***	0.15	0.13, 0.18***	0.06	0.04, 0.09***
>birth - 1 year	0.43	0.41, 0.45***	0.12	0.10, 0.14***	0.06	0.03, 0.08***
>1 - 2 years	0.37	0.34, 0.40***	0.41	0.39, 0.43***	0.32	0.30, 0.35***
>2 - 3 years	0.31	0.29, 0.34***	0.34	0.32, 0.37***	0.28	0.26, 0.30***
>3 - 4 years	0.20	0.17, 0.22***	0.28	0.26, 0.30***	0.25	0.22, 0.29***

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval per standardized residual of a conditionally modelled growth measure. Conditional growth measures are independent of prior growth. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports of the child and mutually for the other growth measures. *P-value <0.05, **P-value <0.01 and ***P-value <0.001

These unadjusted results must be interpreted cautiously as analysis of areal BMD without adjustment for height in a pediatric population may lead to spurious results as a consequence of size artefacts.[25]

Supplement 4.1.5 Associations of conditionally modelled fetal and infant growth with bone mineral content at age 6 - *adjusted for bone area*

	Bone mineral content (SD)			
	(Femur) length		(Estimated fetal) weight	
	ß	95% CI	ß	95% CI
>20 - 30 weeks	0.01	0.00, 0.02	0.02	0.00, 0.03*
>30 weeks - birth	-0.01	-0.03, 0.00	0.01	0.00, 0.03*
>birth - 1 year	0.02	0.01, 0.04**	0.12	0.10, 0.14***
>1 - 2 years	0.05	0.03, 0.06***	0.11	0.09, 0.12***
>2 - 3 years	0.05	0.04, 0.07***	0.10	0.08, 0.12***
>3 - 4 years	0.04	0.02, 0.05***	0.08	0.07, 0.09***

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval per standardized residual of a conditionally modelled growth measure. Conditional growth measures are independent of prior growth. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports, and for current height (weight models) or weight measured as "lean + fat mass" (height models) of the child, bone area and mutually for the other growth measures. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

Supplement 4.1.6 Associations of birth weight with bone measures at age 6 stratified for postnatal growth patterns

		Bone mineral content (SD)		Bone area (SD)		Bone mineral density (SD)	
Growth characteristics	*n*	ß	95% CI	ß	95% CI	ß	95% CI
Small for gestational age							
Decelerated postnatal growth	17	-0.60	-0.95, -0.25***	-0.68	-1.00, -0.35***	-0.48	-0.90, -0.06*
Normal postnatal growth	149	-0.30	-0.42, -0.18***	-0.35	-0.47, -0.24***	-0.21	-0.36, -0.06**
Accelerated postnatal growth	339	0.07	-0.01, 0.15	0.02	-0.06, 0.09	0.12	0.02, 0.22*
Appropriate for gestational age							
Decelerated postnatal growth	989	-0.26	-0.32, -0.21***	-0.25	-0.30, -0.19***	-0.23	-0.29, -0.16***
Normal postnatal growth	2211	Ref		Ref		Ref	
Accelerated postnatal growth	1147	0.36	0.31, 0.41***	0.29	0.24, 0.35***	0.30	0.24, 0.37***
Large for gestational age							
Decelerated postnatal growth	385	0.03	-0.05, 0.10	0.07	0.00, 0.14*	-0.05	-0.14, 0.05
Normal postnatal growth	178	0.44	0.33, 0.55***	0.44	0.34, 0.55***	0.28	0.14, 0.41***
Accelerated postnatal growth	16	0.84	0.48, 1.19***	0.77	0.43, 1.10***	0.52	0.10, 0.95*

Values are based on imputed multiple linear regression models and reflect the coefficients and 95% confidence interval. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking, folic acid supplement use, paternal weight and height, and gender, ethnicity, breastfeeding duration, participation in sports and current height of the child. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

CHAPTER 4.2

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Am J Clin Nutr. 2013 Jul;98(1):224-32.

MATERNAL DIET DURING PREGNANCY AND CHILDHOOD BONE MASS

ABSTRACT

Background Maternal diet during pregnancy has been suggested to influence bone health in later life.

Objective To assess the association of maternal first trimester dietary intake during pregnancy with childhood bone mass.

Design In a prospective cohort study among 2,819 mothers and their children, we measured first trimester daily energy, protein, fat, carbohydrate, calcium, phosphorus and magnesium intake by a food frequency questionnaire and homocysteine, folate and vitamin B12 concentrations in venous blood. We measured childhood total body bone mass by dual-energy x-ray absorptiometry (DXA) at the median age of 6.0 years.

Results Higher first trimester maternal protein, calcium and phosphorus intake, and vitamin B12 concentrations were associated with higher childhood bone mass, while carbohydrate intake and homocysteine concentrations were associated with lower childhood bone mass (all *P* for trend <0.01). Maternal fat, magnesium intake, and folate concentrations were not associated with childhood bone mass. In the fully adjusted regression model including all dietary factors significantly associated with childhood bone mass, maternal phosphorus intake and homocysteine concentrations most strongly predicted childhood bone mineral content (BMC) (ß 2.8 (95% CI 1.1, 4.5) and (ß -1.8 (95% CI -3.6, 0.1) g per SD increase, respectively) whereas maternal protein intake and vitamin B12 concentrations most strongly predicted BMC adjusted for bone area (ß 2.1 (95% CI 0.7, 3.5) and ß 1.8 (95% CI 0.4, 3.2) g per SD increase, respectively).

Conclusions Maternal first trimester dietary factors are associated with childhood bone mass, suggesting that fetal nutritional exposures may permanently influence bone development.

INTRODUCTION

Osteoporosis represents a major public health problem.[1-3] Previous studies have shown the relevance of early life factors for the development of bone health and osteoporosis during the life-course.[4] Maternal diet during pregnancy, the main determinant of fetal nutrition, has been suggested to influence childhood bone mass.[5-9] In mother-offspring cohorts in Tasmania, India and the UK, maternal milk, magnesium, calcium and folate intake during pregnancy were positively associated with bone mass in their offspring,[5-8] whereas fat intake showed an inverse association.[8] Although these finding may reflect an effect of shared genetic or environmental determinants, they support the hypothesis of bone health programming via direct or indirect effects of nutrient availability in early life. Currently, it is unknown which mechanisms may underlie such a programming effect. Nutrients like calcium and phosphorus, the main bone forming minerals, may enhance fetal bone accrual in a direct manner.[10] Additionally, indirect effects of nutrient availability may act trough changes in the growth hormone/insulin-like growth factor (IGF-I) axis or alterations in glucocorticoid levels, such as cortisol.[11,12] Two other candidate hormonal mediators of bone-diet interactions are the adipokine leptin and osteocalcin.[13,14] Further, epigenetic changes due to altered DNA methylation or histone modification have been suggested to be key players in this programming process and should also be considered.[15]

Maternal folate intake and blood concentrations were positively associated with childhood bone mass in two studies focused on this nutrient.[5,7] Folate and other B vitamins (B2, B6 and B12) are involved in the homocysteine metabolism[16] and may reduce the adverse effects of hyperhomocysteinemia, a strong risk factor for osteoporotic fractures in older adults.[17] Alternatively, by acting as methyl donors, a beneficial effect of high maternal folate and B vitamin concentrations on childhood bone mass may support the hypothesis of epigenetic changes programming later bone health. As homocysteine and B vitamin concentrations are potentially modifiable, we investigated the influence of maternal dietary factors on the bone health of their children with particular focus on the homocysteine metabolism. We assessed the associations of maternal first trimester dietary nutrient intake, homocysteine, folate and vitamin B12 concentrations with childhood bone mass in a population-based prospective cohort study including 2,819 Dutch mothers and 6 year old children.

METHODS

Study design

This study is embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.18 All mothers who

were resident in the study area at their delivery date and had a delivery date from April 2002 until January 2006 were eligible. We aimed enrolment in first trimester but we allowed enrolment until delivery of the child. In total, 75% of all mothers enrolled before a gestational age of 18 weeks. Of all eligible children in the study area, 61% participated at birth in the study. We conducted the study according to the guidelines of the Helsinki Declaration and it was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31). We obtained written informed consent from all participants. We restricted the study population to 4,097 mothers of Dutch ethnicity since the food frequency questionnaire (FFQ) was validated for assessment of dietary intake in a Dutch population.[19] We defined ethnicity of the mother according the classification of Statistics Netherlands.[20,21] Of these Dutch mothers, 4,016 mothers enrolled in the first or second trimester of pregnancy. We measured dietary intake and/or homocysteine, folate or vitamin B12 concentrations in 3,859 of these mothers. Their pregnancies resulted in 3,780 singleton live births. Of these, 2,901 children participated in the 6 year visit and we performed DXA-scans in 2,819 children (69%). Since excluding mothers with a second (*n*=241) or third (*n*=2) pregnancy in the study did not change our results, we included these pregnancies in the analyses. (Flowchart is given in Figure 4.2.1).

Maternal dietary intake

We assessed maternal dietary intake at enrolment in the study (median 13.5 weeks of gestation, IQR range 3.3 weeks) using a modified version of the validated semi quantitative food frequency questionnaire (FFQ) of Klipstein-Grobusch *et al.*[19] We validated this modified version of the FFQ against 3day-24hour recalls in 82 Dutch pregnant women visiting their midwives in Rotterdam, the Netherlands. We considered the FFQ valid for studying macro- and micronutrient intake in Dutch pregnant women, as the intra-class correlation coefficients for the (energy-adjusted) nutrients were: 0.65 for protein; 0.54 for fat; 0.54 for carbohydrate; 0.75 for calcium and 0.78 for phosphorus intake, while for total energy intake it was only 0.33. The FFQ considered food intake over the prior three months, thereby mostly covering dietary intake within the first trimester of pregnancy. The FFQ consists of 293 items structured according to meal pattern. Questions included consumption frequency, portion size, preparation method, and additions. We estimated portion sizes using Dutch household measures and photographs of foods showing different portion sizes.[22] To calculate average daily nutritional values we used the Dutch food composition table 2006.[23] In the current study we analyzed intake of macronutrients and intake of calcium, phosphorus, and magnesium following results of earlier studies.[5-8]

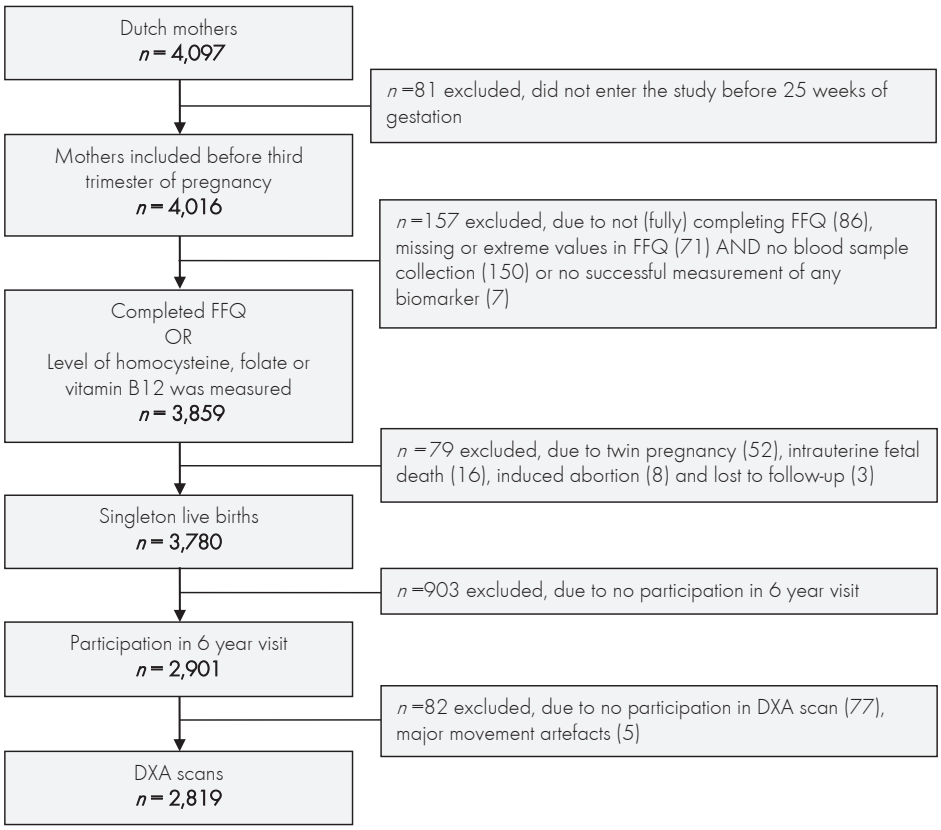

Figure 4.2.1 Flowchart of study participants

Maternal homocysteine, folate and vitamin B12 concentrations

Venous blood samples were drawn at enrolment in early pregnancy (median 12.9 weeks of gestation, IQR range 2.3 weeks), and stored at room temperature for a maximum of 3 hours. Then, blood samples were transported to a dedicated laboratory facility of the regional laboratory in Rotterdam, the Netherlands (STAR-MDC) for further processing and storage at -80°C.[24] To analyze homocysteine, folate and vitamin B12 concentration, we picked and transported the serum samples (vitamin B12) and plasma samples (homocysteine, folate) to the Department of Clinical Chemistry at the Erasmus University Medical Center, Rotterdam in 2008. After thawing, we analyzed homocysteine, folate and vitamin B12 concentrations using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The between-run coefficients of variation for plasma measurements were: 3.1% at 7.2 µmol/L, 3.1% at 12.9 µmol/L, and 2.1% at 26.1 µmol/L, with an analytic range of 1-50 µmol/L for homocysteine; 8.9% at 5.6 nmol/L, 2.5% at 16.6 nmol/L, and 1.5% at 33.6 nmol/L, with an analytic range of 1.8-45.3 nmol/L for plasma folate; and 3.6% at 142 pmol/L,

7.5% at 308 pmol/L, and 3.1% at 633 pmol/L, with an analytic range of 44-1476 pmol/L for serum vitamin B12.

Bone mass measurements

We measured total body bone mineral density, bone mineral content and bone area using a DXA scan (iDXA, General Electrics –Lunar, 2008, Madison, WI, USA) at the median age of 6.0 (IQR 0.37) years. Well-trained research assistants obtained the DXA scans by using the same device and software (enCORE) following standard manufacturer protocols. We performed daily quality assurance using a spine phantom. In 2011, the longitudinal coefficient of variation for bone mineral density was 0.23%. Prior to the scan procedure, we asked participants to take off their shoes, heavy clothes and metal accessories. Then, we measured weight to the nearest 0.2 kg using an electronic personal scale (Seca, Almere, The Netherlands) and height to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K). Thereafter, we placed participants in a supine position on the scan and instructed them to hold their hands flat with the palms down on the scanner table and the arms alongside the body. To ensure that the lines between adjacent sub-regions of the body were placed correctly, scans were evaluated twice; directly after the scanning procedure and at a later time point by a second well-trained research assistant.

In our analyses, we used areal total body less head (TBLH) bone mineral density and bone mineral content as recommended by the International Society for Clinical Densitometry.[25] Bone size effects should be considered in studies focused on BMC and areal BMD measured during periods of growth.[26] In subjects with large bones, areal BMD alone can overestimate the volumetric BMD In studies focused on bone mineral density, Prentice *et al.* suggested to use BMC corrected for bone area (BA), weight and height to correctly adjust for size effects.[27] Therefore, in a sensitivity analysis, we additionally adjusted BMC models for BA, as weight and height were already included.

Covariates

We registered maternal age and measured height and weight, without shoes and heavy clothing, at enrolment. We also collected information about weight just before pregnancy, maternal education, marital status, parity and ethnicity, and ethnicity of the grandparents and father by questionnaire at enrolment in the study. As the enrolment in our study was during pregnancy, we were not able to measure maternal weight before pregnancy. Yet, correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was high (ρ=0.97; P<0.01). Maternal smoking and alcohol habits were assessed by questionnaires in each trimester. We obtained information about offspring sex, gestational age and birth weight from medical records and hospital registries, and about breast feeding from questionnaires at 2, 6 and 12 months after delivery.[28] We assessed

the child's diet around the age of 14 months using a modified version of a validated semi quantitative FFQ[29] consisting of 211 food items. This FFQ has been validated against 3day-24hour recalls in Dutch children aged 14 months giving the following intra-class correlation coefficients: 0.7 for total protein; 0.4 for total fat:, 0.4 for carbohydrate and 0.5 for calcium intake. As this FFQ was implemented in a later stage in the study it was only available in a subgroup of the population (59%).We obtained information about frequency of participation in sports from a questionnaire at 6 years of age.

Statistical analysis

We used t-tests and chi-square tests to compare differences in subject characteristics between boys and girls. We categorized dietary nutrient intake (fat, protein, carbohydrate, calcium, phosphorus and magnesium) and blood concentrations (homocysteine, folate and vitamin B12) into quintiles and used the lowest category as the reference category in all models. We performed multiple linear regression analyses to assess the associations of dietary factors with childhood bone measures at the age of 6 years. We performed tests for trends by using dietary intakes and blood concentrations as continuous variables in the models. We log- and square root transformed blood concentrations that were not normally distributed and lacked goodness of fit. Based on previous studies we adjusted models for maternal age, weight, height, parity, educational level, marital status, alcohol use, and smoking, and gender, birth weight, ethnicity, breastfeeding, participation in sports, and age and height at the time of the measurement of the child.[5-8] We adjusted for weight at the time of the measurement by adding "lean mass + fat mass" to the model, thereby excluding the contribution of bone mass to the child's weight. Since maternal total energy intake and macronutrient intakes were strongly correlated (**Supplement 4.2.1**), we used the energy partition method to adjust for total energy intake in the macronutrient analyses.[30] In the micronutrient analysis we used the residual nutrient method. We entered dietary factors found to be associated with childhood bone mass at $P<0.10$ significance level in a backwards selection regression model in order to identify the major dietary factors of bone mineral accrual in childhood ($P<0.10$). We assessed potential multi-collinearity between nutrients by exploring the change in effect estimates and calculating the variance inflation factor (VIF).[31] We performed a sensitivity analysis to assess whether the associations of maternal nutrient intake with childhood bone mass could be explained by later diet of the child rather than maternal diet. We added available information on protein, fat, carbohydrate or calcium intake of the child at 14 months to each corresponding model. We did not add nutrient intake of the child to our final models as data was missing in 41% of the study population due to the fact that it was only assessed in a subgroup.[18] In another sensitivity analysis, we additionally adjusted models concerning bone mineral content for total body bone area to assure correct size adjustment.[27] To prevent bias associated with missing data, we used multiple imputation

(5 imputations) for covariates with missing values based on the correlation of the missing variables with other participant characteristics, according to the Markov Chain Monte Carlo method.[32] We additionally added variables related to the covariates as predictors to the imputation model to increase the plausibility of the missing at random assumption. The amount of missing values ranged from 1-14%, except for breast feeding duration (26%). We report the pooled results of the analyses performed in each of the 5 imputed datasets. *P*-values are two-sided. We performed analyses using the Predictive Analytic Software version 17.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

Characteristics of study participants

Boys were born with a higher birth weight, were taller and more often participated frequently in sports at age 6 than girls (Table 4.2.1). Bone measures did not differ between boys and girls. Average maternal nutrient intake and homocysteine, folate and vitamin B12 concentrations in the first trimester of pregnancy are shown in Table 4.2.2. Characteristics of the imputed study population are given in Supplement 4.2.2.

Maternal dietary intakes and childhood bone mass

Higher maternal protein intake in the first trimester of pregnancy was associated with a higher childhood bone mineral content (*P* for trend 0.02) and density (*P* for trend <0.001). As shown in Table 4.2.3, the difference in bone mineral content between the highest and lowest (reference) quintile of maternal protein intake was 7.0 g (95%

Table 4.2.1 Characteristics of study participants

	Unit	Boys *n*=1,409	Girls *n*=1,410	*P*-value
Maternal characteristics				
Height	cm	170.9 (6.2)	170.9 (6.6)	0.99
Pre-pregnancy weight	kg	68.1 (12.5)	68.3 (12.2)	0.59
Body mass index	kg/m^2	23.3 (4.0)	23.4 (4.0)	0.50
Age	year	31.6 (4.3)	31.5 (4.1)	0.41
Parity ≥1	No	877 (62)	866 (61)	0.25
	Yes	531 (38)	539 (38)	
	Missing	1 (0)	5 (0)	
Single motherhood	No	1279 (92)	1296 (93)	0.56
	Yes	85 (6)	74 (5)	
	Missing	45 (3)	40 (3)	
Educational status	Primary	24 (2)	38 (3)	0.16

Table 4.2.1 (continued)

	Unit	Boys *n*=1,409	Girls *n*=1,410	*P*-value
	Secondary	495 (35)	519 (37)	
	Higher	866 (62)	835 (59)	
	Missing	24 (2)	18 (1)	
Smoking during pregnancy	Never	958 (68)	988 (70)	0.10
	Until pregnancy was known	116 (8)	136(10)	
	Continued	207 (15)	184 (13)	
	Missing	128 (9)	102 (7)	
Alcohol use during pregnancy	No	597 (42)	640 (45)	0.07
	Yes	694 (49)	680 (48)	
	Missing	118 (8)	90 (6)	
Paternal characteristic				
Ethnicity	Dutch	1115 (79)	1108 (79)	0.90
	Other	266 (19)	271 (19)	
	Missing	28 (2)	31 (20)	
Infant characteristics				
Gestational age at birth	weeks	40.0 (1.6)	40.0 (1.8)	0.26
Birth weight	g	3564 (540)	3434 (539)	<0.001
Birth length	cm	50.9 (2.4)	50.1 (2.3)	<0.001
Breast feeding	Never	103 (7)	106 (8)	0.80
	≤3 months	365 (26)	347 (25)	
	>3 months	583 (41)	587 (42)	
	Missing	358 (25)	370 (26)	
Child characteristics at 6 year visit				
Age	year	6.15 (0.46)	6.14 (0.42)	0.37
Height	cm	119.9 (5.7)	119.2 (5.7)	<0.001
Weight	kg	23.0 (3.4)	22.7 (3.7)	0.05
Body mass index	kg/cm^2	15.9 (1.4)	15.9 (1.6)	0.91
Participation in sports	Never	683 (49)	636 (45)	<0.001
	1/week	445 (32)	613 (44)	
	≥2/week	183 (13)	52 (4)	
	Missing	98 (7)	109 (8)	
Bone mineral density	g/cm^2	0.551 (0.046)	0.548 (0.045)	0.12
Bone mineral content	g	520 (95)	519 (93)	0.78
Bone area	cm^2	940 (111)	942 (107)	0.56

Values reflect the mean (standard deviation) for continuous variables or absolute numbers (percentage) for categorical variables. *P*-values are obtained by Students *t*-tests for continuous variables and chi-square tests for categorical variables.

Table 4.2.2 Maternal first trimester dietary intake and blood concentrations

Dietary intakes	*n*	mean (SD)
Energy – kcal/day	2580	2152 (504)
Fat – g/day	2580	73.6 (10.7)
Protein – g/day	2580	78.5 (11.7)
Carbohydrate – g/day	2580	236 (30)
Calcium – mg/day	2580	1108 (311)
Phosphorus – mg/day	2580	1443 (241)
Magnesium – mg/day	2580	339 (56)
Blood concentrations	***n***	**median (IQR)**
Homocysteine – µmol/L	2260	7.0 (13.3)
Folate – nmol/L	2282	19.5 (1.9)
Vitamin B12 – pmol/L	2173	175 (100)

Dietary intakes are energy adjusted. *SD* denotes standard deviation, *IQR* denotes inter quartile range

CI 0.1, 13.9). Maternal carbohydrate intake was inversely associated with childhood bone mineral content and density (both *P* for trend 0.02).The difference in bone mineral content between the highest quintile and the lowest quintile was -6.9 g (95% CI -12.8, -0.9). Neither maternal total energy intake nor fat intake were associated with bone mass of the child (*P* for trend >0.05) (data total energy not shown).

Higher maternal calcium and phosphorus intake were associated with a higher childhood bone mineral content and density (all *P* for trend <0.005) (Table 4.2.4). The difference in bone mineral content between the highest and lowest quintile was 9.1 g (95% CI 4.1, 14.1) for maternal calcium intake and 8.6 g (95% CI 3.5, 13.6) for maternal phosphorus intake. Maternal magnesium intake was not associated with any bone outcome.

Maternal homocysteine, folate, vitamin B12 concentrations and childhood bone mass

Higher maternal first trimester homocysteine concentrations were associated with a lower childhood bone mineral content (*P* for trend 0.03) and showed a borderline significant association with bone mineral density (*P* for trend 0.06) (Table 4.2.5). The difference in bone mineral content between the highest quintile and the lowest quintile was -5.2 g (95% CI -10.4, 0.1). Maternal folate concentrations were not associated with childhood bone outcomes. Higher maternal vitamin B12 concentrations were not associated with absolute bone mineral accrual, but were associated with bone mineral density (*P* for trend <0.001), suggesting an effect on bone size rather than bone mineral accrual. The first sensitivity analysis showed that the effect estimates did not change when corresponding nutrient intakes of the child at 14 months were added to each of the models (data

Table 4.2.3 Associations of maternal first trimester macronutrient intake with childhood bone mass

Macronutrient intake (kcal/day)	*n*	Bone mineral content (g)		Bone mineral density (mg/cm²)	
		ß	95% CI	ß	95% CI
Energy from proteins – Q1	516	Ref		Ref	
Energy from proteins – Q2	516	**6.4**	**1.3, 11.4***	**6.1**	**2.0, 10.2****
Energy from proteins – Q3	516	**6.3**	**0.9, 11.7***	**8.8**	**4.4, 13.2*****
Energy from proteins – Q4	516	**10.1**	**4.0, 16.1****	**11.4**	**6.5, 16.3*****
Energy from proteins – Q5	516	**7.0**	**0.1, 13.9***	**11.9**	**6.3, 17.5*****
P for trend		***0.02***		***<0.001***	
Energy from fat – Q1	516	Ref		Ref	
Energy from fat – Q2	516	-0.1	-5.1, 4.9	-2.9	-7.0, 1.1
Energy from fat – Q3	516	-0.1	-5.5, 5.3	0.0	-4.4, 4.3
Energy from fat – Q4	516	-1.3	-7.1, 4.5	-3.7	-8.3, 1.0
Energy from fat – Q5	516	0.9	-5.7, 7.5	-3.8	-9.1, 1.5
P for trend		*0.46*		*0.31*	
Energy from carbohydrate – Q1	516	Ref		Ref	
Energy from carbohydrate – Q2	516	-2.6	-7.6, 2.3	-1.8	-5.8, 2.2
Energy from carbohydrate – Q3	516	**-5.4**	**-10.6, -0.2***	-3.7	-7.9, 0.5
Energy from carbohydrate – Q4	516	-3.9	-9.5, 1.6	**-4.9**	**-9.3, -0.5***
Energy from carbohydrate – Q5	516	**-6.9**	**-12.8, -0.9***	-4.6	-9.4, 0.2
P for trend		***0.02***		***0.02***	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily macronutrient intake compared to the lowest reference quintile. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, and smoking; paternal ethnicity; and gender, birth weight, birth length, breastfeeding, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child and mutually for the other macronutrients following the energy-partition method.[30] ***P*-value <0.05**, ****P*-value <0.01** and *****P*-value <0.001**

shown in **Supplement 4.2.3**). Likewise, when we additionally adjusted BMC models for bone area in a second sensitivity analysis, results hardly changed (data shown in **Supplement 4.2.4**).

In order to identify the major dietary factors influencing bone mineral accrual in childhood, taking into account the effect of other dietary factors, we entered all dietary factors associated with childhood bone mass at the $P<0.10$ significance level in a backward selection regression model. When simultaneously analyzed, maternal phosphorus intake was the only dietary factor significantly associated with childhood bone mineral content (*P* for trend 0.002), while maternal homocysteine concentrations showed a borderline significant association (*P* for trend 0.058) (**Table 4.2.6**). Maternal protein intake (*P* for trend 0.003) and vitamin B12 concentrations (*P* for trend 0.01) showed the strongest association with childhood bone mineral density. The other maternal dietary factors did

Table 4.2.4 Associations of maternal first trimester calcium, phosphorus and magnesium intake with childhood bone mass

Micronutrient intake (g/day)	*n*	Bone mineral content (g)		Bone mineral density (mg/cm²)	
		ß	95% CI	ß	95% CI
Calcium – Q1	516	Ref		Ref	
Calcium – Q2	516	5.4	0.6, 10.3*	4.1	0.2, 8.0*
Calcium – Q3	516	5.7	0.8, 10.6*	5.6	1.7, 9.6**
Calcium – Q4	516	5.6	0.7, 10.6*	4.0	0.0, 8.0*
Calcium – Q5	516	9.1	4.1, 14.1***	6.4	2.4, 10.4**
P for trend		*<0.001*		*0.005*	
Phosphorus – Q1	516	Ref		Ref	
Phosphorus – Q2	516	8.6	3.6, 13.5***	6.5	2.5, 10.5**
Phosphorus – Q3	516	4.3	-0.7, 9.3	3.8	-0.2, 7.8
Phosphorus – Q4	516	7.0	2.0, 12.1**	6.1	2.0, 10.1**
Phosphorus – Q5	516	8.6	3.5, 13.6***	7.5	3.4, 11.5***
P for trend		*<0.001*		*<0.001*	
Magnesium – Q1	516	Ref		Ref	
Magnesium – Q2	516	-0.8	-5.7, 4.2	-0.1	-4.0, 3.9
Magnesium – Q3	516	-0.4	-5.4, 4.7	0.5	-3.6, 4.5
Magnesium – Q4	516	4.4	-0.7, 9.5	0.6	-3.5, 4.7
Magnesium – Q5	516	2.3	-3.0, 7.6	0.4	-3.9, 4.6
P for trend		*0.23*		*0.76*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily micronutrient intake compared to the lowest reference quintile. Models are adjusted for maternal total daily energy intake, age, weight, height, parity, educational level, marital status, alcohol use, and smoking; paternal ethnicity; and gender, birth weight, birth length, breastfeeding, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

not add significantly to the explained variation. Multi-collinearity between maternal protein, phosphorus and calcium intake seemed present. Therefore, we explored potential collinearity between nutrients in the final model. We observed that effect estimates for the associations of protein, phosphorus and calcium intake with bone outcomes varied largely when mutually adjusted for one another, and that the variance inflation factors (VIF) were 4.8, 3.8 and 8.0 for protein, calcium and phosphorus intake. These findings suggest that collinearity between these nutrients is present.

Table 4.2.5 Associations of maternal first trimester homocysteine, folate and vitamine B12 concentrations with childhood bone mass

Micronutrient intake (g/day)	*n*	Bone mineral content (g)		Bone mineral density (mg/cm²)	
		ß	95% CI	ß	95% CI
Homocysteine – Q1	485	Ref		Ref	
Homocysteine – Q2	454	-0.9	-6.0, 4.2	-3.3	-7.4, 0.8
Homocysteine – Q3	441	-3.1	-8.3, 2.0	-2.1	-6.3, 2.1
Homocysteine – Q4	436	-3.8	-9.0, 1.3	**-7.1**	**-11.3, -2.9*** **
Homocysteine – Q5	444	-5.2	-10.4, 0.1	**-4.7**	**-9.0, -0.5***
P for trend		*0.03*		*0.06*	
Folate – Q1	457	Ref		Ref	
Folate – Q2	458	-0.1	-5.4, 5.1	0.9	-3.4, 5.2
Folate – Q3	456	0.7	-4.6, 6.1	0.4	-3.9, 4.7
Folate – Q4	456	2.9	-2.5, 8.4	0.6	-3.8, 5.0
Folate – Q5	455	-0.1	-5.6, 5.3	1.1	-3.3, 5.6
P for trend		*0.95*		0.52	
Vitamin B12 – Q1	439	Ref		Ref	
Vitamin B12 – Q2	430	-1.6	-6.9, 3.7	-0.7	-5.0, 3.6
Vitamin B12 – Q3	442	1.8	-3.5, 7.1	2.3	-1.9, 6.6
Vitamin B12 – Q4	434	-1.4	-6.7, 3.9	3.6	-0.7, 7.9
Vitamin B12 – Q5	428	2.5	-2.9, 7.8	**8.9**	**4.6, 13.3*** **
P for trend		*0.39*		*<0.001*	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of blood concentration compared to the lowest reference quintile. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, smoking and gestational age at blood collection; paternal ethnicity; and gender, birth weight, birth length, breastfeeding, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

DISCUSSION

Main findings

In this large population-based prospective cohort study of pregnant women in the Netherlands, we found that maternal dietary factors during pregnancy are associated with childhood bone mass. Maternal daily intake of proteins, calcium and phosphorus and concentrations of vitamin B12 were positively associated with childhood bone mass, whereas carbohydrates intake and homocysteine concentrations were negatively associated. Multiple regression analysis, including all dietary factors associated with childhood bone mass, showed that maternal phosphorus intake and homocysteine concentrations most strongly affected absolute bone mineral accrual, whereas maternal protein intake

Table 4.2.6 Associations of simultaneously analyzed dietary factors with childhood bone mass

	Bone mineral content (g) *n*=2,029			Bone mineral density (mg/cm²) *n*=1,945		
Dietary factors	ß	95% CI	*P for trend*	ß	95% CI	*P for trend*
Protein – SD	-	-	-	2.6	1.2, 4.0	*<0.001*
Carbohydrate – SD	-	-	-	-	-	-
Phosphorus – SD	2.8	1.1, 4.5	*0.002*	-	-	-
Homocysteine – SD	-1.8	-3.6, 0.1	*0.058*	-	-	-
Vitamin B12 – SD	-	-	-	2.3	0.8, 3.7	*0.002*

Values are based on multiple linear regression models using backward selection and reflect the difference and 95% confidence interval for each standard deviation increase in daily nutrient intake or natural-log transformed blood level. **Bone mineral content model** is adjusted for covariates that remained in the model: maternal parity, marital status and gestational age at blood collection; and gender, birth weight, breast feeding duration, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child. **Bone mineral density model** is adjusted covariates that remained in the model: maternal smoking; birth weight, birth length, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child

and vitamin B12 concentrations were the strongest predictors of bone mass adjusted for bone size in childhood.

Interpretation of main results

Maternal phosphorus intake showed the strongest association with childhood bone mass when simultaneously analyzed with other significant dietary factors. However, results from the simultaneous analysis must be carefully interpreted as collinearity between some nutrients was present. Although multi-collinearity does not affect the validity of the model as a whole, it complicates estimation of the individual contribution of predictors in the model. Maternal protein, calcium and phosphorus intake were highly correlated (**Supplement 4.2.1**), which is likely to reflect their shared dietary sources.[33] As a result, we are unable to indicate which of the independent nutrients may be most essential for childhood bone health.

Accordingly, in previous studies regarding the association of maternal dietary intakes with offspring bone health discrepant associations have been described; in the study of Jones *et al.* among 330 Tasmanian mothers and their 8-year old children, maternal phosphorus intake most strongly predicted childhood bone mass,[6] whereas in the study of Ganpule *et al.* among 797 Indian mothers and their 6-year old children intake of calcium-rich foods did.[5] From animal studies, phosphorus and calcium are known to be co-dependent for bone health.[34] Likewise, both may be essential to fetal bone mineral accrual.

In a British study among 4,451 mothers and their 9-year old children, maternal protein intake showed a positive association with childhood bone mass, which attenuated after

simultaneous analysis with mineral and vitamin intake.[7] In the study of Jones *et al.* at 8-years old and of Yin *et al.*, in the same population at the age of 16 years old, of all macronutrients, maternal fat intake was the strongest negative predictor of childhood bone mass. In our study, maternal fat intake did not show an association. However, since intake of macronutrients highly correlates (**Supplement 4.2.1**), it may be that instead of higher or lower intake of one specific macronutrient, a certain balance between intakes of macronutrients is the most optimal for childhood bone development. The existence of an optimal balance needs to be further studied.

Maternal homocysteine concentrations showed a negative association with childhood bone mineral content while vitamin B12 concentrations showed a positive association with bone mineral density. Homocysteine and vitamin B12 concentrations did not show association in the previous Indian study.[5] Their population differed from our study population in size, and, moreover, they reported that 60% of the mothers had low vitamin B12 concentrations (<150 pmol/L), as compared to 26% in our population. Furthermore the children in this study had very low weight, were short and thin according to the NHANES references.[35]

Potential mechanisms

Diet during pregnancy has been shown to be associated with epigenetic changes altering postnatal transcriptional activity of genes that affect childhood body composition[36] and possibly bone mass.[11,12] Protein restriction in pregnant rats has been associated with reduced methylation of the glucocorticoid receptor promoter,[37] and increased expression of this receptor could sensitize osteoblasts to cortisol and thereby reduce offspring bone mass.[12] Recently, in umbilical cord tissue, methylation of the eNOS gene, key to osteocyte, osteoblast and osteoclast function, was found to be associated with childhood bone mass.[38] Further studies are needed to confirm a direct link between maternal diet, epigenetic changes and offspring bone development. Alternatively, higher intake of protein, a component of the organic bone matrix, and calcium and phosphorus, the main bone forming minerals, may lead to increased fetal bone mineral accrual through direct effects.[10,39] In adults, higher protein intake has been shown to increase concentrations of insulin-like growth factor I, an osteotrophic factor, as well as intestinal calcium uptake.[40,41] Vitamin B12 and folate may play a crucial role in fetal bone health by providing methyl donors for DNA methylation following the mechanisms suggested above, or, it may exert a direct effect by acting as a cofactor for osteoblast function[42,43] or for the metabolism of homocysteine.[44,45] Elevated homocysteine concentrations have shown to be a strong risk factor for osteoporotic fractures in adulthood.[17,46] The deleterious effect of homocysteine may involve disturbance of the bone matrix, or a shift of bone metabolism towards bone resorption.[47]

Methodological considerations

The strengths of this study lie in the setting and design. We measured maternal dietary intakes and concentrations combined with childhood bone mass in a large population. Additionally, we collected detailed information on many potential confounding variables. However, as in any observational study, residual confounding due to unmeasured socio-demographic and lifestyle factors should still be considered, especially since a healthy diet is likely to be associated with a health-conscious lifestyle. In total, 88% of all eligible mothers fully completed the FFQ and 79% participated in the blood sample collection. Bone mass in children of mothers who did not have complete data on dietary factors was not different from bone mass in children of mothers who had. However, missing information may have led to loss of power. In mothers whose children did not have DXA data available, intake of protein, calcium, phosphorus and magnesium, and concentrations of folate and vitamin B12 were lower, while homocysteine concentrations were higher ($P<0.05$). Our results would be biased if the effect estimates differ between those included and not included in the analyses. This seems unlikely, as loss to follow-up in a cohort study is unlikely to be related to the research question,[48] but cannot be excluded. Although the FFQ yielded valid estimates of nutrient intakes when validated against 3day-24hour recalls, measurement error may still have occurred. As measurement error in nutrient intake is probably random, it might have led to underestimation of the associations. The sensitivity analysis did not suggest that infant diet explained the associations of maternal diet with childhood bone mass. Nevertheless, we cannot fully exclude that diet of the child in later life, which may be more strongly related to maternal diet and their bone mass, has influenced the results. Lastly, as discussed above, collinearity between protein, calcium and phosphorus intake complicates interpretation of specific effects of the individual nutrients. As a result, we were unable to disentangle the independent contributions of these nutrients to childhood bone health.

Conclusion

In a Dutch population-based cohort, we observed that maternal diet during pregnancy is associated with bone mineral accrual in childhood. In addition to the previously identified role of maternal phosphorus and calcium intake, this study indicates a role for maternal protein intake as well as homocysteine and vitamin B12 blood concentrations in childhood bone development. Additional studies are needed to further explore the components of the most optimal nutrition favoring bone development and to explore whether nutrition during pregnancy may be a potential target for improving bone health during the life course.

REFERENCES

1. Cooper C. Osteoporosis: disease severity and consequent fracture management. *Osteoporos Int*. 2010,21 Suppl 2:S425-9.
2. Cooper C, Cole ZA, Holroyd CR, Earl SC, Harvey NC, Dennison EM, et al. Secular trends in the incidence of hip and other osteoporotic fractures. *Osteoporos Int*. 2011,22(5):1277-88.
3. Harvey N, Dennison E, Cooper C. Osteoporosis: impact on health and economics. *Nat Rev Rheumatol*. 2010,6(2):99-105.
4. Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Review: developmental origins of osteoporotic fracture. *Osteoporos Int*. 2006,17(3):337-47.
5. Ganpule A, Yajnik CS, Fall CH, Rao S, Fisher DJ, Kanade A, et al. Bone mass in Indian children--relationships to maternal nutritional status and diet during pregnancy: the Pune Maternal Nutrition Study. *J Clin Endocrinol Metab*. 2006,91(8):2994-3001.
6. Jones G, Riley MD, Dwyer T. Maternal diet during pregnancy is associated with bone mineral density in children: a longitudinal study. *Eur J Clin Nutr*. 2000,54(10):749-56.
7. Tobias JH, Steer CD, Emmett PM, Tonkin RJ, Cooper C, Ness AR, et al. Bone mass in childhood is related to maternal diet in pregnancy. *Osteoporos Int*. 2005,16(12):1731-41.
8. Yin J, Dwyer T, Riley M, Cochrane J, Jones G. The association between maternal diet during pregnancy and bone mass of the children at age 16. *Eur J Clin Nutr*. 2010,64(2):131-7.
9. Cole ZA, Gale CR, Javaid MK, Robinson SM, Law C, Boucher BJ, et al. Maternal dietary patterns during pregnancy and childhood bone mass: a longitudinal study. *J Bone Miner Res*. 2009,24(4):663-8.
10. Prentice A. Diet, nutrition and the prevention of osteoporosis. *Public Health Nutr*. 2004,7(1A): 227-43.
11. Devlin MJ, Bouxsein ML. Influence of pre- and peri-natal nutrition on skeletal acquisition and maintenance. *Bone*. 2012,50(2):444-51.
12. Goodfellow LR, Cooper C, Harvey NC. Regulation of placental calcium transport and offspring bone health. *Front Endocrinol (Lausanne)*. 2011,2:3.
13. Wong IP, Nguyen AD, Khor EC, Enriquez RF, Eisman JA, Sainsbury A, et al. Neuropeptide Y is a critical modulator of leptin's regulation of cortical bone. *J Bone Miner Res*. 2012.
14. Idelevich A, Sato K, Baron R. What are the effects of leptin on bone and where are they exerted? *J Bone Miner Res*. 2013,28(1):18-21.
15. Holroyd C, Harvey N, Dennison E, Cooper C. Epigenetic influences in the developmental origins of osteoporosis. *Osteoporos Int*. 2012,23(2):401-10.
16. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med*. 1999,340(19): 1449-54.
17. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, et al. Homocysteine levels and the risk of osteoporotic fracture. *N Engl J Med*. 2004,350(20): 2033-41.
18. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. *Eur J Epidemiol*. 2012,27(9): 739-56.
19. Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, Geleijnse JM, Hofman A, Grobbee DE, et al. Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. *Eur J Clin Nutr*. 1998,52(8):588-96.
20. Classification of the population with a foreign background in the Netherlands. www.cbs.nl: Statistics Netherlands, 2001.
21. Survey amongst ethnic minorities [Enquêteonderzoek onder allochtonen]. www.cbs.nl: Statistics Netherlands, 2005.

22. Tabel Maten en gewichten [Internet]. University of Wageningen. 2003.
23. Nevo: Dutch food composition database 2006 [Internet]. Netherlands Nutrition Centre, The Hague, The Netherlands. 2006.
24. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. *Eur J Epidemiol*. 2007,22(12):917-23.
25. Bianchi M, Baim S, Bishop N, Gordon C, Hans D, Langman C, et al. Official positions of the International Society for Clinical Densitometry (ISCD) on DXA evaluation in children and adolescents. *Pediatr Nephrol*. 2010,25(1):37-47.
26. Heaney RP. Bone mineral content, not bone mineral density, is the correct bone measure for growth studies. *Am J Clin Nutr*. 2003,78(2):350-1; author reply 1-2.
27. Prentice A, Parsons TJ, Cole TJ. Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. *Am J Clin Nutr*. 1994,60(6):837-42.
28. Durmus B, van Rossem L, Duijts L, Arends LR, Raat H, Moll HA, et al. Breast-feeding and growth in children until the age of 3 years: the Generation R Study. *Br J Nutr*. 2011,105(11):1704-11.
29. Feunekes GI, Van Staveren WA, De Vries JH, Burema J, Hautvast JG. Relative and biomarker-based validity of a food-frequency questionnaire estimating intake of fats and cholesterol. *Am J Clin Nutr*. 1993,58(4):489-96.
30. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr*. 1997,65(4 Suppl):1220S-8S; discussion 9S-31S.
31. Kleinbaum D, Kupper L, Nizam A, Muller K. Applied Regression Analysis and Other Multivariable Methods. 4 ed. Belmont, California, US: Duxbury Press; 2008.
32. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ*. 2009,338: b2393.
33. Massey LK. Dietary animal and plant protein and human bone health: a whole foods approach. *J Nutr*. 2003,133(3):862S-5S.
34. Shapiro R, Heaney RP. Co-dependence of calcium and phosphorus for growth and bone development under conditions of varying deficiency. *Bone*. 2003,32(5):532-40.
35. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, et al. CDC growth charts: United States. *Adv Data*. 2000 (314):1-27.
36. Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, et al. Epigenetic gene promoter methylation at birth is associated with child's later adiposity. *Diabetes*. 2011,60(5):1528-34.
37. Lillycrop KA, Slater-Jefferies JL, Hanson MA, Godfrey KM, Jackson AA, Burdge GC. Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr*. 2007,97(6):1064-73.
38. Harvey NC, Lillycrop KA, Garratt E, Sheppard A, McLean C, Burdge G, et al. Evaluation of methylation status of the eNOS promoter at birth in relation to childhood bone mineral content. *Calcif Tissue Int*. 2012,90(2):120-7.
39. Palacios C. The role of nutrients in bone health, from A to Z. *Crit Rev Food Sci Nutr*. 2006,46(8): 621-8.
40. Kerstetter JE, O'Brien KO, Caseria DM, Wall DE, Insogna KL. The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women. *J Clin Endocrinol Metab*. 2005,90(1):26-31.

41. Dawson-Hughes B, Harris SS, Rasmussen H, Song L, Dallal GE. Effect of dietary protein supplements on calcium excretion in healthy older men and women. *J Clin Endocrinol Metab*. 2004,89(3):1169-73.
42. Carmel R, Lau KH, Baylink DJ, Saxena S, Singer FR. Cobalamin and osteoblast-specific proteins. *N Engl J Med*. 1988,319(2):70-5.
43. Kim GS, Kim CH, Park JY, Lee KU, Park CS. Effects of vitamin B12 on cell proliferation and cellular alkaline phosphatase activity in human bone marrow stromal osteoprogenitor cells and UMR106 osteoblastic cells. *Metabolism*. 1996,45(12):1443-6.
44. Cashman KD. Homocysteine and osteoporotic fracture risk: a potential role for B vitamins. *Nutr Rev*. 2005,63(1):29-36.
45. Ahmadieh H, Arabi A. Vitamins and bone health: beyond calcium and vitamin D. *Nutr Rev*. 2011,69(10):584-98.
46. McLean RR, Jacques PF, Selhub J, Tucker KL, Samelson EJ, Broe KE, et al. Homocysteine as a predictive factor for hip fracture in older persons. *N Engl J Med*. 2004,350(20):2042-9.
47. Herrmann M, Peter Schmidt J, Umanskaya N, Wagner A, Taban-Shomal O, Widmann T, et al. The role of hyperhomocysteinemia as well as folate, vitamin B(6) and B(12) deficiencies in osteoporosis: a systematic review. *Clin Chem Lab Med*. 2007,45(12):1621-32.
48. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology*. 2006,17(4):413-8.

Supplement 4.2.1 Correlations of maternal daily nutrient intakes during pregnancy

Nutrient	Protein	Fat	Carbohydrate	Calcium	Phosphorus	Magnesium
Protein	-	0.12***	-0.47***	0.68***	0.86***	0.53***
Fat	-	-	-0.93***	-0.09***	0.04*	0.03
Carbohydrate	-	-	-	-0.16***	-0.34***	-0.22***
Calcium	-	-	-	-	0.84***	0.47***
Phosphorus	-	-	-	-	-	0.71***
Magnesium	-	-	-	-	-	-

Nutrient intakes are energy adjusted intakes in g/day. **P*-value <0.05, ***P*-value <0.01 and ****P*-value <0.001

Supplement 4.2.2 Imputed characteristics of study participants

Maternal characteristics	Unit	Boys *n*=1,409	Girls *n*=1,410
Height	cm	170.9 (6.2)	170.9 (6.6)
Pre-pregnancy weight	kg	68.2 (12.4)	68.3 (12.2)
BMI	kg/cm^2	23.3 (4.0)	23.4 (4.0)
Age	yr	31.6 (4.3)	31.5 (4.1)
Parity ≥1	No	878 (62)	869 (62)
	Yes	531 (38)	541 (38)
Single motherhood	No	1321 (94)	1333 (95)
	Yes	88 (6)	77 (5)
Educational status	Primary	25 (2)	40 (3)
	Secondary	509 (36)	529 (37)
	Higher	875 (62)	842 (60)
Smoking during pregnancy	Never	1056 (75)	1068 (76)
	Until pregnancy was known	131 (9)	148 (10)
	Continued	221 (16)	194 (14)
Alcohol use during pregnancy	No	652 (46)	686 (49)
	Yes	757 (54)	724 (51)
Paternal characteristic			
Ethnicity	Dutch	1138 (81)	1134 (80)
	Other	271 (19)	276 (20)
Infant characteristics			
Gestational age at birth	weeks	40.0 (1.6)	40.0 (1.8)
Birth weight	g	3564 (540)	3434 (539)
Birth length	cm	50.9 (2.4)	50.1 (2.3)
Breast feeding	Never	156 (11)	160 (11)
	≤3 months	494 (35)	477 (34)
	>3 months	759 (54)	773 (55)
Child characteristics at 6 year visit			
Age	year	6.15 (0.46)	6.14 (0.42)
Weight	kg	23.0 (3.4)	22.7 (3.7)
Length	cm	120.0 (5.7)	119.2 (5.6)
BMI	kg/cm^2	15.9 (1.4)	15.9 (1.6)
Participation in sports	Never	733 (52)	688 (49)
	1/week	472 (33)	663 (47)
	≥2/week	204 (14)	59 (4)
Bone mineral density	g/cm^2	0.551 (0.046)	0.548 (0.045)
Bone mineral content	g	520 (95)	519 (93)
Bone area	cm^2	940 (111)	942 (107)

Values reflect the mean (standard deviation) for continuous variables or absolute numbers (%) for categorical variables.

Supplement 4.2.3 Associations of maternal first trimester nutrient intake with childhood bone mass - *additionally adjusted for nutrient intake of the child*

Nutrient		Bone mineral content (g)		Bone mineral density (mg/cm²)	
Macronutrient intake (kcal/day)		ß	95% CI	ß	95% CI
Energy from proteins – Q1	516	Ref		Ref	
Energy from proteins – Q2	516	**6.4**	**1.3, 11.5***	**6.0**	**1.9, 10.1****
Energy from proteins – Q3	516	**6.3**	**0.9, 11.8***	**8.7**	**4.3, 13.1*****
Energy from proteins – Q4	516	**10.1**	**4.1, 16.2****	**11.2**	**6.3, 16.1*****
Energy from proteins – Q5	516	**7.1**	**0.1, 14.0***	**11.6**	**6.0, 17.2*****
P for trend		***0.02***		***<0.001***	
Energy from fat – Q1	516	Ref		Ref	
Energy from fat – Q2	516	-0.1	-5.1, 4.9	-3.0	-7.0, 1.0
Energy from fat – Q3	516	-0.1	-5.5, 5.3	-0.1	-4.4, 4.3
Energy from fat – Q4	516	-1.3	-7.1, 4.5	-3.6	-8.3, 1.0
Energy from fat – Q5	516	0.9	-5.7, 7.5	-3.7	-9.0, 1.6
P for trend		*0.46*		0.36	
Energy from carbohydrate – Q1	516	Ref		Ref	
Energy from carbohydrate – Q2	516	-2.6	-7.6, 2.3	-1.8	-5.8, 2.2
Energy from carbohydrate – Q3	516	**-5.4**	**-10.6, -0.2***	-3.8	-8.0, 0.4
Energy from carbohydrate – Q4	516	-4.0	-9.5, 1.6	**-5.0**	**-9.5, -0.6***
Energy from carbohydrate – Q5	516	**-6.9**	**-12.8, -0.9***	-4.8	-9.6, 0.0
P for trend		***0.02***		***0.02***	
Micronutrient intake (g/day)					
Calcium – Q1	516	Ref		Ref	
Calcium – Q2	516	**5.4**	**0.5, 10.3***	**4.1**	**0.2, 8.1***
Calcium – Q3	516	**5.6**	**0.7, 10.5***	**5.7**	**1.7, 9.6****
Calcium – Q4	516	**5.6**	**0.6, 10.5***	**4.1**	**0.1, 8.1***
Calcium – Q5	516	**9.0**	**4.0, 14.0*****	**6.5**	**2.5, 10.5****
P for trend		***0.001***		***0.005***	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily nutrient intake compared to the lowest reference quintile. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, and smoking; paternal ethnicity; and gender, birth weight, birth length, breastfeeding, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child, and mutually for the other macronutrients following the energy-partition method,[30] and for the daily intake of the corresponding nutrient by the child at the age of 14 months. ***P*-value <0.05**, ****P*-value <0.01** and *****P*-value <0.001**

Supplement 4.2.4 Associations of maternal dietary factors with childhood bone mineral content - *additionally adjusted for bone area*

	Bone mineral content (g)		Micronutrient intake	Bone mineral content (g)		Blood concentration	Bone mineral content (g)	
Macronutrient intake	ß	95% CI		ß	95% CI		ß	95% CI
Energy from proteins – Q1	Ref		Calcium – Q1	Ref		Homocysteine – Q1	Ref	
Energy from proteins – Q2	**5.5**	**1.6, 9.4****	Calcium – Q2	3.6	-0.2, 7.3	Homocysteine – Q2	-3.0	-6.9, 0.9
Energy from proteins – Q3	**7.8**	**3.6, 12.0*****	Calcium – Q3	**4.5**	**0.7, 8.3***	Homocysteine – Q3	-2.4	-6.3, 1.6
Energy from proteins – Q4	**10.0**	**5.4, 14.6*****	Calcium – Q4	3.4	-0.4, 7.2	Homocysteine – Q4	**-6.2**	**-10.2, -2.2****
Energy from proteins – Q5	**10.3**	**5.0, 15.6*****	Calcium – Q5	**6.1**	**2.3, 10.0****	Homocysteine – Q5	**-4.6**	**-8.7, -0.6***
P for trend	***<0.001***		*P for trend*	***0.004***		*P for trend*	***0.04***	
Energy from fat – Q1	Ref		Phosphorus – Q1	Ref		Folate – Q1	Ref	
Energy from fat – Q2	-2.1	-5.9, 1.8	Phosphorus – Q2	**5.7**	**1.9, 9.5****	Folate – Q2	0.4	-3.6, 4.4
Energy from fat – Q3	0.4	-3.7, 4.6	Phosphorus – Q3	3.0	-0.9, 6.8	Folate – Q3	-0.4	-4.5, 3.7
Energy from fat – Q4	-3.0	-7.4, 1.4	Phosphorus – Q4	**5.1**	**1.3, 9.0****	Folate – Q4	0.2	-3.9, 4.4
Energy from fat – Q5	-2.6	-7.6, 2.5	Phosphorus – Q5	**6.6**	**2.7, 10.5*****	Folate – Q5	0.7	-3.5, 4.9
P for trend	*0.57*		*P for trend*	***<0.001***		*P for trend*	*0.70*	
Energy from carbohydrate – Q1	Ref		Magnesium – Q1	Ref		Vitamin B12 – Q1	Ref	
Energy from carbohydrate – Q2	-2.1	-5.9, 1.7	Magnesium – Q2	-0.5	-4.3, 3.3	Vitamin B12 – Q4	-1.0	-5.0, 3.1
Energy from carbohydrate – Q3	-3.8	-7.8, 0.2	Magnesium – Q3	0.2	-3.7, 4.1	Vitamin B12 – Q3	1.6	-2.4, 5.6
Energy from carbohydrate – Q4	**-4.6**	**-8.8, -0.3***	Magnesium – Q4	0.1	-3.8, 4.1	Vitamin B12 – Q4	2.3	-1.7, 6.4
Energy from carbohydrate – Q5	-4.2	-8.8, 0.4	Magnesium – Q5	0.1	-3.9, 4.2	Vitamin B12 – Q5	**7.2**	**3.0, 11.3*****
P for trend	***0.03***		*P for trend*	*0.80*		*P for trend*	***<0.001***	

Values are based on multiple linear regression models and reflect the difference and 95% confidence interval for each quintile of daily micronutrient intake compared to the lowest reference quintile. Models are adjusted for maternal age, weight, height, parity, educational level, marital status, alcohol use, and smoking; paternal ethnicity; and gender, birth weight, birth length, breastfeeding, participation in sports, and age, weight measured as "lean mass + fat mass" and height at measurement of the child, and additionally for total body bone area. **Macronutrient model** intakes are mutually adjusted follow the energy partition method.[30] **Micronutrient model** is additionally adjusted for maternal total energy intake. **Blood concentration model** is additionally adjusted for gestational age at blood collection. ***P*-value <0.05**, ****P*-value <0.01** and *****P*-value <0.001**

CHAPTER 4.3

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Osteoporos Int. 2015 Apr;26(4):1319-29.

MATERNAL SMOKING DURING PREGNANCY AND CHILDHOOD BONE MASS

ABSTRACT

Introduction Maternal smoking during pregnancy may adversely affect bone health in later life. By comparing the associations of maternal and paternal smoking and of prenatal and postnatal exposure with childhood bone measures we aimed to explore whether the suggested association could be explained by fetal programming or reflects confounding by familial factors.

Methods In 5,565 mothers, fathers, and children participating in a population-based prospective cohort study, parental smoking habits during pregnancy and current household smoking habits were assessed by postal questionnaires. Total body bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) were measured by dual-energy x-ray absorptiometry (DXA) at the median age of 6.0 (IQR 0.37) years.

Results In confounder adjusted models, maternal smoking during pregnancy was associated with a 11.6 g (95% CI 5.6, 17.5) higher BMC, a 9.7 cm^2 (95% CI 3.0, 16.4) larger BA, a 6.7 g/cm^2 (95% CI 2.4, 11.0) higher BMD and a 5.4 g (95% CI 1.3, 9.6) higher BMC adjusted for BA of the child. Current weight turned out to mediate these associations. Among mothers who did not smoke, paternal smoking did not show evident associations with childhood bone measures. Also, household smoking practices during childhood were not associated with childhood bone measures.

Conclusions Our results do not support the hypothesis of fetal smoke exposure affecting childhood bone mass via intrauterine mechanisms. Maternal smoking or related lifestyle factors may affect childhood weight gain rather than skeletal growth.

INTRODUCTION

Adverse fetal exposures may lead to adaptations that permanently affect skeletal development and, as a result, influence susceptibility for impaired bone health and osteoporosis during the life course.[1] Maternal smoking is one of the most prevalent modifiable adverse fetal exposure[2] and showed a negative association with bone mineral content (BMC) in neonates.[3,4] The mechanisms by which maternal smoking may harm the developing skeleton are unclear. Maternal smoking is well-known to restrict fetal growth in a dose-dependent relationship[5] and reduced fetal growth has been shown to lead to a lower BMC in childhood and adulthood.[6,7] Maternal smoking leads to lower calcium absorption[8] and impaired placental function.[9] Diminished nutrient supply will impair fetal bone formation. Furthermore, cigarette smoke exists of thousands of constituents that may directly harm the developing skeleton.[8,10]

Results from studies on the association of maternal smoking with bone development in later life have been inconclusive. The negative association of maternal smoking with bone measures found in 8-year-old children[11] had disappeared around the age of 16 years.[12] These findings suggest that the effect of maternal smoking may be transient. The observed association of maternal smoking with offspring bone measures may also reflect confounding by shared familial factors rather than direct intrauterine programming effects.[13] Smoking is highly correlated with socio-demographic and lifestyle related factors. Comparison of maternal and paternal smoking might help to disentangle intrauterine from confounding factors.[14] Another issue when studying fetal smoke exposure and bone health in later life may be postnatal exposure of the child to passive smoking. Mothers who could not give up smoking during pregnancy are very likely to continue smoking after giving birth.[15] Household tobacco smoke exposure during adolescence has been associated with lower bone measures in later life[16], but results are conflicting.[11] Therefore, we examined in a population-based prospective cohort study from fetal life onwards, the associations of maternal and paternal smoking during pregnancy along with childhood smoke exposure with bone properties in 5,565 children of school age.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[17] All mothers who were resident in the study area at their delivery date and had a delivery date from April 2002 until January 2006 were eligible. Seventy-five percent of all mothers enrolled before 18 weeks of gestation. Of all eligible children in the study area, 61% participated

in the study at birth. We conducted the study according to the guidelines of the Helsinki Declaration and it was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31).We obtained written informed consent from all participants' parents.

In total, we enrolled 8,879 mothers during pregnancy, leading to 8,633 singleton live births. Any information about smoking habits during pregnancy was available in 8,211 mothers of the singleton live born children. Around the age of 6 years, 5,745 of these children and their parents visited our dedicated research center (response rate 70%). We successfully performed bone measurements in 5,565 of the visiting children. (Flowchart is given in **Figure 4.3.1**). Some mothers participated more than once, as data on the second (*n*=353) or third (*n*=5) pregnancy in our study was available.

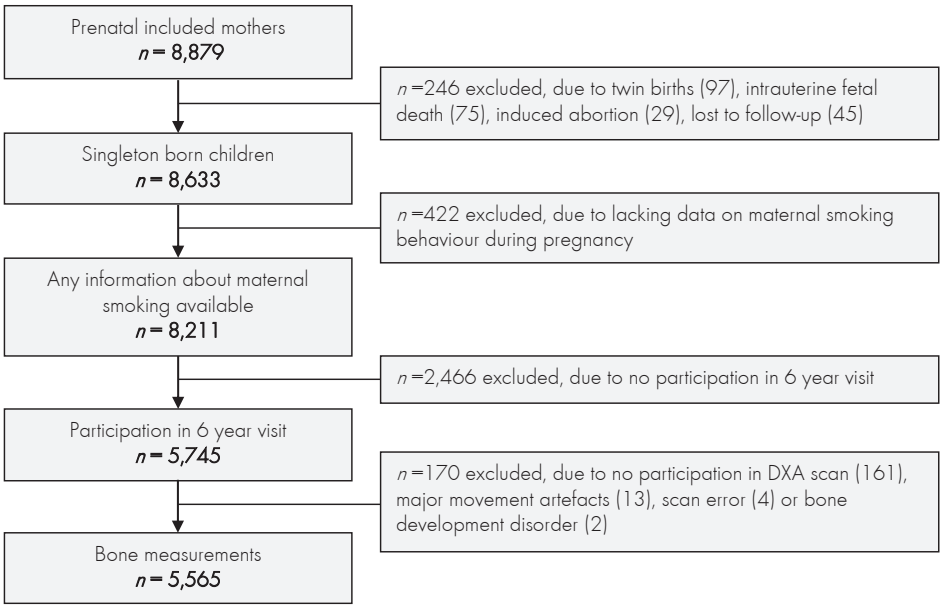

Figure 4.3.1 Flowchart of study participants

Maternal and paternal smoking during pregnancy

As previously described in detail,[17] we obtained information about maternal smoking by postal questionnaires sent in the first, second, and third trimesters of pregnancy. We combined and categorized answers on smoking habits as follows: *1*) never smoked during pregnancy, *2*) smoked until their pregnancy was known, and 3) continued to smoke during pregnancy. We reclassified mothers as "continued smokers" if they reported not to have smoked or to have smoked until their pregnancy was known in the first questionnaire, but reported to have smoked in the second or third questionnaire. We assessed active paternal smoking in the first questionnaire by asking the mother whether

the father smoked during pregnancy. In a subset of 3,671 participants (66%), fathers agreed to participate in the study and completed a questionnaire in which they were asked about their smoking habits. Agreement between paternal smoking reported by the mother and the father himself was high (94% agreement on father does not smoke, 91% on father smokes). We primarily used maternal information on paternal smoking and completed missing information with the partner-questionnaire. In smoking parents, we categorized number of cigarettes smoked as follows: <5, ≥5-10, and ≥10 cigarettes/day. Dose–response analyses in mothers who continued smoking were primarily based on information from the first trimester questionnaire and completed with information from the second or third questionnaire if missing (*n*=15). We assessed the child's current exposure to household smoking by questionnaire.

Bone mineral density measurements

We measured total body bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) using a DXA scan (iDXA, General Electrics –Lunar, 2008, Madison, WI, USA) at the median age of 6.0 (IQR 0.37) years. As described in detail earlier,[18] well-trained research assistants obtained the DXA scans by using the same device and software (enCORE) following standard manufacturer protocols. In our analyses, we used areal total body less head (TBLH) BMC, BA and BMD as recommended by the International Society for Clinical Densitometry.[19] All measures were adjusted for skeletal size by using body height as covariate in the models to correct for artefacts arising caused by rapid periods of growth;[20] areal BMD measured on larger bones overestimates true (volumetric) BMD while on smaller bones it can underestimate BMD across individuals.[21,22] Additionally, we analyzed BMC corrected for bone area to further rule out potential size effects and to obtain a measure that approximates volumetric bone mineral density.[21]

Covariates

We registered maternal age at enrolment and collected information about maternal parity, alcohol use and folic acid supplement use, paternal age and parental education, marital status and ethnicity by questionnaire at enrolment in the study.[23,24] We categorized ethnicity into three main groups: Western (Dutch, Turkish, other European, American, Oceanic), African (Moroccan, other African, Antillean, Surinamese-Creole and Cape Verdean) and Asian (Indonesian, other Asian and Surinamese-Hindu) descent according to the three largest transcontinental ancestral groups. We measured parental height and weight at the research center. As enrolment in our study was during pregnancy, we were not able to measure maternal weight before pregnancy. Yet, correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was high (ρ=0.95; *P*<0.01). We obtained information about the sex and gestational age of the children

at birth from medical records and information about breast feeding[25] and the child's participation in sports from postnatal questionnaires.

Statistical analysis

We used Student's t-tests, Kruskal-Wallis tests, and Chi-square tests to compare characteristics of children whose mothers never smoked, smoked until their pregnancy was known, and continued smoking during pregnancy. We used multiple linear regression analyses to assess the associations of maternal smoking with childhood bone measures in 4 steps: In the first model (crude model), we explored the associations by adjusting models for sex, age and height of the child only. Then, in the second model (confounder model), we additionally adjusted the associations for potential confounding factors: maternal age, weight, height, ethnicity, parity, educational level, marital status, alcohol use and use of folic acid supplements and child's breastfeeding duration and child's participation in sports. To assess potential mediation by birth weight or current weight, we additionally adjusted the confounder model for gestational-age adjusted birth weight (birth weight model) and current weight (current weight model), respectively. As maternal smoking has shown to exert different effects on body composition in boys and girls,[13] we explored a potential interaction of maternal smoking with child sex on childhood bone measures by adding a multiplicative term to the confounder-adjusted model. Similarly, as maternal smoking is known to lead to impaired fetal growth and subsequent postnatal catch-up growth,[26] we explored a potential interaction of maternal smoking with postnatal catch-up growth. In a sensitivity analysis, we tested for clustering effects within families that participated more than once in the study by multi-level modelling. Next, we assessed the associations of paternal smoking during pregnancy with childhood bone measures by multiple linear regression among mothers who did not smoke during pregnancy. We adjusted these models for potential confounders and mediators similar to the maternal models, replacing maternal characteristics by paternal age, weight, height, ethnicity and educational level. Lastly, in order to evaluate a potential influence of passive smoking throughout childhood, we assessed the associations of parental household smoking practices with childhood bone measures using similar models, stratified for prenatal smoke exposure. To prevent biased effect estimates associated with missing data, risk factors with missing values were multiple imputed (5 imputations) based on the correlation of the missing variables with other patient characteristics, according to the Markov Chain Monte Carlo method.[27] The amount of missing values ranged from 0-23% across variables, except for infant breast feeding (36%) and paternal education (32%). *P*-values are two-sided. Analyses were performed using the Predictive Analytic Software version 20.0 for Windows (PASW Inc., Chicago, IL, USA).

RESULTS

Characteristics of study population

In total, 789 (14%) mothers reported that they had continued their smoking habit throughout pregnancy, whereas 430 (8%) mothers reported that they had stopped smoking as soon as their pregnancy was known (Table 4.3.1). Of the mothers who never smoked 1,331 (35%) had a partner who did. As illustrated in Supplement 4.3.1, parents of children who did not participate in the 6-year visit more often smoked, were younger, of smaller statue, lower educated, had lower income, and were more often single mothers and of non-Western descent (all $P<0.05$). Characteristics of the imputed dataset are given in Supplement 4.3.2. As only relatively small percentages of covariates were imputed, descriptive statistics of imputed variables hardly differ from descriptive statistics of non-imputed covariates.

Maternal smoking and childhood bone measures

Table 4.3.2 shows that continued maternal smoking during pregnancy was positively associated with childhood bone measures in models only controlled for sex, age and height of the child (crude models). As compared to children of non-smoking mothers, children of mothers who continued smoking had a 10.2 g higher BMC (95% CI 6.2, 14.4), a 5.5 cm^2 larger BA (95% CI 1.1, 9.9), a 7.8 g/cm^2 higher BMD (95% CI 6.3, 9.3) and a 6.5 g (95% CI 5.0, 8.0) higher BMC adjusted for BA. The associations did not show a dose-response relationship. After adjustment for potential confounders, the associations of maternal smoking with childhood bone measures attenuated, leaving a difference of 11.6 g (95% CI 5.6, 17.5) in BMC, 9.7 cm^2 (95% CI 3.0, 16.4) in BA, 6.7 g/cm^2 (95% CI 2.4, 11.0) in BMD and of 5.4 g (95% CI 1.3, 9.6) in BMC adjusted for BA between children of non-smoking and smoking mothers. Adding birth weight to the confounder adjusted models slightly strengthened the observed associations. In contrast, after adding current weight to the models associations disappeared. We did not find evidence for an interactive effect of smoking neither with sex nor with infant catch-up growth (data not shown). By multi-level modelling we tested for clustering effects within families that participated more than once in the study. As the multilevel analysis did not materially change effect estimates (Supplement 4.3.3), we included data on the second and third pregnancy in the population for analysis.

Paternal smoking and childhood bone measures

As illustrated in Table 4.3.3, analysis of paternal smoking habits during pregnancy in non-smoking mothers did not reveal consistent associations with childhood bone measures at the age of 6 years. In the crude models, adjusted for sex, age and current height of the child, paternal smoking was not associated with BMC, BA or BMD of the child. When

Table 4.3.1 Parental, fetal and child characteristics

		Maternal smoking during pregnancy			
	Unit	Never *n*=3767	Until pregnancy was known *n*=430	Continued *n*=789	*P*-value
Maternal characteristics					
Age	year	30.7 (4.9)	30.1 (5.0)	28.8 (5.8)	<0.001
Height	cm	167.8 (7.4)	168.9 (6.9)	167.2 (7.0)	<0.001
Pre-pregnancy weight	kg	64 (58-72)	65 (58-71)	63 (57-72)	0.19
Pre-pregnancy body mass index	kg/m^2	22.7 (20.8-25.3)	22.3 (20.6-24.9)	22.7 (20.6-25.7)	0.28
Ethnicity	Western	2680 (64)	324 (75)	594 (75)	<0.001
	African	649 (17)	49 (11)	112 (14)	
	Asian	354 (9)	42 (10)	46 (6)	
	Missing	84 (10)	15 (4)	37 (5)	
Parity ≥1	No	2138 (57)	311 (72)	461 (58)	<0.001
	Yes	1624 (43)	118 (27)	326 (41)	
	Missing	5 (0)	1 (0)	2 (0)	
Educational status	Primary	312 (8)	32 (7)	120 (15)	<0.001
	Secondary	1517 (40)	199 (46)	480 (61)	
	Higher	1880 (50)	196 (46)	172 (22)	
	Missing	58 (2)	3 (1)	17 (2)	
Alcohol use	No	2311 (61)	213 (50)	438 (56)	<0.001
	Yes	1451 (39)	215 (50)	346 (44)	
	Missing	5 (0)	2 (0)	5 (1)	
Paternal characteristics					
Age	year	33.7 (5.7)	32.6 (5.7)	31.8 (6.4)	<0.001
Height	cm	182.4 (7.8)	182.9 (8.1)	180.5 (8.2)	<0.001
Weight	kg	84.1 (12.8)	83.8 (13.0)	83.1 (14.0)	0.32
Body mass index	kg/m^2	25.3 (3.4)	25.0 (3.4)	25.4 (3.6)	0.24
Ethnicity	Western	2591 (69)	312 (73)	525 (67)	<0.001
	African	607 (16)	47 (11)	35 (14)	
	Asian	319 (9)	36 (8)	112 (6)	
	Missing	250 (7)	35 (8)	106 (13)	
Educational status	Primary	154 (4)	20 (5)	64 (8)	<0.001
	Secondary	979 (26)	139 (32)	275 (35)	
	Higher	1496 (40)	154 (36)	125 (16)	
	Missing	154 (4)	20 (5)	64 (8)	
Smoking	No	2401 (64)	145 (34)	187 (24)	<0.001
	Yes	1331 (35)	280 (65)	592 (75)	
	Missing	35 (1)	5 (1)	10 (1)	

Table 4.3.1 (continued)

		Maternal smoking during pregnancy			
	Unit	Never *n*=3767	Until pregnancy was known *n*=430	Continued *n*=789	*P*-value
Family characteristics					
Monthly household income	<€1600	714 (19)	80 (19)	266 (34)	<0.001
	≥€1600-2200	459 (12)	54 (13)	119 (15)	
	≥€2200	2002 (53)	225 (52)	244 (31)	
	Missing	592 (16)	71 (17)	160 (20)	
Married/living together	Yes	3385 (90)	358 (83)	587 (74)	<0.001
	No	361 (10)	65 (15)	195 (25)	
	Missing	21 (1)	7 (2)	7 (1)	
In house smoking	No	2624 (70)	264 (61)	263 (33)	<0.001
	Yes	187 (5)	47 (11)	205 (26)	
	Missing	956 (25)	119 (28)	321 (41)	
Infant characteristics					
Sex	Girl	1929 (51)	225 (52)	366 (46)	0.09
	Boy	1838 (49)	205 (48)	423 (54)	
Gestational age at birth	weeks	40.1 (39.1-41.0)	40.1 (39.1-40.0)	40.0 (39.0-40.9)	0.003
Birth weight	grams	3460 (537)	3462 (546)	3278 (561)	<0.001
Breast feeding	Never	183 (5)	26 (6)	77 (10)	<0.001
	>0-3 months	820 (22)	134 (31)	215 (27)	
	≥3 months	1448 (38)	141 (33)	432 (18)	
	Missing	1316 (35)	129 (30)	357 (45)	
Child characteristics at 6 year visit					
Age	year	6.1 (5.9-6.3)	6.0 (5.9-6.3)	6.1 (5.9-6.5)	<0.001
Height	cm	119.5 (5.9)	119.8 (6.3)	119.7 (6.3)	0.25
Weight	kg	22.6 (20.6-25.0)	22.4 (20.4-25.4)	23.0 (20.8-26.0)	0.001
Body mass index	kg/m^2	15.8 (15.0-16.9)	15.8 (14.9-16.9)	16.1 (15.3-17.5)	<0.001
Participation in sports	Never	1764 (47)	205 (48)	358 (45)	<0.001
	1/week	1234 (33)	134 (31)	185 (23)	
	≥2/week	274 (7)	32 (7)	76 (10)	
	Missing	495 (13)	59 (14)	170 (22)	
Bone mineral density	g/cm^2	0.553 (0.050)	0.554 (0.051)	0.563 (0.052)	<0.001
Bone mineral content	g	512 (456-580)	510 (460-579)	523 (465-605)	0.003
Bone area	cm^2	947 (116)	948 (121)	957 (124)	0.09

Values reflect the mean (standard deviation) or median (interquartile range) for continuous variables or absolute numbers (percentage) for categorical variables. *P*-values are obtained by Students *t*-tests for normal-distributed continuous variables, Kruskal-Wallis tests for non-normal distributed continuous variables, and chi-square tests for categorical variables.

Table 4.3.2 Associations of maternal smoking during pregnancy with bone measures at age 6

		Crude model		Confounder model		Birth weight model		Current weight model	
Maternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Bone mineral content (g)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-1.9	-7.4, 3.6	-0.9	-7.0, 5.1	-1.4	-7.5, 4.6	-0.3	-5.1, 4.6
Continued	789	**10.2**	**6.0, 14.4*****	**11.6**	**5.6, 17.5*****	**12.2**	**6.3, 18.2*****	3.1	-1.6, 7.9
<5 cigarettes/day	388	**12.8**	**7.1, 18.5*****	**14.1**	**6.4, 21.8*****	**15.0**	**7.4, 22.7*****	**6.5**	**0.4, 12.7***
≥5-10 cigarettes/day	208	6.3	-1.3, 13.9	8.4	-1.2, 18.0	8.9	-0.7, 18.4	0.9	-6.8, 8.5
≥10 cigarettes/day	137	7.1	-2.3, 16.3	**18.6**	**5.9, 31.3****	**19.4**	**6.7, 32.1****	1.9	-8.2, 12.1
P for trend		***0.03***		***0.008***		***0.006***		*0.38*	
Bone area (cm²)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-3.3	-9.1, 2.6	-0.4	-7.2, 6.4	-1.1	-7.9, 5.7	-0.2	-6.3, 6.0
Continued	789	**5.5**	**1.1, 9.9***	**9.7**	**3.0, 16.4****	**10.6**	**4.0, 17.3****	3.3	-2.7, 9.3
<5 cigarettes/day	388	**9.6**	**3.9, 15.3****	**12.6**	**3.9, 21.3****	**13.9**	**5.2, 22.6****	7.1	-0.8, 15.0
≥5-10 cigarettes/day	208	1.7	-2.5, 5.9	6.3	-4.5, 17.1	6.9	-3.8, 17.7	0.6	-9.2, 10.3
≥10 cigarettes/day	137	-5.2	-10.3, -0.1	7.0	-7.3, 21.4	8.1	-6.2, 22.4	-5.9	-18.8, 7.1
P for trend		*0.28*		*0.10*		*0.09*		*0.78*	
Bone mineral density (g/cm²)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-0.4	-4.3, 3.4	-0.8	-5.1, 3.6	-0.9	-5.3, 3.5	-0.3	-4.2, 3.7
Continued	789	**7.8**	**6.3, 9.3*****	**6.7**	**2.4, 11.0****	**6.9**	**2.6, 11.2****	2.1	-1.8, 5.9
<5 cigarettes/day	388	**7.8**	**5.7, 9.9*****	**7.7**	**2.2, 13.3****	**8.0**	**2.4, 13.6****	3.4	-1.6, 8.4
≥5-10 cigarettes/day	208	**6.6**	**3.9, 9.4***	5.6	-1.3, 12.6	5.7	-1.2, 12.7	1.5	-4.8, 7.7
≥10 cigarettes/day	137	**10.6**	**7.2, 13.9****	**15.2**	**6.0, 24.4****	**15.4**	**6.2, 24.6****	6.0	-2.3, 14.3
P for trend		***<0.001***		***0.02***		***0.02***		*0.33*	
Bone mineral content adjusted for area (g)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	0.3	-3.5, 4.1	-0.7	-4.9, 3.5	-0.7	-4.9, 3.5	-0.2	-3.9, 3.6
Continued	789	**6.5**	**5.0, 8.0*****	**5.4**	**1.3, 9.6****	**5.5**	**1.4, 9.6****	1.5	-2.1, 5.2
<5 cigarettes/day	388	**6.4**	**3.9, 8.8****	**6.1**	**0.8, 11.5***	**6.2**	**0.9, 11.6***	3.0	-1.8, 7.7
≥5-10 cigarettes/day	208	5.2	-0.1, 10.5	4.4	-2.2, 11.1	4.5	-2.2, 11.2	0.6	-5.3, 6.5
≥10 cigarettes/day	137	**10.5**	**7.2, 13.8****	**14.2**	**5.4, 23.0****	**14.3**	**5.4, 23.1****	4.8	-3.0, 12.6
P for trend		***0.001***		***0.03***		***0.03***		*0.36*	

Values are based on multiple linear regression models and reflect the coefficients and 95% confidence interval. **Crude model** is adjusted for sex, current age and height of the child. **Confounder model** is additionally adjusted for current maternal household smoking , maternal age, weight, height, ethnicity, parity, educational level, marital status, alcohol use and folic acid supplement use, and breastfeeding duration and participation in sports of the child. **Birth weight model** is additionally adjusted for birth weight. **Current weight model** is additionally adjusted for current weight. ******P*-value <0.05, *******P*-value <0.01 and ********P*-value <0.001

Table 4.3.3 Associations of paternal smoking during pregnancy, in absence of maternal smoking, with bone measures at age 6

		Crude model		Confounder model		Birth weight model		Current weight model	
Paternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Bone mineral content (g)									
No	2401	Ref		Ref		Ref		Ref	
Yes	1331	1.5	-2.1, 5.1	1.3	-2.3, 4.8	1.3	-2.3, 4.9	-2.5	-5.4, 0.3
<5 cigarettes/day	584	0.0	-4.9, 4.9	-0.2	-5.0, 4.6	-0.1	-4.9, 4.7	-2.7	-6.5, 1.0
≥5-10 cigarettes/day	264	3.9	-2.9, 10.7	2.8	-4.0, 9.6	2.8	-4.0, 9.6	0.6	-4.7, 5.8
≥10 cigarettes/day	461	0.7	-4.6, 6.1	1.5	-3.9, 6.8	1.5	-3.8, 6.9	**-5.1**	**-9.3, -0.9***
P for trend		*0.53*		*0.47*		*0.46*		***0.03***	
Bone area (cm²)									
No	2401	Ref		Ref		Ref		Ref	
Yes	1331	-0.1	-4.0, 3.8	-0.6	-4.5, 3.3	-0.6	-4.5, 3.3	-3.4	-7.0, 0.1
<5 cigarettes/day	584	-4.1	-9.4, 1.1	-4.3	-9.6, 0.9	-4.3	-9.5, 1.0	**-6.2**	**-10.9, -1.5***
≥5-10 cigarettes/day	264	5.6	-1.7, 12.9	5.0	-2.4, 12.4	5.0	-2.4, 12.4	3.3	-3.4, 9.9
≥10 cigarettes/day	461	0.2	-5.6, 6.0	0.1	-5.8, 5.9	0.1	-5.7, 6.0	-4.8	-10.1, 0.5
P for trend		*0.71*		*0.81*		*0.79*		*0.12*	
Bone mineral density (g/cm²)									
No	2401	Ref		Ref		Ref		Ref	
Yes	1331	1.9	-0.7, 4.5	2.0	-0.6, 4.5	2.0	-0.6, 4.5	-0.1	-2.3, 2.2
<5 cigarettes/day	584	2.8	-0.7, 6.3	2.8	-0.6, 6.2	2.9	-0.5, 6.2	1.5	-1.5, 4.5
≥5-10 cigarettes/day	264	1.3	-4.1, 6.7	0.7	-4.1, 5.5	0.7	-4.1, 5.5	-0.5	-4.8, 3.7
≥10 cigarettes/day	461	0.5	-3.1, 4.1	1.3	-2.5, 5.1	1.3	-2.5, 5.1	-2.2	-5.6, 1.2
P for trend		*0.53*		*0.37*		*0.37*		*0.30*	
Bone mineral content adjusted for area (g)									
No	2401	Ref							
Yes	1331	0.3	-0.8, 1.4	1.7	-0.9, 4.2	1.7	-0.9, 4.2	-0.8	-3.0, 1.3
<5 cigarettes/day	584	2.8	-0.7, 6.2	2.7	-0.7, 6.0	2.7	-0.7, 6.0	0.4	-2.5, 3.3
≥5-10 cigarettes/day	264	0.2	-2.3, 2.6	-0.4	-5.2, 4.3	-0.4	-5.2, 4.3	-1.1	-5.2, 3.0
≥10 cigarettes/day	461	0.6	-1.3, 2.5	1.4	-2.3, 5.2	1.4	-2.3, 5.2	-2.7	-6.0, 0.5
P for trend		*0.22*		*0.43*		*0.43*		*0.13*	

Values are based on multiple linear regression models and reflect the coefficients and 95% confidence interval. **Crude model** is adjusted for sex, current age and height of the child. **Confounder model** is additionally adjusted for paternal age, weight, height, ethnicity, educational level, marital status and household income, and breastfeeding duration and participation in sports of the child. **Birth weight model** is additionally adjusted for birth weight. **Current weight model** is additionally adjusted for current weight. ***P-value <0.05**

we additionally adjusted for potential confounders and, subsequently, for birth weight results hardly changed. After adjustment for current weight, paternal smoking showed a negative association with childhood BMC (*P* for trend 0.03). Children of fathers who smoked ≥10 cigarettes/day had a -5.1 g (95% CI -9.3, -0.9) lower BMC than children of fathers who did not smoke. Children of smoking fathers had a -6.2 cm^2 (95% CI -10.9, -1.5) lower BA. Yet, we found no evidence for a dose-response relationship (*P* for trend 0.22). Also, no associations were found for BMD or BMC adjusted for bone area.

Exposure to household smoking and childhood bone measures

In the households in which at least one parent smoked inside the house, 47% of mothers had continued smoking while pregnant. In the households none of the parents smoked inside the house, only 8% of the mothers had smoked throughout pregnancy. Analysis of current exposure to household smoking stratified for maternal smoking status during pregnancy revealed no statistically significant associations with bone outcomes (Table 4.3.4).

DISCUSSION

Main findings

In this large population-based prospective cohort study we found positive associations of maternal smoking with childhood bone BMC, BA and BMD, which were fully explained by differences in weight of the child. Among mothers who did not smoke during pregnancy, paternal smoking was negatively associated with childhood BMC, but not consistently with BA and BMD. Hence, neither paternal smoking nor maternal smoking showed a strong association with childhood bone measures. Also, current exposure to parental household smoking was not associated with childhood bone measures.

Methodological considerations

A major strength of the study is that it is designed as a large-scale, population-based prospective cohort study. We collected detailed information on many potential confounding variables as well as on smoking behavior of both parents. As we were able to compare associations of maternal and paternal smoking on childhood bone measures, we could estimate the likelihood that residual confounding explained the results. We used the well-validated DXA technique to estimate bone mineral density in children. Furthermore, only 5% of the mothers were excluded due to lack of any information on smoking habits. A limitation of the study is that of all children of whom we had any information on prenatal smoke exposure, 70% visited our research center where childhood bone measures were assessed. Parents of children who did not participate in the 6-year visit more often smoked and were of lower socioeconomic status. Missing

information may have led to loss of power and biased estimates. The latter would arise when as a result of selection the absence of an association between maternal smoking

Table 4.3.4 Associations of current parental household smoking practices with bone measures at age 6, stratified for maternal smoking status during pregnancy

Maternal smoking during pregnancy	Household smoking	*n*	Crude model		Confounder model		Birth weight model		Current weight model	
			ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Bone mineral content (g)										
Never	No	2624	Ref		Ref		Ref		Ref	
	Yes	187	5.3	-2.3, 12.9	-0.4	-8.0, 7.2	0.1	-7.5, 7.6	-2.2	-8.3, 3.9
Until pregnancy was known	No	264	Ref		Ref		Ref		Ref	
	Yes	47	6.0	-9.9, 21.9	2.5	-14.6, 19.5	4.1	-13.0, 21.1	0.4	-13.2, 14.0
Continued	No	263	Ref		Ref		Ref		Ref	
	Yes	205	4.7	-5.4, 14.8	1.4	-9.1, 11.9	1.8	-8.7, 12.4	0.5	-7.5, 8.5
Bone area (cm^2)										
Never	No	2624	Ref		Ref		Ref		Ref	
	Yes	187	2.1	-6.3, 10.5	-2.3	-10.9, 6.3	-1.7	-10.2, 6.9	-3.5	-11.3, 4.4
Until pregnancy was known	No	264	Ref		Ref		Ref		Ref	
	Yes	47	2.4	-15.1, 19.9	0.2	-19.3, 19.6	2.2	-17.2, 21.6	-1.2	-18.1, 15.7
Continued	No	263	Ref		Ref		Ref		Ref	
	Yes	205	-2.5	-13.8, 8.9	-5.4	-17.3, 6.4	-4.1	-16.0, 7.7	-5.3	-15.4, 4.7
Bone mineral density (g/cm^2)										
Never	No	2624	Ref		Ref		Ref		Ref	
	Yes	187	4.9	-0.2, 10.1	1.9	-3.7, 7.4	2.0	-3.6, 7.6	0.7	-4.3, 5.7
Until pregnancy was known	No	264	Ref		Ref		Ref		Ref	
	Yes	47	4.8	-7.1, 16.7	2.7	-10.0, 15.4	3.2	-9.5, 16.0	1.5	-10.3, 13.3
Continued	No	263	Ref		Ref		Ref		Ref	
	Yes	205	6.2	-0.7, 13.2	4.8	-2.5, 12,1	4.5	-2.8, 11.9	3.9	-2.7, 10.6
Bone mineral content adjusted for area (g)										
Never	No	2624	Ref		Ref		Ref		Ref	
	Yes	187	3.9	-0.7, 8.5	1.1	-4.3, 6.3	1.1	-4.2, 6.5	-0.5	-5.2, 4.2
Until pregnancy was known	No	264	Ref		Ref		Ref		Ref	
	Yes	47	4.5	-6.8, 15.8	2.4	-9.7, 14.4	2.7	-9.4, 14.8	1.0	-10.1, 12.0
Continued	No	263	Ref		Ref		Ref		Ref	
	Yes	205	6.4	-0.2, 12.9	5.0	-1.9, 11.9	4.6	-2.3, 11.6	3.2	-3.0, 9.4

Values are based on multiple linear regression models and reflect the coefficients and 95% confidence interval. **Crude model** is adjusted for sex, current age and height of the child. **Confounder model** is additionally adjusted for maternal age, weight, height, ethnicity, parity, educational level, marital status, alcohol use and folic acid supplement use, and breastfeeding duration and participation in sports of the child. **Birth weight model** is additionally adjusted for birth weight. **Current weight model** is additionally adjusted for current weight.

and childhood bone mass would not apply to those lost to follow-up. This seems unlikely, as loss to follow-up in our cohort is not likely to be related to the studied research question,[28] but cannot be excluded. Another limitation is that maternal smoking behavior was self-reported. Since pregnant women may be aware of the detrimental effect of smoking on fetal development, underreporting of smoking behavior may have occurred. The prevalence of maternal smoking in the current study was similar to the prevalence reported in a nationwide survey that assessed smoking habits among pregnant women in the Netherlands during the same time period.[29] However, underreporting may have been present in both studies. In our study cotinine levels were not available, yet results from other studies in pregnant women comparing self-reported smoking behavior to measured cotinine levels indicated that self-reported data is quite accurate.[30,31]

Interpretation of results

Adverse exposures during fetal life may lead to adaptations that permanently and unfavorably program skeletal growth.[1] Maternal smoking, still the most prevalent modifiable adverse fetal exposures in developed countries,[2] restricts fetal growth and as a result lowers birth weight in the offspring.[5] Increasing evidence suggests that a low birth weight leads to a lower BMC in childhood and adulthood.[6,7] Consequently, maternal smoking during pregnancy has been suggested to play a role in the adverse programming of offspring bone development. In the study of Jones *et al.*, a Tasmanian cohort of 330 mothers and their 8-year-old children, children whose mothers smoked during pregnancy had lower BMD at the lumbar spine and femoral neck, but not total body, as compared to children whose mothers did not.[11] Their results attenuated, but remained significant when adjusted for current size, however, not when adjusted for placental weight, suggesting that the association may be mediated through placental function. The prevalence of smoking in this study was high (49%) which may have been due to the fact that the study population was selected on the basis of being at high risk of sudden infant death syndrome. Recently, Jones *et al.* reassessed this study population at a follow up age of 16 years.[29] The detrimental effect of maternal smoking on childhood bone mineral density did not endure through adolescence. Godfrey *et al.* showed in 145 British neonates that maternal smoking was associated with a lower neonatal total body BMC and BMD independent of placental weight.[3] The association weakened after adjustment for neonatal length, indicating fetal skeletal growth may have been a potential mediator. Harvey *et al.* re-evaluated the association in a larger set of the same cohort including 841 neonates.[4] This time, the negative association between maternal smoking and neonatal bone measures fully disappeared upon adjustment for birth length. The largest and most recent study investigating the influence of maternal smoking on offspring bone health was performed among 7121 British 10-year-olds.[13] A positive association of maternal smoking with bone measures was observed in girls, not in boys. As paternal

smoking yielded similar associations, the authors attributed these associations to lifestyle related factors associated with smoking, rather than intrauterine mechanisms. Our results are similar to the results of this British study. Both studies showed a positive association between maternal smoking and childhood bone measures that became stronger upon adjustment for birth weight and disappeared after adjustment for current body weight.

In the present study as well as in the British study current weight was higher in children whose mothers smoked during pregnancy, whereas in the study Jones *et al.* children of smoking mothers weighed less.

Bone measures highly correlate with body weight. The strong influence of adjustment for current weight may reflect the impact that maternal smoking has on fetal and subsequent childhood growth. Earlier studies within the Generation R cohort clearly demonstrated that maternal smoking led to impaired fetal growth, lower birth weight and birth length.[5] In infancy, children of mothers who smoked during pregnancy quickly caught-up in weight, resulting in a normal to high weight from 3 months onwards.[26] In contrast, their height deficit appeared to persist throughout childhood, which led to a substantially higher BMI in children of smoking mothers. Although possibly through shared family lifestyle-related factors rather than intrauterine effects, daughters of smoking mothers were found to have a more adverse body and abdominal fat distribution.[32] In pre-pubertal children, a higher BMI or fat mass is thought to lead to increased bone strength resulting from larger mechanical strain on weight-bearing bones[33] or possibly through other endocrine stimulatory effects of fat mass.[34,35]

Household smoke exposure was found to lead to high cotinine levels and markers of oxidative stress in infants.[15,36] In line with these findings, household smoke exposure during adolescence has been associated with lower femoral neck BMD in adulthood.[16] However, the only previous study in children did not find an association.[11] Unfortunately, postnatal smoke exposure was not studied in the largest study on maternal smoking and childhood bone health.[13] Comparable to the results of the only previous study, our results did not provide evidence for an influence of passive smoking on childhood bone health.

Conclusion

In this large population-based prospective cohort study, we observed a positive association of maternal smoking during pregnancy with bone measures at 6 years of age that was fully explained by a higher childhood body weight. As paternal smoking, rather than maternal smoking showed a tendency towards a negative association with childhood bone measures, the previously suggested association between maternal smoking during pregnancy and childhood bone measures is more likely to be explained by shared familial lifestyle related factors than intrauterine effects. Further studies should focus on the consequences of fetal smoke exposure on later body composition rather than on bone health.

REFERENCES

1. Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Review: developmental origins of osteoporotic fracture. *Osteoporos Int.* 2006,17(3):337-47.
2. Salihu HM, Wilson RE. Epidemiology of prenatal smoking and perinatal outcomes. *Early Hum Dev.* 2007,83(11):713-20.
3. Godfrey K, Walker-Bone K, Robinson S, Taylor P, Shore S, Wheeler T, et al. Neonatal bone mass: influence of parental birthweight, maternal smoking, body composition, and activity during pregnancy. *J Bone Miner Res.* 2001,16(9):1694-703.
4. Harvey N, K. JM, Arden N. K., Poole JR, Crozier SR, Robinson SM, et al. Maternal predictors of neonatal bone size and geometry: the Southampton Women's Survey. *Journal of Developmental Origins of Health and Disease.* 2010,1(1):35-41.
5. Jaddoe VW, Verburg BO, de Ridder MA, Hofman A, Mackenbach JP, Moll HA, et al. Maternal smoking and fetal growth characteristics in different periods of pregnancy: the generation R study. *American journal of epidemiology.* 2007,165(10):1207-15.
6. Baird J, Kurshid MA, Kim M, Harvey N, Dennison E, Cooper C. Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis. *Osteoporos Int.* 2011,22(5): 1323-34.
7. Martinez-Mesa J, Restrepo-Mendez MC, Gonzalez DA, Wehrmeister FC, Horta BL, Domingues MR, et al. Life-course evidence of birth weight effects on bone mass: systematic review and meta-analysis. *Osteoporos Int.* 2012.
8. Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. *Clinical science.* 2007,113(5):233-41.
9. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev.* 2007,83(11):699-706.
10. Yan C, Avadhani NG, Iqbal J. The effects of smoke carcinogens on bone. *Curr Osteoporos Rep.* 2011,9(4):202-9.
11. Jones G, Riley M, Dwyer T. Maternal smoking during pregnancy, growth, and bone mass in prepubertal children. *J Bone Miner Res.* 1999,14(1):146-51.
12. Jones G, Hynes KL, Dwyer T. The association between breastfeeding, maternal smoking in utero, and birth weight with bone mass and fractures in adolescents: a 16-year longitudinal study. *Osteoporos Int.* 2012.
13. Macdonald-Wallis C, Tobias JH, Davey Smith G, Lawlor DA. Parental smoking during pregnancy and offspring bone mass at age 10 years: findings from a prospective birth cohort. *Osteoporos Int.* 2011,22(6):1809-19.
14. Smith GD. Assessing intrauterine influences on offspring health outcomes: can epidemiological studies yield robust findings? *Basic Clin Pharmacol Toxicol.* 2008,102(2):245-56.
15. Noakes PS, Thomas R, Lane C, Mori TA, Barden AE, Devadason SG, et al. Association of maternal smoking with increased infant oxidative stress at 3 months of age. *Thorax.* 2007,62(8): 714-7.
16. Blum M, Harris SS, Must A, Phillips SM, Rand WM, Dawson-Hughes B. Household tobacco smoke exposure is negatively associated with premenopausal bone mass. *Osteoporos Int.* 2002,13(8):663-8.
17. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. *Eur J Epidemiol.* 2012,27(9): 739-56.
18. Heppe DH, Medina-Gomez C, Hofman A, Franco OH, Rivadeneira F, Jaddoe VW. Maternal first-trimester diet and childhood bone mass: the Generation R Study. *Am J Clin Nutr.* 2013,98(1): 224-32.

19. Lewiecki EM, Gordon CM, Baim S, Binkley N, Bilezikian JP, Kendler DL, et al. Special report on the 2007 adult and pediatric Position Development Conferences of the International Society for Clinical Densitometry. *Osteoporos Int*. 2008,19(10):1369-78.
20. Heaney RP. Bone mineral content, not bone mineral density, is the correct bone measure for growth studies. *Am J Clin Nutr*. 2003,78(2):350-1; author reply 1-2.
21. Prentice A, Parsons TJ, Cole TJ. Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. *Am J Clin Nutr*. 1994,60(6):837-42.
22. Warner JT, Cowan FJ, Dunstan FD, Evans WD, Webb DK, Gregory JW. Measured and predicted bone mineral content in healthy boys and girls aged 6-18 years: adjustment for body size and puberty. *Acta Paediatr*. 1998,87(3):244-9.
23. Netherlands S. [Allochtonen in Nederland]. Voorburg/Heerlen: 2004.
24. http://www.cos.rotterdam.nl [Internet]. Centre for Research and Statistics. 2005.
25. Durmus B, van Rossem L, Duijts L, Arends LR, Raat H, Moll HA, et al. Breast-feeding and growth in children until the age of 3 years: the Generation R Study. *Br J Nutr*. 2011,105(11):1704-11.
26. Durmus B, Kruithof CJ, Gillman MH, Willemsen SP, Hofman A, Raat H, et al. Parental smoking during pregnancy, early growth, and risk of obesity in preschool children: the Generation R Study. *Am J Clin Nutr*. 2011,94(1):164-71.
27. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. *BMJ*. 2009,338: b2393.
28. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology*. 2006,17(4):413-8.
29. Lanting CI, Segaar D, Crone MR, van Wouwe JP. [Slight decrease in the prevalence of smoking around pregnancy]. *Nederlands tijdschrift voor geneeskunde*. 2007,151(46):2566-9.
30. Klebanoff MA, Levine RJ, Morris CD, Hauth JC, Sibai BM, Ben Curet L, et al. Accuracy of self-reported cigarette smoking among pregnant women in the 1990s. *Paediatr Perinat Epidemiol*. 2001,15(2):140-3.
31. George L, Granath F, Johansson AL, Cnattingius S. Self-reported nicotine exposure and plasma levels of cotinine in early and late pregnancy. *Acta obstetricia et gynecologica Scandinavica*. 2006,85(11):1331-7.
32. Durmus B, Heppe DH, Taal HR, Manniesing R, Raat H, Hofman A, et al. Parental smoking during pregnancy and total and abdominal fat distribution in school-age children: the Generation R Study. *Int J Obes (Lond)*. 2014.
33. Rocher E, Chappard C, Jaffre C, Benhamou CL, Courteix D. Bone mineral density in prepubertal obese and control children: relation to body weight, lean mass, and fat mass. *J Bone Miner Metab*. 2008,26(1):73-8.
34. Timpson NJ, Sayers A, Davey-Smith G, Tobias JH. How does body fat influence bone mass in childhood? A Mendelian randomization approach. *J Bone Miner Res*. 2009,24(3):522-33.
35. Clark EM, Ness AR, Tobias JH. Adipose tissue stimulates bone growth in prepubertal children. *J Clin Endocrinol Metab*. 2006,91(7):2534-41.
36. Blackburn CM, Bonas S, Spencer NJ, Coe CJ, Dolan A, Moy R. Parental smoking and passive smoking in infants: fathers matter too. *Health education research*. 2005,20(2):185-94.

Supplement 4.3.1 Characteristics of participants lost to follow-up for the 6-year research center visit

Maternal characteristics	Unit	No follow-up (*n*=2,466)	Follow-up (*n*=5,745)	*P*-value
Age	year	28.3 (5.4)	30.3 (5.1)	<0.001
Height	cm	166.4 (7.3)	167.6 (7.4)	<0.001
Pre-pregnancy weight	kg	65.8 (13.4)	66.5 (12.6)	0.04
Pre-pregnancy body mass index	kg/m^2	23.7 (4.7)	23.6 (4.2)	0.43
Ethnicity	Western	1417 (58)	4076 (71)	<0.001
	African	596 (24)	949 (17)	
	Asian	233 (9)	488 (9)	
	Missing	230 (9)	602 (10)	
Parity ≥1	No	1307 (53)	3289 (57)	<0.001
	Yes	1135 (46)	2424 (42)	
	Missing	24 (1)	32 (1)	
Educational status	Primary	354 (14)	532 (9)	<0.001
	Secondary	1172 (48)	2434 (42)	
	Higher	748 (30)	2528 (44)	
	Missing	192 (8)	255 (4)	
Smoking	Never	461 (19)	807 (14)	<0.001
	First trimester only	165 (7)	443 (8)	
	Continued	1610 (65)	3897 (68)	
	Missing	228 (9)	593 (10)	
Alcohol use	No	1595 (65)	3091 (54)	<0.001
	Yes	656 (27)	2147 (37)	
	Missing	215 (9)	507 (9)	
Paternal characteristics				
Age	year	31.6 (6.3)	33.4 (5.9)	<0.001
Height	cm	180.7 (7.7)	182.1 (7.9)	<0.001
Weight	kg	82.8 (13.2)	83.9 (13.0)	0.003
Body mass index	kg/m^2	25.3 (3.7)	25.3 (3.4)	0.52
Ethnicity	Western	1217 (49)	3866 (67)	<0.001
	African	523 (21)	989 (16)	
	Asian	179 (7)	440 (8)	
	Missing	547 (22)	541 (9)	
Educational status	Primary	152 (6)	270 (5)	<0.001
	Secondary	515 (21)	1586 (28)	
	Higher	566 (23)	2025 (35)	
	Missing	1233 (50)	1864 (32)	

Supplement 4.3.1 (continued)

	Unit	No follow-up (*n*=2,466)	Follow-up (*n*=5,745)	*P*-value
Smoking	No	1220 (50)	3047 (53)	<0.001
	Yes	1092 (44)	2447 (43)	
	Missing	154 (6)	251 (4)	
Family characteristics				
Monthly household income	<€1600	700 (28)	1282 (22)	<0.001
	≥€1600-2200	230 (9)	744 (13)	
	≥€2200	724 (29)	2803 (49)	
	Missing	812 (33)	916 (16)	
Married/living together	Yes	1834 (74)	4793 (83)	<0.001
	No	452 (18)	680 (12)	
	Missing	180 (7)	272 (5)	
Infant characteristics				
Sex	Girl	1194 (48)	2885 (50)	0.14
	Boy	1271 (52)	2860 (50)	
Gestational age at birth	weeks	39.7 (2.1)	39.9 (1.7)	<0.001
Birth weight	grams	3380 (580)	3430 (548)	<0.001

Values reflect the mean (standard deviation) for continuous variables or absolute numbers (%) for categorical variables.

Supplement 4.3.2 Imputed characteristics of study participants

		Maternal smoking during pregnancy			
Maternal characteristics	Unit	Never *n*=3767	First trimester only *n*=430	Continued *n*=789	*P*-value
Age	year	30.7 (4.9)	30.1 (5.0)	28.8 (5.8)	<0.001
Height	cm	167.8 (7.4)	168.9 (6.9)	167.2 (7.0)	<0.001
Pre-pregnancy weight	kg	64 (58-72)	65 (58-71)	63 (57-72)	0.60
Pre-pregnancy body mass index	kg/m²	22.7 (20.8-25.3)	22.3 (20.6-24.9)	22.7 (20.6-25.7)	0.15
Ethnicity	Western	2714 (72)	329 (77)	607 (77)	<0.001
	African	675 (18)	55 (13)	133 (17)	
	Asian	378 (10)	46 (10)	47 (6)	
Parity ≥1	No	2141 (57)	312 (73)	462 (59)	<0.001
	Yes	1626 (43)	118 (27)	327 (41)	
Educational status	Primary	330 (9)	32 (7)	125 (16)	<0.001
	Secondary	1545 (41)	201 (47)	490 (62)	
	Higher	1891 (50)	196 (46)	174 (22)	
Alcohol use	No	2315 (61)	215 (50)	441 (56)	<0.001
	Yes	1451 (39)	215 (50)	348 (44)	
Paternal characteristics					
Age	year	33.6 (5.8)	32.4 (5.8)	31.6 (6.4)	<0.001
Height	cm	181.6 (8.0)	182.5 (8.0)	179.9 (8.0)	<0.001
Weight	kg	83.6 (13.1)	83.6 (13.6)	82.7 (14.2)	0.32
Body mass index	kg/m²	25.3 (3.4)	25.0 (3.4)	25.4 (3.6)	0.18
Ethnicity	Western	2717 (72)	332 (77)	587 (74)	<0.001
	African	695 (18)	56 (13)	149 (19)	
	Asian	354 (9)	42 (10)	53 (7)	
Educational status	Primary	365 (10)	35 (8)	137 (17)	<0.001
	Secondary	1523 (40)	205 (48)	477 (60)	
	Higher	1879 (50)	190 (44)	175 (22)	
Family characteristics					
Monthly household income	<€1600	1031 (27)	117 (27)	367 (47)	<0.001
	≥€1600-2200	553 (15)	64 (15)	146 (19)	
	≥€2200	2183 (58)	249 (58)	276 (35)	
Married/living together	Yes	3403 (90)	363 (84)	591 (75)	<0.001
	No	364 (10)	67 (16)	198 (25)	
Infant characteristics					
Sex	Girl	1929 (51)	225 (52)	366 (46)	0.09
	Boy	1838 (49)	205 (48)	423 (54)	
Gestational age at birth	weeks	40.1 (39.1-41.0)	40.1 (39.1-40.0)	40.0 (39.0-40.9)	0.001
Birth weight	grams	3460 (537)	3462 (546)	3278 (561)	<0.001

Supplement 4.3.2 (continued)

		Maternal smoking during pregnancy			
	Unit	Never *n*=3767	First trimester only *n*=430	Continued *n*=789	*P*-value
Breast feeding	Never	337 (9)	44 (10)	157 (20)	<0.001
	>0-3 months	1363 (36)	194 (45)	399 (51)	
	≥3 months	2068 (55)	192 (45)	232 (29)	
Child characteristics at 6 year visit					
Age	year	6.2 (0.5)	6.2 (0.5)	6.3 (0.6)	<0.001
Height	cm	119.5 (5.9)	119.8 (6.3)	119.7 (6.3)	0.57
Weight	kg	22.6 (20.6-25.0)	22.4 (20.4-25.4)	23.0 (20.8-26.0)	0.001
Body mass index	kg/m^2	15.8 (15.0-16.9)	15.8 (14.9-16.9)	16.1 (15.3-17.5)	<0.001
Participation in sports	Never	2062 (55)	239 (56)	458 (58)	<0.001
	1/week	1371 (36)	150 (35)	226 (29)	
	≥2/week	334 (9)	41 (10)	105 (13)	

Values reflect the mean (standard deviation) or median (interquartile range) for continuous variables or absolute numbers (%) for categorical variables. *P*-values are obtained by Students *t*-tests for normal-distributed continuous variables, Kruskal-Wallis tests for non-normal distributed continuous variables, and chi-square tests for categorical variables.

Supplement 4.3.3 Associations of maternal smoking during pregnancy with bone measures at age 6, corrected for clustering effects within families

		Crude model		Confounder model		Birth weight model		Current weight model	
Maternal smoking	*n*	ß	95% CI	ß	95% CI	ß	95% CI	ß	95% CI
Bone mineral content (g)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-1.5	-7.0, 4.0	-0.6	-6.7, 5.4	-1.1	-7.1, 4.9	0.0	-4.8, 4.8
Continued	789	**10.4**	**6.2, 14.7*** **	**11.6**	**5.7, 17.5*** **	**12.3**	**6.4, 18.2*** **	3.2	-1.5, 7.9
<5 cigarettes/day	388	**13.0**	**7.3, 18.7*** **	**14.2**	**6.5, 21.8*** **	**15.1**	**7.5, 22.8*** **	**6.6**	**0.5, 12.8***
≥5-10 cigarettes/day	208	6.5	-1.1, 14.2	8.2	-1.3, 17.8	8.7	-0.8, 18.2	0.8	-6.9, 8.4
≥10 cigarettes/day	137	7.3	-2.0, 16.7	**18.6**	**5.9, 31.3** **	**19.4**	**6.7, 32.0** **	1.9	-8.2, 12.0
P for trend		***0.02***		***0.007***		***0.006***		*0.36*	
Bone area (cm²)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-2.0	-7.8, 3.9	0.4	-6.4, 7.2	-0.3	-7.0, 6.5	0.6	-5.5, 6.7
Continued	789	**6.2**	**1.8, 10.6** **	**9.8**	**3.1, 16.4** **	**10.7**	**4.1, 17.4** **	3.4	-2.5, 9.4
<5 cigarettes/day	388	**10.3**	**4.4, 16.1** **	**12.8**	**4.2, 21.4** **	**14.2**	**5.6, 22.8** **	7.4	-0.4, 15.2
≥5-10 cigarettes/day	208	2.4	-1.8, 6.5	5.8	-4.9, 16.5	6.5	-4.2, 17.2	0.2	-9.5, 9.9
≥10 cigarettes/day	137	-4.2	-9.3, 0.8	6.9	-7.3, 21.1	8.0	-6.2, 22.2	-5.9	-18.7, 7.0
P for trend		*0.69*		*0.09*		*0.08*		*0.73*	
Bone mineral density (g/cm²)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-0.7	-4.5, 3.2	-0.9	-5.3, 3.5	-1.0	-5.4, 3.4	-0.4	-4.4, 3.5
Continued	789	**7.7**	**6.0, 9.3*** **	**6.7**	**2.4, 11.0** **	**6.9**	**2.6, 11.2** **	2.0	-1.8, 5.9
<5 cigarettes/day	388	**7.7**	**5.6, 9.7*** **	**7.7**	**2.1, 13.3** **	**7.9**	**2.3, 13.5** **	3.3	-1.7, 8.4
≥5-10 cigarettes/day	208	**6.5**	**3.7, 9.2***	5.7	-1.2, 12.6	5.8	-1.1, 12.7	1.6	-4.7, 7.8
≥10 cigarettes/day	137	**10.4**	**7.0, 13.7** **	**15.2**	**6.0, 24.4** **	**15.4**	**6.2, 24.6** **	6.0	-2.2, 14.3
P for trend		***<0.001***		***0.02***		***0.02***		*0.34*	
Bone mineral content adjusted for area (g)									
Never	3767	Ref		Ref		Ref		Ref	
Until pregnancy was known	430	-0.1	-3.8, 3.7	-0.8	-5.0, 3.4	-0.9	-5.1, 3.3	-0.3	-4.0, 3.5
Continued	789	**6.3**	**4.8, 7.8*** **	**5.4**	**1.3, 9.5***	**5.5**	**1.3, 9.6** **	1.5	-2.2, 5.1
<5 cigarettes/day	388	**6.1**	**2.7, 9.5** **	**6.0**	**0.7, 11.4***	**6.1**	**0.8, 11.5***	2.9	-1.8, 7.7
≥5-10 cigarettes/day	208	5.0	-0.3, 10.3	4.5	-2.1, 11.2	4.6	-2.1, 11.2	0.7	-5.2, 6.6
≥10 cigarettes/day	137	**10.3**	**7.0, 13.5** **	**14.2**	**5.4, 23.0** **	**14.3**	**5.5, 23.1** **	4.8	-3.0, 12.6
P for trend		***0.002***		***0.03***		***0.03***		*0.37*	

Values are based on linear mixed models including rank of the child in the study as grouping variable. Values reflect the estimates and 95% confidence interval for the fixed effects of maternal smoking during pregnancy. **Crude model** is adjusted for sex, current age and height of the child. **Confounder model** is additionally adjusted for current maternal household smoking, maternal age, weight, height, ethnicity, parity, educational level, marital status, alcohol use and folic acid supplement use, and breastfeeding duration and participation in sports of the child. **Birth weight model** is additionally adjusted for birth weight. **Current weight model** is additionally adjusted for current weight.

CHAPTER 5

GENERAL DISCUSSION

The aim of this thesis was to identify early life determinants possibly explaining variation in fetal growth as well as childhood adiposity and bone mass. Four main aspects were addressed:

1. Maternal dietary factors in relation to fetal growth and neonatal complications.
2. Parental, fetal and infant determinants of childhood adiposity.
3. Parental, fetal and infant determinants of childhood bone mass.

In the previous chapters, the main results, merits and shortcomings of the studies were already discussed in detail. This chapter provides a more general discussion of the main findings, potential underlying mechanisms, methodological considerations and implications for further research.

MATERNAL DIETARY FACTORS AND FETAL GROWTH

Fetal growth depends on fetal oxygen and nutrient availability. Nutrient availability is mainly determined by maternal dietary nutrient intake during pregnancy and the transport of these nutrients across the placenta.[1] The influence of maternal diet on fetal growth has been extensively studied. Although some specific nutrients were granted growth promoting potential, results on dietary products have been less consistent.[2] A few dietary products have shown promising results worth of further research.[2] In this thesis, milk, a commonly consumed product in the Netherlands, and fish, the most common dietary source of *n*-3 polyunsaturated fatty acids (PUFA), have been thoroughly studied in relation to fetal growth patterns. In the following paragraphs the findings of these studies are discussed in the light of the current literature.

Maternal fish consumption and fetal growth

Over the past years the potential harms and benefits of fish consumption during pregnancy gained substantial interest. As said, fish is the most common dietary source of *n*-3 PUFA.[3] The other essential long-chain PUFA, *n*-6, is mainly derived from vegetable oils.[4] The levels and ratio of *n*-3 and *n*-6 PUFA can influence cell membrane properties, cell-to-cell signaling, expression of genes regulating cell differentiation and growth, and synthesis of eicosanoids.[5] Eicosanoids are biologically active lipids and include prostaglandins, thromboxanes and leukotrienes. Prostaglandins play a major role in the onset of delivery, and, together with thromboxanes, regulate placental blood flow by balancing platelet aggregation and vasoconstriction or –dilatation.[6] Trough differences in biologic activity in prostaglandins and thromboxanes derived from *n*-3 PUFA or *n*-6 PUFA, high maternal *n*-3 PUFA intake was suggested to prolong gestation and enhance fetal growth through increased placental blood flow.[7,8] As promising as these hypotheses are, to date, results of numerous studies assessing maternal fish consumption and gestational

duration or fetal growth have been largely inconsistent. Strong positive associations of fish consumption with birth size have mainly been described in ecological and cohort studies conducted in the 1980s and 1990s in countries high in fish consumption.[7,9-11] Results of studies conducted more recently in countries with lower fish consumption, showed either positive,[12,13] no,[14] or negative associations.[15] Maternal fatty fish and canned tuna consumption showed both positive and negative associations with birth size.[16,17] Yet, maternal shellfish consumption only showed a negative association.[18,19] In the study presented in this thesis, associations between maternal fish consumption and fetal growth fully disappeared after adjustment for lifestyle related confounders. Interestingly, maternal shell fish consumption showed a negative association with birth weight. Fatty fish and lean fish consumption did not show an association with any fetal growth parameters. More recently, a meta-analysis of more than 150,000 European mother-child pairs, including those of the Generation R Study, was performed.[20] Its results showed maternal fish consumption, especially fatty fish, decreased the risk of preterm birth, but only slightly increased birth weight. Altogether, these results indicate that the role of maternal fish consumption during pregnancy in promoting fetal growth is limited. The potential adverse effects of shellfish consumption require further study. Meanwhile, similar to fatty fish consumption, shellfish consumption should be advised to be limited to a maximum of twice weekly.[21]

Maternal milk consumption and fetal growth

Since cow's milk has become globally available, cow's milk consumption has become a common component of human diet.[22] Compared to human milk, cow's milk contains about three times more protein, four times more calcium, and overall more minerals. Through its high levels of protein, minerals and B vitamins, maternal milk consumption during pregnancy was expected to be beneficial for fetal growth.[23] A positive association between maternal milk consumption and birth size was first reported within a rural population in India, and later confirmed in Western countries.[24-27] Within a cohort of nearly 50,000 pregnant women living in Denmark, maternal milk consumption not only contributed to birth weight, but also birth length and head circumference.[28] Results presented in this thesis show maternal milk consumption (>1 glass daily) to be positively associated with birth weight and head circumference, but not with birth length. Unlike previous studies, fetal growth was measured in different trimesters and longitudinally modelled. By doing so, maternal milk consumption appeared to affect fetal growth from the third trimester of pregnancy onwards, when absolute fetal growth is at its peak. In the most recent study, maternal milk consumption was positively associated with birth weight and length.[29] After 20 years of follow-up, although very weakly, the association with adult weight, height, but not BMI, persisted. These findings suggest maternal milk consumption during pregnancy may promote fetal growth from the third trimester onwards.

Our findings suggest that pregnant women should be encouraged to drink 2-3 glasses of milk per day, as recommended by the Netherlands Nutrition Center (*Voedingscentrum Nederland*).[21] Intervention studies are needed to determine the potential of maternal milk consumption in improving fetal outcomes.

Potential underlying mechanisms

A number of factors may explain the inconsistency between results from previous studies and results from this thesis concerning maternal fish consumption. First of all, fish consumption tends to be related to a healthier lifestyle.[30] Hence, residual confounding factors related to a healthy lifestyle may have led to false positive associations. However, in some studies the observed association was not influenced by adjustment for confounders.[12,13] Second, quantity and quality of consumed fish differs between countries.[31] If a threshold exists for the effect of fish consumption, the low level of fish consumption in the Netherlands, may have led to null findings.[32,33] Furthermore, besides beneficial nutrients, fish contains pollutants such as methylmercury, dioxins and polychlorinated biphenyls (PCB). Levels of contamination differ between countries, areas and within fish species.[34] Fatty fish and shellfish contain higher levels of pollutants than lean fish.[35] Besides, shellfish contains relatively low levels of beneficial *n*-3.[36] Most chemicals are able to pass the placental barrier and enter the fetal blood stream.[37] Methylmercury levels were found to be even higher in cord blood than in maternal blood samples.[38] Due to their immature organ systems and detoxification enzymes, the fetus is highly vulnerable to pollutant exposure. Consequently, methylmercury, dioxins and PCB's levels in maternal and cord blood have been associated with lower birth weight.[17,39,40] Formation of reactive oxygen species (ROS) may affect placentation and fetal development; directly through disruption of cellular structures and processes, or indirectly through disruption of endocrine, thyroid or immune functions.[18] Yet, the mechanisms linking such pollutants to fetal growth remain uncertain.

Quite strong evidence indicates regular cow's milk consumption promotes linear growth during childhood, especially during periods of fast growth.[41] Several milk constituents have been suggested to underlie this growth promoting effect and may also promote fetal growth during pregnancy: First of all, milk is a source of energy. Results from this thesis were adjusted for total energy intake, thus increased energy intake is unlikely to explain the findings. Calcium is a major component of skeletal tissue. However, calcium supplementation during childhood has not been proven to enhance childhood growth,[22] and during pregnancy, it only slightly increases birth weight.[42] Furthermore, milk consumption increases endogenous circulating levels of insulin-like growth factor-I (IGF-I).[41] Especially, the casein protein from milk, rather than whey, seemed to raise circulating IGF-I levels.[43] Accordingly, results presented in this thesis showed the protein fraction from milk drove the association between maternal milk consumption and fetal growth. IGF-I is a peptide

hormone which induces cell division and proliferation, and prevents apoptosis. IGF-I is a key regulator of feto-placental growth. Maternal IGF-I does not cross the placenta.[44] Yet, possibly through regulating placental function, higher maternal IGF-I levels lead to higher birth weight, especially in late pregnancy.[45] In an intervention study among children, increased milk consumption stimulated insulin secretion.[46] Hence, plasma insulin was suggested to mediate the increase in IGF-I levels, or vice versa. The influence of maternal milk consumption on postnatal offspring IGF-I or insulin levels has not been studied yet. Milk supplementation during pregnancy along with the first five years of life did lower offspring IGF-I levels in adulthood.[47] However, a recent observational study only found a very weak association between maternal milk consumption and adult IGF-I and insulin levels.[29] Especially since IGF-I levels in later life may impact hormonal cancer and cardiovascular disease risk, the long-term consequences of maternal milk consumption require further study. Also, more research is needed on short-term consequences of maternal milk consumption, such as risk of birth complications.

PARENTAL, FETAL AND INFANT DETERMINANTS OF CHILDHOOD ADIPOSITY

Along with the rising prevalence of childhood overweight, a growing number of studies assessed and identified potential risk factors of overweight.[48] The development of overweight results from a complex interaction between (epi)genetic, nutritional, behavioral and environmental risk factors.[49] Therefore, risk factors often coexist and, thus, are correlated.[50] The study presented in this thesis aimed to identify the early risk factors most strongly related to preschool overweight. The much debated role of maternal smoking in childhood adiposity was studied in more detail. The findings of these studies are evaluated against the existing knowledge in the following paragraphs.

Risk factors of preschool overweight

Risk factors of early overweight have been extensively studied. The risk factors identified thus far seem to be socially patterned, and often coexist.[48] As a result, estimating the individual contribution of a risk factor to overweight development is challenging. Obviously, prevention of overweight should target children at risk of developing overweight. To identify these children, we need to identify the strongest predictors of overweight by simultaneous analysis of known risk factors. By doing so, high parental BMI and low socio-economic status, female sex, rapid fetal and infant weight gain, high birth weight and a toddler diet low in PUFA were the strongest risk factors for developing preschool overweight in this thesis. All five previous studies using a similar approach indicated maternal BMI, paternal BMI (when available), and infant weight gain strongly and

independently affected preschool overweight risk.[51-55] All studies, except for one study,[55] showed low parental socio-economic status and high birth weight were independent risk factors of preschool overweight.[51-54] Maternal smoking was an independent risk factor in some,[51,54] but not all studies.[52] Infant feeding practices and female sex showed inconsistent results.[51-55] The findings on fetal growth patterns, in the context of other risk factors, are novel and cannot be compared to existing literature yet. A meta-analysis focused on early risk factors of childhood overweight showed strong evidence indicating that maternal pre-pregnancy overweight, maternal smoking in pregnancy, high birth weight, and rapid infant weight gain increase childhood overweight risk, whereas there is some evidence for a moderate protective effect of breastfeeding and late introduction of solid foods.[56] Results of a recent study showed having at least four out of five early risk factors - being maternal obesity, excess gestational weight gain, smoking in pregnancy, low maternal vitamin D status, and short duration of breastfeeding - led to a 4-fold increase in preschool overweight risk and to a 19% increase in fat mass at 4 years and a 47% increase at 6 years of age.[48] Breastfeeding was not a predictor of preschool overweight in this thesis. As a large randomized controlled trial (RCT) showed breastfeeding did not protect against overweight at 6 or 11 years of age, previous results are likely affected by residual confounding.[57,58] Taken together, these findings suggest that a risk score based on parental BMI and socio-economic status, birth weight, and fetal and infant weight gain may help identifying children for targeted overweight interventions. Such a risk score may be particularly useful in preventive care, for instance the Community Health Centers (*Consultatiebureau's*). The relevance of maternal smoking and infant feeding practices herein is disputable. As maternal smoking has shown to be one of the most prevalent modifiable risk factors, its relation with adiposity was studied in further detail in Chapter 3.2.

Parental smoking and childhood adiposity

Despite the risks, around 15% of pregnant women in the Netherlands smoked throughout their pregnancy in 2012.[59] Fetal smoke exposure is known to limit fetal growth.[60] Maternal smoking during pregnancy was hypothesized to lead to childhood overweight through postnatal catch-up growth following fetal growth restriction.[61] Yet, in some studies birth weight and catch-up growth did not mediate the association between maternal smoking and offspring overweight.[62,63] Cigarette smoke contains numerous toxic elements, such as nicotine, carbon monoxide, cadmium and reactive oxygen species (ROS) that may directly harm the growing fetus or indirectly impair placental function and fetal nutrient supply.[64] Yet, the existence of an association between fetal smoke exposure and childhood overweight remained disputed, as it may also result from uncontrolled residual confounding. In the study presented in this thesis, the influence of intrauterine exposure to maternal smoking on childhood body composition was compared to the influence of

paternal smoking among non-smoking mothers, to disentangle the intrauterine effect of maternal smoking from environmental effects.[65] Girls, but not boys, born from mothers who continued smoking throughout pregnancy had higher BMI, total fat mass, android/gynoid fat ratios, subcutaneous abdominal fat mass, and preperitoneal abdominal fat mass than girls of mothers who did not smoke. As, also paternal smoking showed a positive association with body fat accumulation, intrauterine mechanisms are unlikely to fully explain this relation. A large amount of previous studies, summarized by meta-analyses, showed maternal smoking leads to a higher childhood BMI.[64,66,67] One of those meta-analyses limited its inclusion to studies that also took paternal smoking into account.[66] After mutual adjustment, maternal smoking showed stronger adipogenic effects than paternal smoking. However, when restricting to high quality studies, this difference lost significance.[66] Childhood fat mass and distribution have hardly been measured accurately in previous studies.[68] In the few studies that did, maternal smoking was positively associated with total fat mass in childhood and late puberty.[69-71] Yet, the association of maternal smoking with abdominal fat mass, observed in the study presented in this thesis, had not been described before and requires further study. Although the possibility of residual confounding in the association between maternal smoking and childhood adiposity cannot be ruled out, maternal as well as paternal smoking during pregnancy should be discouraged to avoid known adverse effects for maternal and offspring health, and possible side effects of passive smoke exposure.[72]

Potential underlying mechanisms

Maternal BMI and weight gain during pregnancy are accepted indicators of maternal and, hence fetal, (macro)nutrient availability.[73] Increased fetal nutrient supply leads to fetal overgrowth and subsequent infant fatness.[74] However, in the study presented in this thesis, both maternal and paternal BMI strongly predicted preschool overweight. Hence, the effect of maternal BMI is more likely explained by shared familial factors than by intrauterine mechanisms.[65] Furthermore, low household income was a strong risk factor of preschool overweight, even slightly stronger than educational level. Income is considered to determine access to resources, whereas education relates to knowledge and beliefs.[75] Perhaps in the studied area, reduced financial possibilities more likely predispose to overweight development than limited health-related knowledge. Low income may also be a more accurate indicator of low SES in the Generation R Study. Interestingly, higher birth weight and accelerated growth during the third trimester and infancy independently predicted preschool overweight. Accelerated fetal growth may result from fetal over-nutrition due to maternal over-nutrition or hyperglycemia.[74] Or, from relative fetal growth restriction during an earlier period.[76] The higher overweight prevalence in girls may reflect artefacts of the used *International Obesity Taskforce* criteria,[77] or, result from biological differences. From early infancy, girls and boys differ in fat mass quantity and

distribution,[78] resulting from differences in sex steroids and hormones like leptin and insulin, involved in feeding behavior and metabolism,[78-80] and, possibly, differences in vulnerability to environmental queues.[81] Although not confirmed by a systematic review,[82] early introduction of solid food was hypothesized to increase energy intake and lead to rapid weight gain.[83] Early solid food introduction may differently affect formula-fed and breast fed infants.[84] Contrary to formula-fed infants, solid food intake displaced milk intake among breastfed children.[85] High intake of *n*-3 versus *n*-6 PUFA during infancy may beneficially affect adipose tissue through the adipogenic and lipolytic effects of eiconsanoids derived from *n*-6 and *n*-3 PUFA, respectively.[86] However, these first results require further study.[87] Above all, due its strong relation with lifestyle, identification of risk factors of overweight is prone to the consequences of residual confounding.[58]

The pathophysiology underlying the relation between maternal smoking and offspring adiposity is far from being elucidated. Nicotine can cross the placenta and is only slowly metabolized by the fetus. Fetal nicotine exposure was hypothesized to lead to increased appetite via alterations in the hypothalamus, the most important central regulator of body weight homeostasis.[88] Exposure to carbon monoxide and ROS may affect placental vascular function and cause fetal oxidative stress.[89] The fact that paternal smoking was also positively associated with childhood fat mass can be explained by three mechanisms: 1) uncontrolled, residual confounding of the association; 2) a genuine effect of exposure to passive smoking in pregnancy; or 3) a genuine effect of postnatal smoking of father or mother.[90] Although exposure to passive smoking slightly increases cotinine levels, a measure of cumulative nicotine exposure and marker of oxidative stress, cotinine levels in neonates exposed to active smoking during pregnancy are by far higher.[91,92] Moreover, since household smoke exposure has not shown a consistent relationship with childhood adiposity,[93] residual confounding most likely explains the association between maternal smoking and childhood adiposity. For example, the fact that children of smoking mothers tend to be less physically active and have poorer diet quality may play a role.[94,95] These findings warrant the need of study designs less prone to the consequences of residual confounding, which will be described in the "Future research" section.

PARENTAL, FETAL AND INFANT DETERMINANTS OF CHILDHOOD BONE MASS

Bone mass increases through early life, childhood and adolescence, and peaks in early adulthood. Optimizing bone mineral accrual during early life, childhood and adolescence may increase the peak bone mass achieved and, hence, reduce later osteoporosis risk.[96] Evidence showing bone mineral accrual during childhood and adolescence may be modified by early life influences is growing.[97] In the following paragraphs,

the relationship between early growth patterns and childhood bone mass, including a potential role for maternal diet and maternal smoking during pregnancy herein, will be demonstrated and put in the context of current research perspectives.

Fetal growth, childhood growth and childhood bone mass

The rapid rate of mineral apposition during fetal life and infancy, along with the plasticity of skeletal development in utero, make the early skeleton susceptible to interactions with environmental queues. Over the past two decades, several studies have investigated and convincingly confirmed an association between early growth and later bone health.[98,99] The majority of studies focused on associations between birth or infant weight and bone mass at later age. Only one previous study simultaneously considered fetal and infant growth.[100] Identification of a critical period is challenged by correlation between repeatedly measured growth measures. Also, given sufficient nutrition, fetal growth restriction is often followed by postnatal catch-up growth.[101] By using conditional growth modeling, a technique that handles correlation between growth measures, both fetal growth and infant growth were found to independently contribute to childhood bone mass in the study presented in this thesis. Growth in the first year of life (both height and weight growth) appeared to have the largest impact on bone mineral accrual. After the first year, the impact of growth slowly decreased every year. These results were in agreement with results of the only previous study that used a similar longitudinal approach to investigate this association.[100] Furthermore, this thesis showed that, if present, catch-up growth in the first two postnatal years compensated for the adverse consequences of fetal growth restriction on childhood bone mass. Thus, children who were growth restricted during fetal life, but showed postnatal catch-up growth had similar bone measures to children who showed normal growth during fetal and postnatal life. None of the previous studies had evaluated the influence of postnatal growth realignment. These findings highlight the importance of preventing fetal growth restriction or postnatal stunting. As postnatal catch-up growth is risk factor for developing overweight, the postnatal growth trajectory should be carefully monitored.

Maternal diet and childhood bone mass

Fetal growth largely depends on the availability of oxygen and nutrients. The main determinant of fetal nutrient availability is the maternal diet during pregnancy. Consequently, unravelling the potential role of maternal diet in fetal bone mass acquisition has been the aim of several previous studies. Due to differences across findings, the authors of these studies attributed potential beneficial or detrimental actions to different nutrients. Potentially positive effects were attributed to maternal phosphorus, magnesium, potassium, calcium, and folate intake while fat intake was attributed negative effects.[24,102-104] In the study presented in this thesis, maternal protein intake showed a positive association

with childhood bone mass. Carbohydrate intake showed a negative association, which is likely explained by its codependence of protein intake. As every diet exists of protein, fat and carbohydrate, intakes are highly correlated. Consequently, the risk of collinearity, which occurs when two or more predictor variables in a regression model are highly correlated, increases. Although (multi)collinearity does not affect the validity of the model per se, it may have complicated the estimation of the individual contribution of nutrients. Likewise, the positive association of maternal calcium intake found in the study presented this thesis, depended on maternal phosphorus intake. Due to their similar dietary sources, i.e. animal and vegetable products rich in protein, such as green leafy vegetables, beans, nuts, dairy, whole grains, intake of these minerals are highly correlated. Calcium and phosphorus are also co-dependent in their bone forming actions.[105] Maternal homocysteine levels were inversely and maternal vitamin B12 levels positively associated with childhood bone measures. The association of vitamin B12, essential to the metabolism of homocysteine,[106] was only partly independent of the association of homocysteine with childhood bone measures. These novel findings require further replication. Altogether, the findings of this study indicate the importance of a balanced diet, rich in protein, minerals and vitamin B12 may support healthy childhood bone development.

Parental smoking and childhood bone mass

Reduced fetal growth has been shown to lead to lower bone mineral content in childhood and adulthood.[98,107] Consequently, maternal smoking has also been suggested to adversely affect offspring bone mass. In line with these suggestions, children of mothers that smoked during pregnancy were found to have lower BMD at the lumbar spine and femoral neck than children of mothers who did not.[108] As the association was explained by differences in placental weight, impaired placental function was the suggested mediator. However, in another study population the association was better explained by neonatal length, indicating (skeletal) growth restriction may be a mediator.[109,110] As described above, cigarette smoke contains numerous toxic elements that may harm the developing skeleton. These toxins may directly affect osteoblast and osteoclast activity,[111,112] or lead to diminished nutrient supply due to lower intestinal calcium absorption[112] or impaired placental function.[113] Or, since smokers tend to engage in a less healthy lifestyle, this association may be prone to the risks of uncontrolled residual confounding. Again, the influence of maternal and paternal smoking were compared to disentangle intrauterine from confounding factors.[65] In the study presented in this thesis, maternal smoking seemed positively associated with childhood bone measures. Yet, when the analysis was adjusted for current weight, this association disappeared. Interestingly, the largest and most recent previous study, had shown a similar phenomenon.[114] Heavy paternal smoking was associated with lower childhood bone mass, but not density. Household smoking was not associated with childhood bone measures. According to these findings,

maternal, or paternal, smoking does not directly influence childhood bone development. However, because of the already known adverse effects of exposure to maternal or passive smoking, maternal and paternal smoking should be discouraged.[72]

Potential underlying mechanisms

Bone growth is regulated by growth factors, cytokines, hormones and mechanical stimuli, under the influence of genetic and epigenetic regulation, availability of nutrients and exposure to adverse events.[115] Among others, alterations to the GH-IGF-axis, the hypothalamic-pituitary-adrenal (HPA) axis, or adipokine levels such as leptin and osteocalcin, have been suggested to play a prominent role in the early programming of bone mineral accrual.[116,117] Yet, thus far, the exact mechanism has not been clarified. In adults, higher protein intake increases intestinal calcium uptake and raises IGF-I levels. IGF-I promotes osteoblast genesis and reduces osteoblast apoptosis. IGF-I levels are lower in fetal growth restricted neonates[115] and are positively correlated with bone mass in both neonates and children.[118,119] Maternal protein restriction was associated with reduced methylation of the glucocorticoid receptor (GR) promotor.[120] Increased expression of the GR could sensitize osteoblasts to cortisol, which inhibits its function, and thereby reduces offspring bone mass.[117] Serum cortisol levels are higher in infants born SGA,[121] especially in those who do not achieve catch-up growth.[122] High normal endogenous cortisol levels have been negatively associated with bone mass, predominantly in boys.[123,124] Altered leptin levels, resulting from low or high nutrient availability, may program bone development by stimulating cortical bone formation and differentiation of mesenchymal stem cells into bone precursors (osteoblasts), over adipocyte precursors.[116] Leptin levels are positively correlated with fetal growth,[125] postnatal catch-up growth and neonatal BA and BMC.[126,127] Also, results on altered transcriptional activity of other genes, such as the *eNOS* gene, key to osteocyte, osteoblast and osteoclast function, the IGF-2 gene, key to fetal and placental growth, the *PMCA3* gene, involved in placental calcium transport, seem promising and require further study.[116,117] Vitamin B12 and folate may support fetal bone health by providing methyl donors for DNA methylation, or by directly acting as a cofactor in osteoblast function[128] or in the metabolism of homocysteine.[129] Elevated homocysteine concentrations are a strong risk factor for osteoporotic fractures in adulthood,[130] through disturbing bone matrix, or shifting bone metabolism towards bone resorption.[131] Lastly, higher intake of protein, a component of the organic bone matrix, and calcium and phosphorus, the main bone forming minerals, may lead to increased fetal bone mineral accrual through the direct effect of increased availability.[132,133]

Bone measures highly correlate with body weight. Results presented in this thesis showed a positive association of maternal smoking with childhood bone measures that disappeared after adjustment for current weight. It may have reflected the association of maternal smoking, or factors correlated to maternal smoking, with childhood adiposity

described in the previous paragraphs. In pre-pubertal children, a higher fat mass is thought to increase bone strength through larger mechanical strain on weight-bearing bones[134] or through endocrine stimulatory effects of fat mass.[135,136] Leptin, produced by adipocytes, has shown to stimulate periosteal growth through stimulating osteoblast differentiation.[137] Fat mass in prepubertal children is also related to serum levels of IGF-I and estrogen, both of which can influence skeletal growth.[78] Although the underlying mechanisms are not fully clarified, early nutrition and growth patterns may program later bone mineralization through altered regulation of bone metabolism.

METHODOLOGICAL CONSIDERATIONS

The studies presented in this thesis were conducted within the Generation R Study, a population-based prospective cohort study. In epidemiology, a *cohort* is defined as "any designated group of individuals who are followed or traced over a period of time".[138] Within a prospective cohort study individuals who share a common characteristic within a defined period, but who differ with respect to certain exposures under study, are followed over time to determine how these exposures affect the risk of developing the outcome of interest. In the Generation R Study the shared common characteristic of the participants is being born within a certain period of time and area. Thanks to its longitudinal data collection a temporal relation between multiple exposures and outcomes can be assessed. Recall bias are reduced by data collection at regular intervals. Disadvantages of prospective cohort studies are the fact that they are expensive to conduct, time-consuming and sensitive to bias that may harm the internal validity, such as selection bias, information bias and confounding. Therefore, with respect to the level of evidence, results from a cohort study are considered inferior to results from randomized clinical trials (RCT) and cannot prove causal relationships. Nevertheless, observational cohort studies are the best source for formulating sound research questions that can be addressed by RCT's, or for studying research questions that cannot be addressed by RCT's due to ethical reasons. In the following paragraphs the potential influence of the above mentioned bias of cohort studies will be discussed together with the approximations used to minimize their effects.

Selection bias

Selection bias is a systematic error and a consequence of the procedures used to select study participants or of factors that influence study participation.[138] Selective participation may affect generalizability of the study results to other populations. Moreover, when the association between exposure and outcome differs between those who participate and those who do not participate in the study, selective participation may impair inter-

nal validity of the study. As the association between exposure and outcome is usually unknown in nonparticipants, the presence of selection bias must be inferred. Within the Generation R Study, estimation of the precise number of eligible pregnant women in the study area was hampered by the lack of a pregnancy registry. Based on the number of children born in the study area during the inclusion period the estimated response rate was 61 %,[139] which is quite high compared to other birth cohorts.[140] As compared to people living in the study area, both the average household income as well as the educational level of participating mothers suggested a selection towards mothers with a higher socioeconomic status. Ethnic distribution was fairly comparable to the population of the study area.[141] The number of medical complications, such as preterm birth and low birth weight was lower among participants than expected from the Rotterdam population.[141] Selection towards a healthier study population typically occurs in large cohort studies. It may have led to reduced statistical power as a result of lower prevalence rates of adverse exposures and outcomes. Thanks to the prospective design of the study, selective participation is unlikely to be related to the specific associations studied within this thesis. On the other hand, its longitudinal design is particularly prone to the consequences of loss to follow-up and missing data. Hence, biased results in large cohort studies have been suggested to arise from selective loss to follow-up rather than selective participation at baseline.[140] In part of the studies presented in this thesis children were followed up until 6 years of age. Participation in the 6-year visit averaged 70%. Selective loss to follow-up may have occurred; since mothers who did not participate were lower educated, more often belonged to an ethnic minority and more often engaged in unhealthy lifestyle habits. Selective loss to follow-up may have led to biased effect estimates, yet to what extent is difficult to quantify. In order to reduce selection bias due to missing data multiple imputation was used to impute missing values in covariates. In Chapter 4.1 also missing growth variables (exposure) were imputed. Multiple imputation is a technique that fills in missing data based on a predictive model. This predictive model should and did include variables associated with the missing variables and the studied outcome.[142] When the predictive model describes the missing variables accurately and multiple datasets with imputed values are generated (≥5), the difference between the imputed values equals the uncertainty due to missing data. Hence, no extra certainty is created by applying this technique. By applying this technique, potential selection bias due to missing values in participant data were reduced under the assumption that data was missing at random.

Information bias

Information bias may arise from systematic error in the collection of information about or from study participants. This error, also referred to as misclassification, can be either differential or non-differential, depending on the underlying mechanism.[138] Misclassification of the exposure variable is differential when the misclassification is related to the studied

outcome. When unrelated, it is called random error or non-differential misclassification. Likewise, misclassification of the outcome is differential when related to the exposure, and non-differential when unrelated. Differential misclassification can either lead to over- or underestimation of effect estimates. Non-differential misclassification, however, leads to a dilution or underestimation of effect estimates. In the studies included in this thesis, information about or from participants was collected prospectively through postal questionnaires or through hands-on physical examinations performed at a dedicated research center, the hospital or at the Community Health Centers (*Consultatiebureau's*).

Questionnaires

In the majority of the studies presented in this thesis self-administered questionnaires were used to assess exposure of the fetus to maternal diet, parental smoking, or other parental or infant characteristics. Both the data collectors and the parents were unaware of the research questions under study, which makes differential misclassification of the exposure less likely. However, studying adverse lifestyle habits by self-report is prone to under- or over-reporting. Pregnant women may under-report their smoking behavior due to feeling it is socially unacceptable. As exposure to these lifestyle habits was assessed prior to measuring fetal growth and childhood body composition, under- or over reporting of lifestyle habits was unlikely to be related to the studied outcomes. Yet, non-differential misclassification of maternal smoking may have led to underestimated or attenuated results. Unfortunately, biomarkers of smoking, such as urinary cotinine excretion were not available within the Generation R Study. Nevertheless, studying biomarkers has not shown to be superior in reducing misclassification.[143]

Dietary assessment in epidemiological studies can be particularly challenging. Questioning respondents on the frequency and amount of consumption of regularly eaten foods by a food frequency questionnaire (FFQ) or a 24-hour recall has been the most commonly used method. It is relatively inexpensive and easy to administer. A disadvantage of these methods is its dependence on recall. In principal, recall bias, a form of differential misclassification, occurs when the outcome is measured prior to the exposure. In studies presented in this study the outcome was measured subsequent to the exposure. Yet, validation studies have shown that reported values from FFQs are subject to substantial error, in cohort studies in principle non-differential.[144] Especially overweight women tend to underestimate their intake. To overcome this issue, all dietary analyses were corrected for pre-pregnancy BMI. Energy adjustment may also help to resolve the issue of under- or over-reporting.[145] Overestimation of fish consumption for example, a generally considered healthy product, may have led to underestimation of effect estimates in a dose-response analysis. Studying biomarkers that describe nutritional status may help to reduce the issue of misclassification. Within the Generation R Study maternal PUFA-levels were not available at the time. Fortunately, biomarkers of vitamin B12, folic

acid levels and homocysteine were. Furthermore, prospectively collecting dietary data through keeping food records, with or without weighing of food items, would yield more precise estimates of dietary intake. However, due to the fact that it is very time-consuming and expensive, it is not suitable for implementation in large epidemiologic studies. Lastly, the issue of collinearity between nutrients has been described in detail in the previous paragraphs and is not expected to affect the interpretation of the results.

Measures of growth and body composition

Fetal growth was measured by ultrasound in each trimester at either of the two research centers. First trimester fetal growth measures were used to establish gestational age. A disadvantage of establishing gestational age by ultrasound is that variation in fetal growth in the first trimester of pregnancy is assumed to be zero, which may not be fully justified.[146,147] The fact that any already existing variation in fetal growth is neglected may lead to underestimation of effect estimates in early pregnancy. The accuracy of fetal growth estimation by ultrasound is much debated. In a recent systematic review of 42 studies that had measured fetal weight, measurements were shown to lack systematic error.[148] Nonetheless, a mean random error of 10% was determined. Therefore, in all studies including fetal growth, random error, or non-differential misclassification, may have yielded underestimated effects. Yet, establishing gestational age by ultrasound has been shown to be more accurate than calculations based on the last menstrual period.[149] Besides, in a previous study within the Generation R Study, 39% of pregnant women either had an unknown or irregular last menstrual period or had recently used oral contraceptives.[150] Birth anthropometrics and anthropometric measurements from 1 to 4 years of age were acquired from hospital records and community health centers, respectively. As these measurements were performed in clinical settings using standardized protocols carefully reviewed by the research staff, misclassification was assumed not to differ substantially from measurements performed in the research center.

Analysis of repeatedly measured growth is challenged by the high correlation between the growth measures within each individual. The choice of statistical methods with which to model repeatedly measured growth continues to be debated. Different methods, such as linear regression, fitted linear and polynomial equations or splines all have their merits and benefits.[151] For example, in Chapter 2.2, to study the existence of a critical period for the effect of maternal milk consumption on fetal growth, a natural cubic spline was fitted to the data. This technique allowed assessing non-linear associations that differ over time, yet requires the use of advanced statistical software. In Chapter 4.1 we used repeatedly measured growth as determinant instead of as outcome. In this situation, conditional growth modeling was used to identify the period most critical to bone mineral accrual, as it adequately considers correlation between follow-up measurements while

it is easy to apply and interpret. However, due to the fact that it does not consider a non-linear pattern of growth, it does not allow for precise interpolation.

Childhood body composition was measured by DXA, a widely used and well-validated technique to estimate fat mass and bone mass in children. The iDXA device (GE-Lunar, Madison, US) provides benefits of very low dose radiation (5-10 micro Sv), quick scan time (7 minutes), high precision and multiple measurement options.[152] Short-term imprecision, reflecting imprecision of the equipment and imprecision resulting from positioning and motion artefacts, is less than 1-2% for whole body measurements.[153] Long-term imprecision of the equipment used in the studies presented in this thesis was only 0.23% (measured daily by using a phantom). To reduce motion artefacts, children were carefully instructed to remain motionless prior and during the scanning procedure. Despite substantial efforts, motion artefacts affecting the measurements could not be fully prevented. Such type of measurement error is unrelated to the studied exposures; it will have led to non-differential misclassification and, hence, underestimated effect estimates.

A major disadvantage of the DXA technique is that body composition is estimated by a two-dimensional approach while it evaluates a three-dimensional structure.[153] Hence, when scanning the whole body, the measurement of bone area is hampered by the variation in depth and thickness of all bones throughout the body. Especially in a pediatric population undergoing periods of rapid growth, small changes in bone dimensions may have large impact on areal measurements. In growing bones, bone volume increases at a faster rate than bone area. As a result, areal bone density in growing bone will increase even if the volumetric density remains stable. Or, in children with relatively large bones, areal BMD can overestimate true volumetric BMD. To overcome this issue, all analysis in the studies presented in this thesis were corrected for age, height and weight, as advocated by the International Society for Clinical Densitometry (ISCD) for pediatric measurements.[154] Furthermore, all whole-body measurements were performed using total-body less head. As suggested by Prentice *et al.*,[155] but not agreed upon by others,[156] BMC was additionally adjusted for bone area in sensitivity analyses to further diminish possible size effects.

Due to its two-dimensional approach fat mass can only be measured at areas where no bone is present. Moreover, increased tissue depth leads to increased attenuation of the X-ray.[157] To overcome subsequent overestimation of fat mass and underestimation of lean mass, obese children were scanned by a using a higher dose of radiation. Alternative techniques to measure body fat mass have been the four-compartment model, multiple skinfold anthropometry, bioelectrical impedance analysis, and air displacement plethysmography.[158] None of these methods have proved to be more accurate than DXA. Besides, as they are time-consuming, technically demanding and require patient cooperation, those techniques are less suitable for children. Although CT provides accurate assessment of subcutaneous and visceral fat within the area imaged, it is expensive,

requires fairly high radiation doses and does not provide total body assessment.[158] MRI provides highly detailed images of fat and lean tissue, from which fat volume can be derived. As it is relatively expensive and very time consuming it was not applicable to the young children included in the studies presented in this thesis. It will, however, be of interest for use in older children.

Confounding

Confounding is a major issue in observational, non-randomized studies. A confounding factor is an extraneous factor that is both related to the studied determinant and outcome and interferes with the relationship between the determinant and outcome. Failure to account for this confounding factor may lead to spurious results. To deal with the issue of confounding, restriction, stratification, adjustment in multivariable models or propensity scores can be applied. Essentially, the goal of these methods is to make groups comparable with respect to the confounding variable. In the studies presented in this thesis adjustment was applied by multivariable models. Owing to the extensive data collection on many different topics over the years within the Generation R study, information about a wide range potential confounding factors was available. Potential confounding factors were mainly considered a confounder based on the previous literature, or a significant change in effect estimates. Only, the above mentioned methods used to reduce the potential impact of confounding reduce the impact of "measured confounding". Residual confounding due to unmeasured factors may still have influenced the results. Especially since consuming a healthy diet and not-smoking is likely to be related to a health-conscious lifestyle, the analysis of such data may be prone to the consequences of residual confounding. For example, no information was available on maternal physical activity during pregnancy. To reduce the impact of unmeasured confounding, socio-demographic and lifestyle-related variables were added to the models that were possibly related to and descriptive of unmeasured confounding. To do so, parental educational level, household income, marital status, folic acid supplement use, alcohol use, fruit and vegetable consumption, physical activity of the child, etc. were added. Second, when studying the influence of maternal smoking, effect estimates of maternal and paternal smoking were compared to disentangle intrauterine from shared familial factors.[65] In that way, paternal smoking was used as a negative control.

FUTURE RESEARCH

Fetal dietary and smoke exposure

The studies presented in this thesis showed associations between maternal diet, maternal smoking, fetal growth and body composition in childhood. Novel evidence was provided

for early determinants of fetal growth and childhood body composition. The epidemiological studies included in this thesis offer useful insight in potential etiological pathways. However, due to the limitations of its observational design, the existence of causality in these associations cannot be inferred. To establish causality a randomized controlled trial design is preferred and provides the highest degree of evidence. In a trial in which women with low habitual fish consumption were randomly assigned to continue their habitual diet or being provided with 2 portions of salmon per week, the latter effectively increased EPA and DHA intake.[159] Such a trial design could also be used to investigate whether maternal fish intake effectively enhances fetal growth, postnatal growth and later adiposity. Thus far, randomized clinical trials have shown a beneficial effect of fish oil supplementation on gestational duration, and indirectly on fetal growth.[160] Yet, whether such an effect can also be achieved through dietary fish intake remains uncertain. In a small trial of pregnant teens, counselling of consuming at least four dairy products per day effectively increased birth weight.[161] A similar study design or a trial design in which women are provided with milk tokens could be used to investigate the effectivity of increasing maternal milk consumption in a more representative population.

Due to ethical issues conducting an RCT is not always suitable. Assessing the influence of fetal smoke exposure in human trials is impossible. Instead, comparing effect sizes of maternal and paternal smoking exposure provides a way of disentangling intrauterine from confounding factors and was the approach used in this thesis. Another approach to deal with confounding factors would be the comparison of siblings, exposed to similar family-based confounders.[162] Or, using Mendelian randomization, a method that uses common genetic polymorphisms with a well-known, strong relation to the exposure as instruments to evaluate causality. As genetic material is randomly interchanged between maternal and paternal chromosomes when passed on from parents to offspring, the genotype distribution is assumed to be unrelated to environmental confounders. Under the condition that the genotype(s) only affects the outcome via the exposure of interest, the genetic marker(s) constitute(s) an instrumental variable to examine a causal relationship. Previously, a genotype strongly associated with smoking behavior was used as a proxy for fetal smoke exposure to study its causal relation with offspring smoking initiation.[163] Such an approach may also help to study the existence of a causal relation between maternal smoking and later offspring health. It is, however, less suitable for studying the effects of maternal diet as genetic variants describing maternal dietary intake adequately have not yet been identified.

The studies part of this thesis were not designed to thoroughly study the potential mechanisms by which fetal nutrition or smoke exposure act in the etiology of fetal growth restriction, overweight or low bone mineral density. Therefore, further research on the underlying mechanisms is still necessary. Measuring biomarkers such as levels of *n*-3 PUFA, contaminants and cotinine, or hormonal levels such as IGF-I, leptin and cortisol

may offer an improved method to study the mechanisms explaining the effects of early exposures on offspring growth patterns. Furthermore, it will be interesting to study the effect of longitudinal diet and smoke exposure on (skeletal) growth and adiposity. In the study presented in this thesis, the associations between maternal diet and childhood bone mass were not better explained by longitudinal dietary exposures through infant diet. However, this analysis was only performed in a small subgroup and diet in later life may play a more prominent role. Furthermore, to help identify a specific diet beneficial to bone development dietary pattern analysis, a technique that estimates the influence of overall diet based on the correlation between consumed food items, may gather results that may be easier translated into dietary measures. Besides diet other determinants of body composition seem promising and worth further study, such as the potential role of maternal vitamin D levels. Finally, epigenetics has gained increasing interest as a potential underlying mechanism in the developmental origins hypothesis.[164] Until recently, epigenetic variation was studied by a candidate gene approach. However, lately, new technologies to measure DNA methylation have been developed, enabling epigenome-wide association studies (EWAS).[165] The first EWAS identified epigenetic variation related to adolescent and adult BMI among African- and European-Americans.[166-168] Although results, thus far, are promising, more studies are needed to obtain insight in the magnitude of the role of epigenetics in growth, fat mass distribution and bone health, and the existence of critical periods for epigenetic modifications. Especially, longitudinal DNA methylation measurements will help to gain more insight on time-specific effects and will enable distinguishing cause and effect.

Growth outcomes

In the studies presented in this thesis, first trimester fetal growth measures were used to establish gestational age. As described, a drawback of establishing gestational age by ultrasound is that variation in fetal growth before the first ultrasound examination is assumed to be zero. Any already existing variation in fetal growth was thereby erased. Yet, maternal characteristics were already suggested to influence growth propensities in the first trimester.[146] In order to study the potential influences of maternal diet on embryonic growth studies should be focused on women with a reliable last menstrual period. Also, although body composition tends to track in childhood and adulthood, the findings of this thesis need to be followed over time. Recently, results were published from a study in Denmark that investigated the long-term effect of maternal milk consumption. Although not statistically significant, it provided some evidence for an effect on offspring's height, as well as IGF-I and insulin blood levels 20 years later.[29] More prospective studies with long follow-up time are needed to confirm this long-term effect and to investigate whether the described associations of maternal diet and smoking with childhood body composition will last into adult life and old age.

A major disadvantage of the DXA technique is its estimation of bone mineral density by a two-dimensional approach. Unlike DXA, quantitative computed tomography (QCT), a low dose CT scan, is able to accurately measure volumetric bone mineral density. It also distinguishes cortical from trabecular bone and it can assess geometric properties of long bones. In pediatric research, peripheral QCT (pQCT) devices are becoming more widely used to measure bone mineral density of the radius, tibia, or femur. Thus far, both consensus on the optimal measurement techniques and reference values are lacking. Further exploration of this technique, or high-resolution peripheral QCT (HR-pQCT), capable of assessing bone microstructure, may help to identify determinants of bone health irrespective of these methodological issues.

CLINICAL IMPLICATIONS

In the studies presented in this thesis, maternal, paternal, fetal and infant characteristics were related to growth, adiposity and bone mass in early life. The findings of this thesis suggest maternal diet, in particular maternal milk consumption, may promote fetal growth from the third trimester onwards. Pregnant mothers should therefore be encouraged to drink 2-3 glasses of milk per day as recommended by the Netherlands Nutrition Center,[21] especially when growth of their unborn child appears to be below average. According to the findings of this thesis, maternal fish consumption does not promote fetal growth. However, a recent meta-analysis, including data presented in this thesis, showed maternal fish consumption decreased the risk of preterm birth and slightly increased birth weight. Fish is the main dietary source of n-3 PUFA. As recommended by the Netherlands Nutrition Center, pregnant women should be encouraged to consume fish at least twice weekly, preferably fatty fish.[21] In view of the findings presented in this thesis as well as the existing literature, shellfish intake should be minimized during pregnancy. Furthermore, the findings of this thesis indicate that several parental, fetal and infant characteristics, such as parental BMI and socio-economic status, birth weight, and fetal and infant weight, predict the risk of early overweight development. A risk score based on these characteristics should be developed and may help identifying children for targeted overweight interventions in preventive care, for instance the Community Health Centers (*Consultatiebureau's*). Shared familial factors related to maternal smoking during pregnancy, rather than maternal smoking itself, seemed to adversely affect childhood adiposity. However, given the existing evidence concerning negative health effects of (passive) tobacco exposure,[72] parents should be strongly discouraged to smoke during pregnancy. Finally, maternal diet in the first trimester as well as fetal and infant growth patterns were associated with offspring bone health. Especially, a diet rich in protein, minerals, such as phosphorus and calcium, and vitamin B12 may support offspring bone

development. Meat, poultry, fish, eggs, milk, milk products, green leafy vegetables, whole grain, nuts, and legumes are good sources of protein, phosphorus, calcium and vitamin B12, and should therefore be well represented in a pregnant women's diet.

These results highlight the importance of a healthy lifestyle during pregnancy, assigning a key role to preventive care from early pregnancy onwards. Thus, to optimize fetal growth and later body composition in the offspring, achieving a healthy body weight prior to pregnancy as well as consuming a healthy diet during pregnancy should be strongly encouraged in antenatal care. As antenatal care does not usually start before the 8th gestational week, the general public as well as general practitioners, midwifes and obstetricians should be educated on the importance of achieving a healthy weight, consuming a healthy diet and smoking cessation during the periconceptional period. The preconception period as well as pregnancy may be periods in which women are more motivated to make lifestyle changes. Developing preventive strategies focused on consuming a healthy diet and smoking cessation for pregnant women, or women in the preconception period, may help to improve offspring body composition and prevent future overweight and osteoporosis.

CONCLUSION

The findings of this thesis suggest maternal dietary factors during pregnancy influence fetal growth, while maternal diet and nutritional status, as well as subsequent fetal and infant growth patterns may influence childhood adiposity and bone mass. Shared familial factors related to maternal smoking behavior, rather than maternal smoking itself, seemed to lead to increased adiposity and adverse body fat distribution in childhood. This thesis highlights the importance of engaging in a healthy lifestyle during pregnancy. Structural and functional adaptations in response to a suboptimal fetal environment may have lasting effects on the risk of overweight and low bone mineral density in later life. Therefore, prevention of future overweight and osteoporosis should start from early life onwards.

Future studies should focus on elucidating the underlying mechanisms and confirming the long-term consequences of these findings. Studies in this thesis also repeatedly demonstrated the large impact residual confounding may have in epidemiological studies focused on lifestyle-related determinants. Hence, it clearly stresses the need of randomized clinical trials to study the relation between consumption of specific food products, such as fish or milk, and fetal growth or childhood body composition. Such studies will yield results solid enough to translate into preventive health care policies.

REFERENCES

1. Barker DJ. The malnourished baby and infant. *Br Med Bull*. 2001,60:69-88.
2. Grieger JA, Clifton VL. A review of the impact of dietary intakes in human pregnancy on infant birthweight. *Nutrients*. 2015,*7*(1):153-78.
3. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. n-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. *Am J Clin Nutr*. 2006,83(6 Suppl):1526S-35S.
4. Dommels YE, Alink GM, van Bladeren PJ, van Ommen B. Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies. *Environ Toxicol Pharmacol*. 2002,11(3-4):297-308.
5. Groh-Wargo S, Jacobs J, Auestad N, O'Connor DL, Moore JJ, Lerner E. Body composition in preterm infants who are fed long-chain polyunsaturated fatty acids: a prospective, randomized, controlled trial. *Pediatr Res*. 2005,*57*(5 Pt 1):712-8.
6. Lange AP. Induction of labour. *Danish medical bulletin*. 1984,31(2):89-108.
7. Olsen SF, Hansen HS, Sorensen TI, Jensen B, Secher NJ, Sommer S, et al. Intake of marine fat, rich in (n-3)-polyunsaturated fatty acids, may increase birthweight by prolonging gestation. *Lancet*. 1986,2(8503):367-9.
8. Andersen HJ, Andersen LF, Fuchs AR. Diet, pre-eclampsia, and intrauterine growth retardation. *Lancet*. 1989,1(8647):1146.
9. Olsen SF, Hansen HS, Sommer S, Jensen B, Sorensen TI, Secher NJ, et al. Gestational age in relation to marine n-3 fatty acids in maternal erythrocytes: a study of women in the Faroe Islands and Denmark. *Am J Obstet Gynecol*. 1991,164(5 Pt 1):1203-9.
10. Olsen SF, Joensen HD. High liveborn birth weights in the Faroes: a comparison between birth weights in the Faroes and in Denmark. *J Epidemiol Community Health*. 1985,39(1):27-32.
11. Olsen SF, Grandjean P, Weihe P, Videro T. Frequency of seafood intake in pregnancy as a determinant of birth weight: evidence for a dose dependent relationship. *J Epidemiol Community Health*. 1993,*47*(6):436-40.
12. Olsen SF, Secher NJ. Low consumption of seafood in early pregnancy as a risk factor for preterm delivery: prospective cohort study. *Bmj*. 2002,324(7335):447.
13. Rogers I, Emmett P, Ness A, Golding J. Maternal fish intake in late pregnancy and the frequency of low birth weight and intrauterine growth retardation in a cohort of British infants. *J Epidemiol Community Health*. 2004,58(6):486-92.
14. Drouillet P, Kaminski M, De Lauzon-Guillain B, Forhan A, Ducimetiere P, Schweitzer M, et al. Association between maternal seafood consumption before pregnancy and fetal growth: evidence for an association in overweight women. The EDEN mother-child cohort. *Paediatr Perinat Epidemiol*. 2009,23(1):76-86.
15. Elwood PC, Haley TJ, Hughes SJ, Sweetnam PM, Gray OP, Davies DP. Child growth (0-5 years), and the effect of entitlement to a milk supplement. *Arch Dis Child*. 1981,56(11):831-5.
16. Halldorsson TI, Meltzer HM, Thorsdottir I, Knudsen V, Olsen SF. Is high consumption of fatty fish during pregnancy a risk factor for fetal growth retardation? A study of 44,824 Danish pregnant women. *American journal of epidemiology*. 2007,166(6):687-96.
17. Ramon R, Ballester F, Aguinagalde X, Amurrio A, Vioque J, Lacasana M, et al. Fish consumption during pregnancy, prenatal mercury exposure, and anthropometric measures at birth in a prospective mother-infant cohort study in Spain. *Am J Clin Nutr*. 2009,90(4):1047-55.
18. Mendez MA, Plana E, Guxens M, Foradada Morillo CM, Albareda RM, Garcia-Esteban R, et al. Seafood consumption in pregnancy and infant size at birth: results from a prospective Spanish cohort. *J Epidemiol Community Health*. 2010,64(3):216-22.
19. Guldner L, Monfort C, Rouget F, Garlantezec R, Cordier S. Maternal fish and shellfish intake and pregnancy outcomes: a prospective cohort study in Brittany, France. *Environ Health*. 2007,6:33.

20. Leventakou V, Roumeliotaki T, Martinez D, Barros H, Brantsaeter AL, Casas M, et al. Fish intake during pregnancy, fetal growth, and gestational length in 19 European birth cohort studies. *Am J Clin Nutr*. 2014,99(3):506-16.
21. Stafleu A, Postma-Smeets A, van der Vossen W, Peters S. Voedingscentrum Factsheet Voeding & Zwangerschap. 2015.
22. Wiley AS. Cow milk consumption, insulin-like growth factor-I, and human biology: a life history approach. *American journal of human biology : the official journal of the Human Biology Council*. 2012,24(2):130-8.
23. Nevo: Dutch food composition database 2006 [Internet]. Netherlands Nutrition Centre, The Hague, The Netherlands. 2006.
24. Ganpule A, Yajnik CS, Fall CH, Rao S, Fisher DJ, Kanade A, et al. Bone mass in Indian children--relationships to maternal nutritional status and diet during pregnancy: the Pune Maternal Nutrition Study. *J Clin Endocrinol Metab*. 2006,91(8):2994-3001.
25. Moore VM, Davies MJ, Willson KJ, Worsley A, Robinson JS. Dietary composition of pregnant women is related to size of the baby at birth. *J Nutr*. 2004,134(7):1820-6.
26. Mannion CA, Gray-Donald K, Koski KG. Association of low intake of milk and vitamin D during pregnancy with decreased birth weight. *CMAJ*. 2006,174(9):1273-7.
27. Ludvigsson JF, Ludvigsson J. Milk consumption during pregnancy and infant birthweight. *Acta Paediatr*. 2004,93(11):1474-8.
28. Olsen SF, Halldorsson TI, Willett WC, Knudsen VK, Gillman MW, Mikkelsen TB, et al. Milk consumption during pregnancy is associated with increased infant size at birth: prospective cohort study. *Am J Clin Nutr*. 2007,86(4):1104-10.
29. Hrolfsdottir L, Rytter D, Hammer Bech B, Brink Henriksen T, Danielsen I, Steingrimsdottir L, et al. Maternal milk consumption, birth size and adult height of offspring: a prospective cohort study with 20 years of follow-up. *Eur J Clin Nutr*. 2013,67(10):1036-41.
30. Wennberg M, Tornevi A, Johansson I, Hornell A, Norberg M, Bergdahl IA. Diet and lifestyle factors associated with fish consumption in men and women: a study of whether gender differences can result in gender-specific confounding. *Nutr J*. 2012,11:101.
31. Welch AA, Lund E, Amiano P, Dorronsoro M, Brustad M, Kumle M, et al. Variability of fish consumption within the 10 European countries participating in the European Investigation into Cancer and Nutrition (EPIC) study. *Public Health Nutr*. 2002,5(6B):1273-85.
32. de Goede J, Geleijnse JM, Boer JM, Kromhout D, Verschuren WM. Marine (n-3) fatty acids, fish consumption, and the 10-year risk of fatal and nonfatal coronary heart disease in a large population of Dutch adults with low fish intake. *J Nutr*. 2010,140(5):1023-8.
33. Basisrapportage en tabellen voedselconsumptiepeiling 1997-1998. [Internet]. TNO. 1998.
34. De Mul A, Bakker MI, Zeilmaker MJ, Traag WA, Leeuwen SP, Hoogenboom RL, et al. Dietary exposure to dioxins and dioxin-like PCBs in The Netherlands anno 2004. *Regul Toxicol Pharmacol*. 2008,51(3):278-87.
35. Baars AJ, Bakker MI, Baumann RA, Boon PE, Freijer JI, Hoogenboom LA, et al. Dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs: occurrence and dietary intake in The Netherlands. *Toxicology letters*. 2004,151(1):51-61.
36. Mahaffey KR. Fish and shellfish as dietary sources of methylmercury and the omega-3 fatty acids, eicosahexaenoic acid and docosahexaenoic acid: risks and benefits. *Environ Res*. 2004,95(3):414-28.
37. Barr DB, Bishop A, Needham LL. Concentrations of xenobiotic chemicals in the maternal-fetal unit. *Reproductive toxicology*. 2007,23(3):260-6.
38. Stern AH, Smith AE. An assessment of the cord blood:maternal blood methylmercury ratio: implications for risk assessment. *Environ Health Perspect*. 2003,111(12):1465-70.

39. Patandin S, Koopman-Esseboom C, de Ridder MA, Weisglas-Kuperus N, Sauer PJ. Effects of environmental exposure to polychlorinated biphenyls and dioxins on birth size and growth in Dutch children. *Pediatr Res*. 1998,44(4):538-45.
40. Halldorsson TI, Thorsdottir I, Meltzer HM, Nielsen F, Olsen SF. Linking exposure to polychlorinated biphenyls with fatty fish consumption and reduced fetal growth among Danish pregnant women: a cause for concern? *American journal of epidemiology*. 2008,168(8):958-65.
41. Molgaard C, Larnkjaer A, Arnberg K, Michaelsen KF. Milk and growth in children: effects of whey and casein. *Nestle Nutrition workshop series Paediatric programme*. 2011,67:67-78.
42. Buppasiri P, Lumbiganon P, Thinkhamrop J, Ngamjarus C, Laopaiboon M, Medley N. Calcium supplementation (other than for preventing or treating hypertension) for improving pregnancy and infant outcomes. *The Cochrane database of systematic reviews*. 2015,2:CD007079.
43. Hoppe C, Molgaard C, Dalum C, Vaag A, Michaelsen KF. Differential effects of casein versus whey on fasting plasma levels of insulin, IGF-1 and IGF-1/IGFBP-3: results from a randomized 7-day supplementation study in prepubertal boys. *Eur J Clin Nutr*. 2009,63(9):1076-83.
44. Hayati AR, Cheah FC, Tan AE, Tan GC. Insulin-like growth factor-1 receptor expression in the placentae of diabetic and normal pregnancies. *Early Hum Dev*. 2007,83(1):41-6.
45. Boyne MS, Thame M, Bennett FI, Osmond C, Miell JP, Forrester TE. The relationship among circulating insulin-like growth factor (IGF)-I, IGF-binding proteins-1 and -2, and birth anthropometry: a prospective study. *J Clin Endocrinol Metab*. 2003,88(4):1687-91.
46. Hoppe C, Molgaard C, Vaag A, Barkholt V, Michaelsen KF. High intakes of milk, but not meat, increase s-insulin and insulin resistance in 8-year-old boys. *Eur J Clin Nutr*. 2005,59(3):393-8.
47. Ben-Shlomo Y, Holly J, McCarthy A, Savage P, Davies D, Davey Smith G. Prenatal and postnatal milk supplementation and adult insulin-like growth factor I: long-term follow-up of a randomized controlled trial. *Cancer Epidemiol Biomarkers Prev*. 2005,14(5):1336-9.
48. Robinson SM, Crozier SR, Harvey NC, Barton BD, Law CM, Godfrey KM, et al. Modifiable early-life risk factors for childhood adiposity and overweight: an analysis of their combined impact and potential for prevention. *Am J Clin Nutr*. 2015,101(2):368-75.
49. Gillman MW, Rifas-Shiman SL, Kleinman K, Oken E, Rich-Edwards JW, Taveras EM. Developmental origins of childhood overweight: potential public health impact. *Obesity*. 2008,16(7):1651-6.
50. Gillman MW, Ludwig DS. How early should obesity prevention start? *N Engl J Med*. 2013,369(23):2173-5.
51. Dubois L, Girard M. Early determinants of overweight at 4.5 years in a population-based longitudinal study. *Int J Obes (Lond)*. 2006,30(4):610-7.
52. Kitsantas P, Gaffney KF. Risk profiles for overweight/obesity among preschoolers. *Early Hum Dev*. 2010,86(9):563-8.
53. Huus K, Ludvigsson JF, Enskar K, Ludvigsson J. Risk factors in childhood obesity-findings from the All Babies In Southeast Sweden (ABIS) cohort. *Acta Paediatr*. 2007,96(9):1321-5.
54. Beyerlein A, Toschke AM, von Kries R. Risk factors for childhood overweight: shift of the mean body mass index and shift of the upper percentiles: results from a cross-sectional study. *Int J Obes (Lond)*. 2010,34(4):642-8.
55. Jouret B, Ahluwalia N, Cristini C, Dupuy M, Negre-Pages L, Grandjean H, et al. Factors associated with overweight in preschool-age children in southwestern France. *Am J Clin Nutr*. 2007,85(6):1643-9.
56. Weng SF, Redsell SA, Swift JA, Yang M, Glazebrook CP. Systematic review and meta-analyses of risk factors for childhood overweight identifiable during infancy. *Arch Dis Child*. 2012,97(12):1019-26.
57. Kramer MS, Matush L, Vanilovich I, Platt RW, Bogdanovich N, Sevkovskaya Z, et al. A randomized breast-feeding promotion intervention did not reduce child obesity in Belarus. *J Nutr*. 2009,139(2):417S-21S.

58. Martin RM, Patel R, Kramer MS, Vilchuck K, Bogdanovich N, Sergeichick N, et al. Effects of promoting longer-term and exclusive breastfeeding on cardiometabolic risk factors at age 11.5 years: a cluster-randomized, controlled trial. *Circulation*. 2014,129(3):321-9.
59. Smedberg J, Lupattelli A, Mardby AC, Nordeng H. Characteristics of women who continue smoking during pregnancy: a cross-sectional study of pregnant women and new mothers in 15 European countries. *BMC Pregnancy Childbirth*. 2014,14:213.
60. Jaddoe VW, Troe EJ, Hofman A, Mackenbach JP, Moll HA, Steegers EA, et al. Active and passive maternal smoking during pregnancy and the risks of low birthweight and preterm birth: the Generation R Study. *Paediatr Perinat Epidemiol*. 2008,22(2):162-71.
61. Durmus B, Kruithof CJ, Gillman MH, Willemsen SP, Hofman A, Raat H, et al. Parental smoking during pregnancy, early growth, and risk of obesity in preschool children: the Generation R Study. *Am J Clin Nutr*. 2011,94(1):164-71.
62. Beyerlein A, Ruckinger S, Toschke AM, Schaffrath Rosario A, von Kries R. Is low birth weight in the causal pathway of the association between maternal smoking in pregnancy and higher BMI in the offspring? *Eur J Epidemiol*. 2011,26(5):413-20.
63. Ruckinger S, Beyerlein A, Jacobsen G, von Kries R, Vik T. Growth in utero and body mass index at age 5 years in children of smoking and non-smoking mothers. *Early Hum Dev*. 2010,86(12): 773-7.
64. Oken E, Levitan EB, Gillman MW. Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J Obes (Lond)*. 2008,32(2):201-10.
65. Smith GD. Assessing intrauterine influences on offspring health outcomes: can epidemiological studies yield robust findings? *Basic Clin Pharmacol Toxicol*. 2008,102(2):245-56.
66. Riedel C, Schonberger K, Yang S, Koshy G, Chen YC, Gopinath B, et al. Parental smoking and childhood obesity: higher effect estimates for maternal smoking in pregnancy compared with paternal smoking–a meta-analysis. *Int J Epidemiol*. 2014,43(5):1593-606.
67. Ino T. Maternal smoking during pregnancy and offspring obesity: meta-analysis. *Pediatr Int*. 2010,52(1):94-9.
68. Kaul S, Rothney MP, Peters DM, Wacker WK, Davis CE, Shapiro MD, et al. Dual-energy X-ray absorptiometry for quantification of visceral fat. *Obesity*. 2012,20(6):1313-8.
69. Leary SD, Smith GD, Rogers IS, Reilly JJ, Wells JC, Ness AR. Smoking during pregnancy and offspring fat and lean mass in childhood. *Obesity*. 2006,14(12):2284-93.
70. Syme C, Abrahamowicz M, Mahboubi A, Leonard GT, Perron M, Richer L, et al. Prenatal exposure to maternal cigarette smoking and accumulation of intra-abdominal fat during adolescence. *Obesity*. 2010,18(5):1021-5.
71. Leary SD, Lawlor DA, Davey Smith G, Brion MJ, Ness AR. Behavioural early-life exposures and body composition at age 15 years. *Nutr Diabetes*. 2015,5:e150.
72. Zhou S, Rosenthal DG, Sherman S, Zelikoff J, Gordon T, Weitzman M. Physical, behavioral, and cognitive effects of prenatal tobacco and postnatal secondhand smoke exposure. *Current problems in pediatric and adolescent health care*. 2014,44(8):219-41.
73. McMillen IC, MacLaughlin SM, Muhlhausler BS, Gentili S, Duffield JL, Morrison JL. Developmental origins of adult health and disease: the role of periconceptional and foetal nutrition. *Basic Clin Pharmacol Toxicol*. 2008,102(2):82-9.
74. Dabelea D, Crume T. Maternal environment and the transgenerational cycle of obesity and diabetes. *Diabetes*. 2011,60(7):1849-55.
75. Sobal J. Obesity and socioeconomic status: a framework for examining relationships between physical and social variables. *Medical anthropology*. 1991,13(3):231-47.
76. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol*. 2010,25(4):669-77.

77. Reilly JJ, Dorosty AR, Emmett PM, Avon Longitudinal Study of P, Childhood Study T. Identification of the obese child: adequacy of the body mass index for clinical practice and epidemiology. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 2000,24(12):1623-7.
78. Garnett SP, Hogler W, Blades B, Baur LA, Peat J, Lee J, et al. Relation between hormones and body composition, including bone, in prepubertal children. *Am J Clin Nutr*. 2004,80(4):966-72.
79. Murphy MJ, Metcalf BS, Voss LD, Jeffery AN, Kirkby J, Mallam KM, et al. Girls at five are intrinsically more insulin resistant than boys: The Programming Hypotheses Revisited–The EarlyBird Study (EarlyBird 6). *Pediatrics*. 2004,113(1 Pt 1):82-6.
80. Petridou E, Mantzoros CS, Belechri M, Skalkidou A, Dessypris N, Papathoma E, et al. Neonatal leptin levels are strongly associated with female gender, birth length, IGF-I levels and formula feeding. *Clin Endocrinol (Oxf)*. 2005,62(3):366-71.
81. Wisniewski AB, Chernausek SD. Gender in childhood obesity: family environment, hormones, and genes. *Gender medicine*. 2009,6 Suppl 1:76-85.
82. Moorcroft KE, Marshall JL, McCormick FM. Association between timing of introducing solid foods and obesity in infancy and childhood: a systematic review. *Matern Child Nutr*. 2011,7(1):3-26.
83. Ong KK, Emmett PM, Noble S, Ness A, Dunger DB, Team AS. Dietary energy intake at the age of 4 months predicts postnatal weight gain and childhood body mass index. *Pediatrics*. 2006,117(3):e503-8.
84. Huh SY, Rifas-Shiman SL, Taveras EM, Oken E, Gillman MW. Timing of solid food introduction and risk of obesity in preschool-aged children. *Pediatrics*. 2011,127(3):e544-51.
85. Heinig MJ, Nommsen LA, Peerson JM, Lonnerdal B, Dewey KG. Intake and growth of breast-fed and formula-fed infants in relation to the timing of introduction of complementary foods: the DARLING study. Davis Area Research on Lactation, Infant Nutrition and Growth. *Acta Paediatr*. 1993,82(12):999-1006.
86. Tai CC, Ding ST. N-3 polyunsaturated fatty acids regulate lipid metabolism through several inflammation mediators: mechanisms and implications for obesity prevention. *J Nutr Biochem*. 2010,21(5):357-63.
87. Rodriguez G, Iglesia I, Bel-Serrat S, Moreno LA. Effect of n-3 long chain polyunsaturated fatty acids during the perinatal period on later body composition. *Br J Nutr*. 2012,107 Suppl 2: S117-28.
88. Bruin JE, Gerstein HC, Holloway AC. Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicological sciences : an official journal of the Society of Toxicology*. 2010,116(2):364-74.
89. Stone WL, Bailey B, Khraisha N. The pathophysiology of smoking during pregnancy: a systems biology approach. *Front Biosci (Elite Ed)*. 2014,6:318-28.
90. Smith GD. Negative control exposures in epidemiologic studies. *Epidemiology*. 2012,23(2): 350-1; author reply 1-2.
91. Eliopoulos C, Klein J, Phan MK, Knie B, Greenwald M, Chitayat D, et al. Hair concentrations of nicotine and cotinine in women and their newborn infants. *JAMA*. 1994,271(8):621-3.
92. Florescu A, Ferrence R, Einarson TR, Selby P, Kramer M, Woodruff S, et al. Reference values for hair cotinine as a biomarker of active and passive smoking in women of reproductive age, pregnant women, children, and neonates: systematic review and meta-analysis. *Ther Drug Monit*. 2007,29(4):437-46.
93. von Kries R, Bolte G, Baghi L, Toschke AM, Group GMES. Parental smoking and childhood obesity–is maternal smoking in pregnancy the critical exposure? *Int J Epidemiol*. 2008,37(1): 210-6.
94. Burke V, Gracey MP, Milligan RA, Thompson C, Taggart AC, Beilin LJ. Parental smoking and risk factors for cardiovascular disease in 10- to 12-year-old children. *J Pediatr*. 1998,133(2): 206-13.

95. Johnson RK, Wang MQ, Smith MJ, Connolly G. The association between parental smoking and the diet quality of low-income children. *Pediatrics*. 1996,97(3):312-7.
96. Hernandez CJ, Beaupre GS, Carter DR. A theoretical analysis of the relative influences of peak BMD, age-related bone loss and menopause on the development of osteoporosis. *Osteoporos Int*. 2003,14(10):843-7.
97. Cooper C, Westlake S, Harvey N, Dennison E. Developmental origins of osteoporotic fracture. *Adv Exp Med Biol*. 2009,639:217-36.
98. Baird J, Kurshid MA, Kim M, Harvey N, Dennison E, Cooper C. Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis. *Osteoporos Int*. 2011,22(5): 1323-34.
99. Schlussel MM, Dos Santos Vaz J, Kac G. Birth weight and adult bone mass: a systematic literature review. *Osteoporos Int*. 2010,21(12):1981-91.
100. Harvey NC, Mahon PA, Kim M, Cole ZA, Robinson SM, Javaid K, et al. Intrauterine growth and postnatal skeletal development: findings from the Southampton Women's Survey. *Paediatr Perinat Epidemiol*. 2012,26(1):34-44.
101. Taal HR, Vd Heijden AJ, Steegers EA, Hofman A, Jaddoe VW. Small and large size for gestational age at birth, infant growth, and childhood overweight. *Obesity*. 2013,21(6):1261-8.
102. Jones G, Riley MD, Dwyer T. Maternal diet during pregnancy is associated with bone mineral density in children: a longitudinal study. *Eur J Clin Nutr*. 2000,54(10):749-56.
103. Tobias JH, Steer CD, Emmett PM, Tonkin RJ, Cooper C, Ness AR, et al. Bone mass in childhood is related to maternal diet in pregnancy. *Osteoporos Int*. 2005,16(12):1731-41.
104. Yin J, Dwyer T, Riley M, Cochrane J, Jones G. The association between maternal diet during pregnancy and bone mass of the children at age 16. *Eur J Clin Nutr*. 2010,64(2):131-7.
105. Shapiro R, Heaney RP. Co-dependence of calcium and phosphorus for growth and bone development under conditions of varying deficiency. *Bone*. 2003,32(5):532-40.
106. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med*. 1999,340(19): 1449-54.
107. Martinez-Mesa J, Restrepo-Mendez MC, Gonzalez DA, Wehrmeister FC, Horta BL, Domingues MR, et al. Life-course evidence of birth weight effects on bone mass: systematic review and meta-analysis. *Osteoporos Int*. 2012.
108. Jones G, Riley M, Dwyer T. Maternal smoking during pregnancy, growth, and bone mass in prepubertal children. *J Bone Miner Res*. 1999,14(1):146-51.
109. Godfrey K, Walker-Bone K, Robinson S, Taylor P, Shore S, Wheeler T, et al. Neonatal bone mass: influence of parental birthweight, maternal smoking, body composition, and activity during pregnancy. *J Bone Miner Res*. 2001,16(9):1694-703.
110. Harvey N, K. JM, Arden N. K., Poole JR, Crozier SR, Robinson SM, et al. Maternal predictors of neonatal bone size and geometry: the Southampton Women's Survey. *Journal of Developmental Origins of Health and Disease*. 2010,1(1):35-41.
111. Yan C, Avadhani NG, Iqbal J. The effects of smoke carcinogens on bone. *Curr Osteoporos Rep*. 2011,9(4):202-9.
112. Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. *Clinical science*. 2007,113(5):233-41.
113. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev*. 2007,83(11):699-706.
114. Macdonald-Wallis C, Tobias JH, Davey Smith G, Lawlor DA. Parental smoking during pregnancy and offspring bone mass at age 10 years: findings from a prospective birth cohort. *Osteoporos Int*. 2011,22(6):1809-19.
115. Setia S, Sridhar MG. Changes in GH/IGF-1 axis in intrauterine growth retardation: consequences of fetal programming? *Horm Metab Res*. 2009,41(11):791-8.

116. Devlin MJ, Bouxsein ML. Influence of pre- and peri-natal nutrition on skeletal acquisition and maintenance. *Bone*. 2012,50(2):444-51.
117. Goodfellow LR, Cooper C, Harvey NC. Regulation of placental calcium transport and offspring bone health. *Front Endocrinol (Lausanne)*. 2011,2:3.
118. Javaid MK, Godfrey KM, Taylor P, Shore SR, Breier B, Arden NK, et al. Umbilical venous IGF-1 concentration, neonatal bone mass, and body composition. *J Bone Miner Res*. 2004,19(1): 56-63.
119. van Coeverden SC, Netelenbos JC, de Ridder CM, Roos JC, Popp-Snijders C, Delemarre-van de Waal HA. Bone metabolism markers and bone mass in healthy pubertal boys and girls. *Clin Endocrinol (Oxf)*. 2002,57(1):107-16.
120. Lillycrop KA, Slater-Jefferies JL, Hanson MA, Godfrey KM, Jackson AA, Burdge GC. Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr*. 2007,97(6):1064-73.
121. Verkauskiene R, Jaquet D, Deghmoun S, Chevenne D, Czernichow P, Levy-Marchal C. Smallness for gestational age is associated with persistent change in insulin-like growth factor I (IGF-I) and the ratio of IGF-I/IGF-binding protein-3 in adulthood. *The Journal of clinical endocrinology and metabolism*. 2005,90(10):5672-6.
122. Cianfarani S, Geremia C, Scott CD, Germani D. Growth, IGF system, and cortisol in children with intrauterine growth retardation: is catch-up growth affected by reprogramming of the hypothalamic-pituitary-adrenal axis? *Pediatr Res*. 2002,51(1):94-9.
123. Remer T, Boye KR, Hartmann MF, Neu C, Schoenau E, Manz F, et al. Adrenal steroid hormones and metaphyseal bone in children. *Hormone research*. 2004,62(5):221-6.
124. Remer T, Boye KR, Hartmann M, Neu CM, Schoenau E, Manz F, et al. Adrenarche and bone modeling and remodeling at the proximal radius: weak androgens make stronger cortical bone in healthy children. *J Bone Miner Res*. 2003,18(8):1539-46.
125. Jaquet D, Leger J, Levy-Marchal C, Oury JF, Czernichow P. Ontogeny of leptin in human fetuses and newborns: effect of intrauterine growth retardation on serum leptin concentrations. *J Clin Endocrinol Metab*. 1998,83(4):1243-6.
126. Javaid MK, Godfrey KM, Taylor P, Robinson SM, Crozier SR, Dennison EM, et al. Umbilical cord leptin predicts neonatal bone mass. *Calcif Tissue Int*. 2005,76(5):341-7.
127. Jaquet D, Leger J, Tabone MD, Czernichow P, Levy-Marchal C. High serum leptin concentrations during catch-up growth of children born with intrauterine growth retardation. *J Clin Endocrinol Metab*. 1999,84(6):1949-53.
128. Kim GS, Kim CH, Park JY, Lee KU, Park CS. Effects of vitamin B12 on cell proliferation and cellular alkaline phosphatase activity in human bone marrow stromal osteoprogenitor cells and UMR106 osteoblastic cells. *Metabolism*. 1996,45(12):1443-6.
129. Cashman KD. Homocysteine and osteoporotic fracture risk: a potential role for B vitamins. *Nutr Rev*. 2005,63(1):29-36.
130. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, et al. Homocysteine levels and the risk of osteoporotic fracture. *N Engl J Med*. 2004,350(20): 2033-41.
131. Herrmann M, Peter Schmidt J, Umanskaya N, Wagner A, Taban-Shomal O, Widmann T, et al. The role of hyperhomocysteinemia as well as folate, vitamin B(6) and B(12) deficiencies in osteoporosis: a systematic review. *Clin Chem Lab Med*. 2007,45(12):1621-32.
132. Prentice A. Diet, nutrition and the prevention of osteoporosis. *Public Health Nutr*. 2004,7(1A): 227-43.
133. Palacios C. The role of nutrients in bone health, from A to Z. *Crit Rev Food Sci Nutr*. 2006,46(8): 621-8.

134. Rocher E, Chappard C, Jaffre C, Benhamou CL, Courteix D. Bone mineral density in prepubertal obese and control children: relation to body weight, lean mass, and fat mass. *J Bone Miner Metab*. 2008,26(1):73-8.
135. Timpson NJ, Sayers A, Davey-Smith G, Tobias JH. How does body fat influence bone mass in childhood? A Mendelian randomization approach. *J Bone Miner Res*. 2009,24(3):522-33.
136. Clark EM, Ness AR, Tobias JH. Adipose tissue stimulates bone growth in prepubertal children. *J Clin Endocrinol Metab*. 2006,91(7):2534-41.
137. Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Endocrinology*. 1999,140(4):1630-8.
138. Rothman KJ. Epidemiology: an introduction. New York: Oxford University Press Inc.; 2002.
139. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. *Eur J Epidemiol*. 2012,27(9):739-56.
140. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? *Epidemiology*. 2006,17(4):413-8.
141. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. *Eur J Epidemiol*. 2006,21(6):475-84.
142. Rubin DB. Multiple imputation for nonresponse in surveys. New Jersey: 1987.
143. Haddow JE, Knight GJ, Palomaki GE, Kloza EM, Wald NJ. Cigarette consumption and serum cotinine in relation to birthweight. *British journal of obstetrics and gynaecology*. 1987,94(7):678-81.
144. Freedman LS, Schatzkin A, Midthune D, Kipnis V. Dealing with dietary measurement error in nutritional cohort studies. *J Natl Cancer Inst*. 2011,103(14):1086-92.
145. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr*. 1997,65(4 Suppl):1220S-8S; discussion 9S-31S.
146. Mook-Kanamori DO, Steegers EA, Eilers PH, Raat H, Hofman A, Jaddoe VW. Risk factors and outcomes associated with first-trimester fetal growth restriction. *JAMA*. 2010,303(6):527-34.
147. van Uitert EM, van der Elst-Otte N, Wilbers JJ, Exalto N, Willemsen SP, Eilers PH, et al. Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study. *Human reproduction*. 2013,28(12):3188-96.
148. Dudley NJ. A systematic review of the ultrasound estimation of fetal weight. *Ultrasound Obstet Gynecol*. 2005,25(1):80-9.
149. Tunon K, Eik-Nes SH, Grottum P. A comparison between ultrasound and a reliable last menstrual period as predictors of the day of delivery in 15,000 examinations. *Ultrasound Obstet Gynecol*. 1996,8(3):178-85.
150. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, et al. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol*. 2008,31(4):388-96.
151. Tilling K, Macdonald-Wallis C, Lawlor DA, Hughes RA, Howe LD. Modelling childhood growth using fractional polynomials and linear splines. *Ann Nutr Metab*. 2014,65(2-3):129-38.
152. Blake GM, Naeem M, Boutros M. Comparison of effective dose to children and adults from dual X-ray absorptiometry examinations. *Bone*. 2006,38(6):935-42.
153. Binkovitz LA, Henwood MJ. Pediatric DXA: technique and interpretation. *Pediatr Radiol*. 2007,37(1):21-31.
154. Bianchi M, Baim S, Bishop N, Gordon C, Hans D, Langman C, et al. Official positions of the International Society for Clinical Densitometry (ISCD) on DXA evaluation in children and adolescents. *Pediatr Nephrol*. 2010,25(1):37-47.

155. Prentice A, Parsons TJ, Cole TJ. Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. *Am J Clin Nutr*. 1994,60(6):837-42.
156. Leonard MB, Shults J, Elliott DM, Stallings VA, Zemel BS. Interpretation of whole body dual energy X-ray absorptiometry measures in children: comparison with peripheral quantitative computed tomography. *Bone*. 2004,34(6):1044-52.
157. Jebb SA, Goldberg GR, Elia M. DXA measurements of fat and bone mineral density in relation to depth and adiposity. *Basic life sciences*. 1993,60:115-9.
158. Helba M, Binkovitz LA. Pediatric body composition analysis with dual-energy X-ray absorptiometry. *Pediatr Radiol*. 2009,39(7):647-56.
159. Miles EA, Noakes PS, Kremmyda LS, Vlachava M, Diaper ND, Rosenlund G, et al. The Salmon in Pregnancy Study: study design, subject characteristics, maternal fish and marine n-3 fatty acid intake, and marine n-3 fatty acid status in maternal and umbilical cord blood. *Am J Clin Nutr*. 2011,94(6 Suppl):1986S-92S.
160. Makrides M, Collins CT, Gibson RA. Impact of fatty acid status on growth and neurobehavioural development in humans. *Matern Child Nutr*. 2011,7 Suppl 2:80-8.
161. Chan GM, McElligott K, McNaught T, Gill G. Effects of dietary calcium intervention on adolescent mothers and newborns: A randomized controlled trial. *Obstet Gynecol*. 2006,108(3 Pt 1): 565-71.
162. Frisell T, Oberg S, Kuja-Halkola R, Sjolander A. Sibling comparison designs: bias from non-shared confounders and measurement error. *Epidemiology*. 2012,23(5):713-20.
163. Taylor AE, Howe LD, Heron JE, Ware JJ, Hickman M, Munafo MR. Maternal smoking during pregnancy and offspring smoking initiation: assessing the role of intrauterine exposure. *Addiction*. 2014,109(6):1013-21.
164. Hanson M, Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD. Developmental plasticity and developmental origins of non-communicable disease: theoretical considerations and epigenetic mechanisms. *Progress in biophysics and molecular biology*. 2011,106(1):272-80.
165. Murphy TM, Mill J. Epigenetics in health and disease: heralding the EWAS era. *Lancet*. 2014,383(9933):1952-4.
166. Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou YH, et al. Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. *Hum Mol Genet*. 2015,24(15):4464-79.
167. Aslibekyan S, Demerath EW, Mendelson M, Zhi D, Guan W, Liang L, et al. Epigenome-wide study identifies novel methylation loci associated with body mass index and waist circumference. *Obesity*. 2015,23(7):1493-501.
168. Xu X, Su S, Barnes VA, De Miguel C, Pollock J, Ownby D, et al. A genome-wide methylation study on obesity: differential variability and differential methylation. *Epigenetics*. 2013,8(5):522-33.

CHAPTER 6

SUMMARY / SAMENVATTING

SUMMARY

Environmental influences during fetal life and early infancy have been suggested to influence body composition throughout the life-course. Especially poor fetal nutrition and fetal growth restriction have been designated important risk factors for gaining high fat mass or low bone mass during later life. As body composition tends to track from childhood into adulthood, the fight against widespread overweight and osteoporosis should focus on prevention from early life onwards. Although, to date a considerable amount of literature has been published on this topic, the influence of maternal diet, fetal growth patterns and maternal smoking (the main cause of fetal growth restriction) on offspring body composition remains disputed. Therefore, as described in Chapter 1 this thesis aimed to assess the impact of maternal diet, more specific fish and milk consumption during pregnancy on fetal growth; define the strongest early maternal, paternal, fetal and infant risk factors of preschool overweight, study the influence of maternal smoking on childhood body composition and explore the associations of maternal diet as well as fetal and infant growth patterns with childhood bone mass. To address these aims the studies included in this thesis were conducted within the Generation R Study, a population-based prospective cohort study from fetal life onwards.

In Chapter 2 maternal fish and milk consumption during pregnancy are related to fetal growth. Except for shell fish consumption, positive associations were found between maternal fish consumption and fetal growth in Chapter 2.1. However, those associations fully disappeared after adjustment for lifestyle-related confounders. Maternal shell fish consumption continued to show a negative association with birth weight. In Chapter 2.2, maternal milk consumption was associated with increased fetal growth leading to a higher weight and head circumference at birth. Drinking more than one glass of milk daily increased fetal growth from the third trimester of pregnancy onwards. The protein fraction from milk, rather than the fat or carbohydrate fraction seemed to drive the association. These findings suggest that maternal milk consumption during pregnancy influences fetal growth patterns, whereas fish consumption may play a lesser role.

In Chapter 3 early determinants of childhood adiposity are studied. Chapter 3.1 shows that when 34 previously reported risk factors were simultaneously analyzed parental anthropometrics, household income, fetal and infant growth, and infant diet were most strongly predicted preschool overweight. In Chapter 3.2 6-year-old girls, but not boys, born from mothers who continued smoking throughout pregnancy were found to have higher BMI, total fat mass, android/gynoid fat ratios, subcutaneous abdominal fat mass, and preperitoneal abdominal fat mass than girls of mothers who did not smoke. Remarkably, paternal smoking showed a similar association. Hence, these findings indicate that fetal and infant nutrition, as well as subsequent growth patterns may play an important role in the risk of developing overweight. The increased adiposity and adverse fat mass

distribution found in girls exposed to smoking during pregnancy is more likely explained by familial factors related to smoking behavior than by intrauterine mechanisms.

Chapter 4 is dedicated to early life determinants of childhood bone mass. In Chapter 4.1 both fetal growth and infant growth were found to independently contribute to childhood bone mass. Growth in the first year of life appeared to have the largest impact on bone mineral accrual. After the first year, the impact of height and weight growth slowly decreased every year. Furthermore, if present, catch-up growth in the first two postnatal years compensated for the consequences fetal growth restriction had on childhood bone mass. Chapter 4.2 indicates that maternal nutrient intakes during pregnancy are associated with childhood bone measures. Maternal phosphorus intake and homocysteine concentrations most-strongly predicted absolute bone mineral accrual in childhood, whereas maternal protein intake and vitamin B-12 concentrations were the strongest predictors of actual bone mineral density. In Chapter 4.3 maternal smoking appeared to be positively associated with childhood bone measures. However, this association was fully explained by increased body weight. Paternal and household smoking were not associated with childhood bone health. Taken together, these results show maternal diet during pregnancy as well as fetal and infant growth patterns may influence childhood bone health. Yet, maternal smoking does not seem to a play a role.

Finally, in Chapter 5 the main findings of this thesis are evaluated against the current knowledge. Both potential underlying mechanisms of the findings as well as methodological issues of the included studies are discussed. The chapter concludes with suggestions for future research and clinical application.

In conclusion, the findings of this thesis suggest maternal dietary factors during pregnancy influence fetal growth, while maternal diet and nutritional status, as well as subsequent fetal and infant growth patterns may influence childhood adiposity and bone mass. Shared familial factors related to maternal smoking, rather than maternal smoking itself, seemed to adversely affect childhood adiposity. Prevention of future overweight and osteoporosis should therefore start from early pregnancy onwards.

SAMENVATTING

Omgevingsfactoren gedurende de foetale- en zuigelingenperiode hebben mogelijk invloed op lichaamssamenstelling in het verdere leven. Vooral suboptimale foetale voeding en groeirestrictie worden gezien als belangrijke risicofactoren voor het ontwikkelen van een vetzucht en een lage botdichtheid. Omdat een ongezonde lichaamssamenstelling op kinderleeftijd blijft voortbestaan tot in het volwassen leven, moet de strijd tegen het wijdverspreide overgewicht en osteoporose zich richten op preventie in het vroege leven. Ondanks dat er veel literatuur gepubliceerd is over dit onderwerp, is de precieze invloed van maternale voeding, foetale groei patronen en roken tijdens de zwangerschap (de belangrijkste oorzaak van foetale groeirestrictie) op lichaamssamenstelling van het kind nog onduidelijk. Daarom, zoals beschreven in **Hoofdstuk 1**, is het doel van dit proefschrift de invloed van maternale voeding, of meer specifiek vis en melk consumptie, op foetale groei te onderzoeken, de belangrijkste vroege risicofactoren van peuter-overgewicht te identificeren, de invloed van roken tijdens de zwangerschap op lichaamssamenstelling van het kind te bestuderen en de rol van maternale voeding en vroege groeipatronen in botdichtheid op de kinderleeftijd in kaart te brengen. Om tot dit doel te komen, werden verschillende studies uitgevoerd binnen de Generation R Study, een prospectief cohort onderzoek in de Rotterdamse populatie.

In **Hoofdstuk 2** wordt maternale vis en melk consumptie tijdens de zwangerschap onderzocht in relatie tot foetale groei. In **Hoofdstuk 2.1** werden, behalve voor schelpdieren, positieve associaties gevonden tussen maternale visconsumptie en foetale groei. Echter, na het corrigeren voor levensstijl gerelateerde factoren, verdwenen deze associaties. Consumptie van schelpdieren was negatief geassocieerd met geboortegewicht, ondanks de correcties. In **Hoofdstuk 2.2** toonde maternale melkconsumptie een positieve associatie met foetale groei, wat leidde tot een hoger gewicht en grotere hoofdomtrek bij de geboorte. Vanaf het derde trimester van de zwangerschap verhoogde het drinken van meer dan 1 glas melk per dag de foetale groeisnelheid. De eiwitfractie van melk, in tegenstelling tot de vet- of koolhydratenfractie, leek verantwoordelijk voor dit fenomeen. Deze bevindingen suggereren dat melk consumptie, in tegenstelling tot vis een groei bevorderend effect heeft tijdens de zwangerschap.

In **Hoofdstuk 3** worden vroege determinanten van vetzucht op de kindertijd bestudeerd. **Hoofdstuk 3.1** laat zien dat wanneer 34 bekende risicofactoren tegelijkertijd geanalyseerd worden antropometrie van ouders, huishoudinkomen, groei in de foetale- en zuigelingenperiode, en zuigelingenvoeding de sterkste invloed op peuter-overgewicht hebben. In **Hoofdstuk 3.2** bleken 6-jaar-oude meisjes, maar niet jongens, blootgesteld aan roken gedurende de hele zwangerschap, een hoger BMI, een hoger vetpercentage, meer subcutaan abdominaal vet en een hoger androïde/gynoïde vet ratio te hebben dan kinderen die niet blootgesteld zijn aan roken. Opvallend genoeg, liet roken van de

vader tijdens de zwangerschap hetzelfde verband zien. Dus deze bevindingen geven aan dat zowel voeding tijdens de foetale- en zuigelingenperiode, als de daaropvolgende groeipatronen een belangrijke rol spelen in de ontwikkeling van overgewicht. De toegenomen vetzucht en ongunstige vetverdeling gemeten bij meisjes blootgesteld aan roken tijdens de zwangerschap wordt waarschijnlijk beter verklaard door familiaire factoren samenhangend met het rookgedrag dan door intra-uteriene mechanismen.

Hoofdstuk 4 is gewijd aan vroege determinanten van botdichtheid op de kinderleeftijd. In **Hoofdstuk 4.1** bleken zowel groei in de foetale- als in de zuigelingenperiode, onafhankelijk van elkaar, bij te dragen aan botdichtheid op kinderleeftijd. Groei in lengte en gewicht gedurende het eerste levensjaar had de grootste impact op botdichtheid. Na het eerste jaar, nam de invloed van groei langzaam af. Verder, bleek inhaalgroei in de eerste twee levensjaren, indien aanwezig, te kunnen compenseren voor de gevolgen van foetale groei restrictie op botdichtheid. **Hoofdstuk 4.2** laat zien dat voeding tijdens de zwangerschap is geassocieerd met botdichtheid op de kinderleeftijd. Maternale fosfor inname en serum homocysteine waren de sterkste voorspellers van toegenomen botmassa, terwijl maternale eiwit inname en serum vitamine B12 de werkelijke botdichtheid het best voorspelden. In **Hoofdstuk 4.3** leek roken tijdens de zwangerschap positief geassocieerd met botdichtheid op kinderleeftijd. Echter, deze associatie werd volledig verklaard door toegenomen lichaamsgewicht. Blootstelling aan roken door vader of in huis was niet gerelateerd aan botdichtheid op kinderleeftijd. Tezamen genomen, tonen deze resultaten dat zowel voeding tijdens de zwangerschap als groei in de foetale- en zuigelingenperiode mogelijk botdichtheid op kinderleeftijd beïnvloeden. Roken tijdens de zwangerschap lijkt hierin geen rol te spelen.

Ten slotte worden in **Hoofdstuk 5** de belangrijkste bevindingen van dit proefschrift vergeleken met de bestaande literatuur. Zowel de mogelijke onderliggende mechanismen van de bevindingen als de beperkingen van de geïncludeerde studies worden besproken. Het hoofdstuk sluit af met suggesties voor toekomstig onderzoek en toepassing in de praktijk.

Concluderend, de bevindingen van dit proefschrift suggereren dat maternale voedingsfactoren tijdens de zwangerschap foetale groei beïnvloeden, terwijl maternale voeding en voedingsstatus, evenals groei tijdens de foetale- en zuigelingenperiode vetzucht en botdichtheid op de kinderleeftijd beïnvloeden. Familiaire factoren gerelateerd aan roken tijdens de zwangerschap, meer dan het roken zelf, lijken vetzucht en ongunstige vetdistributie tijdens de kindertijd te verergeren. Preventie van overgewicht en osteoporose moet daarom al vanaf de vroege zwangerschap gestart worden.

CHAPTER 7

APPENDICES

LIST OF ABBREVIATIONS

AC	abdominal circumference
AGA	appropriate for gestational age
BA	bone area
BAT	brown adipose tissue
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BPD	bi-parietal diameter
CI	confidence interval
CT	computertomografie
d	day
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DXA	dual-energy x-ray absorptiometry
ECTS	european credit transfer system
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EFW	estimated fetal weight
FFQ	food frequency questionnaire
FL	femur length
g	gram
GA	gestational age
GH	growth hormone
GR	glucocorticoid receptor
HC	head circumference
HPA	hypothalamic-pituitary-adrenal
HR-pQCT	high resolution-peripheral quantitative computed tomography
IGF	insulin-like growth factor
IOTF	international obesity taskforce
IQR	interquartile range
ISCD	international society for clinical densitometry
IUFD	intrauterine fetal death
IUGR	intrauterine growth retardation
kcal	kilocalorie
kg	kilogram
L	liter
LMP	last menstrual period

m	meter
MEC	medical ethical committee
mg	milligram
mL	milliliter
mm	millimeter
MRI	magnetic resonance imaging
n	number
n-3	omega-3
n-6	omega-6
Nevo	nederlands voedingsstoffenbestand
ng	nanogram
nmol	nanomol
OR	odds ratio
PASW	predictive analytic software (or SPSS)
PCB	polychlorinated biphenyls
PMCA	plasma membrane calcium ATPase
pQCT	peripheral quantitative computed tomography
PTB	preterm birth
PUFA	poly-unsaturated fatty acids
Q	quintile
RCT	randomized clinical trial
ROS	reactive oxygen species
SD	standard deviation
SDS	standard deviation score
SES	socioeconomic status
SGA	small for gestational age
SPSS	statistical package social sciences
TV	television
WAT	white adipose tissue
WHO	world health organization

LIST OF PUBLICATIONS

1. Heppe DH, Steegers EA, Timmermans S, Breeijen H, Tiemeier H, Hofman A, Jaddoe VW. **Maternal fish consumption, fetal growth and the risks of neonatal complications. the Generation R Study.** *Br J Nutr.* 2011 Mar;105(6):938-49.
2. Heppe DH, van Dam RM, Willemsen SP, den Breeijen H, Raat H, Hofman A, Steegers EA, Jaddoe VW. **Maternal milk consumption, fetal growth, and the risks of neonatal complications. the Generation R Study.** *Am J Clin Nutr.* 2011 Aug;94(2):501-9.
3. Heppe DH, Taal HR, Ernst GD, Van Den Akker EL, Lequin MM, Hokken-Koelega AC, Geelhoed JJ, Jaddoe VW. **Bone age assessment by dual-energy X-ray absorptiometry in children: an alternative for X-ray?** *Br J Radiol.* 2012 Feb;85(1010):114-20.
4. Medina-Gomez C, Kemp JP, Estrada K, Eriksson J, Liu J, Reppe S, Evans DM, Heppe DH, Vandenput L, Herrera L, Ring SM, Kruithof CJ, Timpson NJ, Zillikens MC, Olstad OK, Zheng HF, Richards JB, St Pourcain B, Hofman A, Jaddoe VW, Smith GD, Lorentzon M, Gautvik KM, Uitterlinden AG, Brommage R, Ohlsson C, Tobias JH, Rivadeneira F. **Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus.** *PLoS Genet.* 2012 Jul;8(7):e1002718.
5. Heppe DH, Kiefte-de Jong JC, Durmuş D, Moll HA, Raat H, Hofman A, Jaddoe VW. **Parental, fetal and infant risk factors of preschool overweight. The Generation R Study.** *Pediatr Res.* 2013 Jan;73(1):120-7.
6. Van den Hil LC, Rob Taal H, de Jonge LL, Heppe DH, Steegers EA, Hofman A, van der Heijden AJ, Jaddoe VW. **Maternal first-trimester dietary intake and childhood blood pressure: the Generation R Study.** *Br J Nutr.* 2013 Mar 26:1-11.
7. Leermakers ET, Sonnenschein-van der Voort AM, Heppe DH, de Jongste JC, Moll HA, Franco OH, Hofman A, Jaddoe VW, Duijts L. **Maternal fish consumption during pregnancy and risks of wheezing and eczema in childhood: the Generation R Study.** *Eur J Clin Nutr.* 2013 Apr;67(4):353-9.
8. Heppe DH, Medina-Gomez C, Hofman A, Franco OH, Rivadeneira F, Jaddoe VW. **Maternal first-trimester diet and childhood bone mass: the Generation R Study**. *Am J Clin Nutr.* 2013 Jul;98(1):224-32.
9. Leventakou V, Roumeliotaki T, Martinez D, Barros H, Brantsaeter AL, Casas M, Charles MA, Cordier S, Eggesbø M, van Eijsden M, Forastiere F, Gehring U, Govarts E, Halldórsson TI, Hanke W, Haugen M, Heppe DH, Heude B, Inskip HM, Jaddoe VW, Jansen M, Kelleher C, Meltzer HM, Merletti F, Moltó-Puigmartí C, Mommers M, Murcia M, Oliveira A, Olsen SF, Pele F, Polanska K, Porta D, Richiardi L, Robinson SM, Stigum H, Strøm M, Sunyer J, Thijs C, Viljoen K, Vrijkotte TG, Wijga AH, Kogevinas M, Vrijheid M, Chatzi L. **Fish intake during pregnancy, fetal growth, and gestational length in 19 European birth cohort studies**. *Am J Clin Nutr.* 2014;99(3):506-16.
10. Durmuş B, Heppe DH, Taal HR, Manniesing R, Raat H, Hofman A, Steegers EA, Gaillard R, Jaddoe VW. **Parental smoking during pregnancy and total and abdominal fat distribution in school-age children. the Generation R Study**. *Int J Obes (Lond).* 2014 Jul;38(7):966-72.
11. Durmuş B, Heppe DH, Gishti O, Manniesing R, Abrahamse-Berkeveld M, van der Beek EM, Hofman A, Duijts L, Gaillard R, Jaddoe VW. **General and abdominal fat outcomes in school-age children associated with infant breastfeeding patterns**. *Am J Clin Nutr.* 2014 Jun;99(6):1351-8.
12. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, Oei L, Ring SM, Kruithof CJ, Timpson NJ, Wolber LE, Reppe S, Gautvik K, Grundberg E, Ge B, van der Eerden B, van de Peppel J, Hibbs MA, Ackert-Bicknell CL, Choi K, Koller DL, Econs MJ, Williams FM, Foroud T, Zillikens MC, Ohlsson C, Hofman A, Uitterlinden AG, Davey Smith G, Jaddoe VW, Tobias JH, Rivadeneira F, Evans DM. **Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment**. *PLoS Genet.* 2014 Jun 19;10(6):e1004423.

13. Gishti O, Gaillard R, Manniesing R, Abrahamse-Berkeveld M, van der Beek EM, Heppe DH, Steegers EA, Hofman A, Duijts L, Durmuş B, Jaddoe VW. **Fetal and infant growth patterns associated with total and abdominal fat distribution in school-age children**. *J Clin Endocrinol Metab.* 2014 Jul;99(7):2557-66
14. Heppe DH, Medina-Gomez C, de Jongste JC, Raat H, Steegers EA, Hofman A, Rivadeneira F, Jaddoe VW. **Fetal and childhood growth patterns associated with bone mass in school-age children: the Generation R Study**. *J Bone Miner Res.* 2014 Dec;29(12):2584-93
15. Qi Q, Kilpeläinen TO, Downer MK, Tanaka T, Smith CE, Sluijs I, Sonestedt E, Chu AY, Renström F, Lin X, Ängquist LH, Huang J, Liu Z, Li Y, Asif Ali M, Xu M, Ahluwalia TS, Boer JM, Chen P, Daimon M, Eriksson J, Perola M, Friedlander Y, Gao YT, Heppe DH, Holloway JW, Houston DK, Kanoni S, Kim YM, Laaksonen MA, Jääskeläinen T, Lee NR, Lehtimäki T, Lemaitre RN, Lu W, Luben RN, Manichaikul A, Männistö S, Marques-Vidal P, Monda KL, Ngwa JS, Perusse L, van Rooij FJ, Xiang YB, Wen W, Wojczynski MK, Zhu J, Borecki IB, Bouchard C, Cai Q, Cooper C, Dedoussis GV, Deloukas P, Ferrucci L, Forouhi NG, Hansen T, Christiansen L, Hofman A, Johansson I, Jørgensen T, Karasawa S, Khaw KT, Kim MK, Kristiansson K, Li H, Lin X, Liu Y, Lohman KK, Long J, Mikkilä V, Mozaffarian D, North K, Pedersen O, Raitakari O, Rissanen H, Tuomilehto J, van der Schouw YT, Uitterlinden AG, Zillikens MC, Franco OH, Shyong Tai E, Ou Shu X, Siscovick DS, Toft U, Verschuren WM, Vollenweider P, Wareham NJ, Witteman JC, Zheng W, Ridker PM, Kang JH, Liang L, Jensen MK, Curhan GC, Pasquale LR, Hunter DJ, Mohlke KL, Uusitupa M, Cupples LA, Rankinen T, Orho-Melander M, Wang T, Chasman DI, Franks PW, Sørensen TI, Hu FB, Loos RJ, Nettleton JA, Qi L. **FTO genetic variants, dietary intake and body mass index: insights from 177,330 individuals**. *Hum Mol Genet.* 2014 Dec 20;23(25):6961-72.
16. Heppe DH, Medina-Gomez C, Hofman A, Rivadeneira F, Jaddoe VW. **Does fetal smoke exposure affect childhood bone mass? The Generation R Study**. *Osteoporos Int.* 2015 Apr;26(4):1319-29
17. van den Hooven EH, Heppe DH, Kiefte-de Jong JC, Medina-Gomez C, Moll HA, Hofman A, Jaddoe VW, Rivadeneira F, Franco OH. **Infant dietary patterns and bone mass in childhood: the Generation R Study.** *Osteoporos Int.* 2015 May;26(5):1595-604.
18. Medina-Gómez C, Chesi A, Heppe DH, Zemel BS, Yin JL, Kalkwarf HJ, Hofman A, Lappe JM, Kelly A, Kayser M, Oberfield SE, Gilsanz V, Uitterlinden AG, Shepherd JA, Jaddoe VW, Grant SF, Lao O, Rivadeneira F. **BMD Loci Contribute to Ethnic And Developmental Differences in Skeletal Fragility Across Populations: Assessment of Evolutionary Selection Pressures**. *Mol Biol Evol.* 2015 Nov;32(11):2961-72.
19. Medina-Gómez C, Heppe DH, Yin YL, Trajanoska K, Uitterlinden AG, Beck TJ, Jaddoe VWV, Rivadeneira F. **Bone mass and strength in school age children exhibit sexual dimorphism related to differences in lean mass: the Generation R Study**. *J Bone Miner Res. [In press]*

Submitted

20. Van den Hooven EH, Gharsalli M, Heppe DH, Hofman A, Franco OH, Rivadeneira F, Jaddoe VW. **Associations of breastfeeding patterns and introduction of solid foods with childhood bone mass: The Generation R Study**. *Br J Nutr. [Under revision]*
21. Ong YL, Quah PL, Tint MT, Aris IM, van Dam RM, Heppe DH, Saw SM, Godfrey KM, Gluckman PD, Seng Chong Y, Yap F, Seng Lee Y, Chong MF. **Maternal vitamin D status is not associated with infant birth outcomes, postnatal growth and adiposity in a multi-ethnic Asian population: the GUSTO Study**. *Br J Nutr. [Under revision]*

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Department of Radiology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands. Rashindra Manniesing.

PhD PORTFOLIO

Name PhD student:	Denise Helena Maria Heppe
Department:	Epidemiology, Erasmus Medical Center, Rotterdam
Medical School:	Erasmus University Rotterdam, Sept 2004 - Aug 2007, Sept 2008 - Jan 2010, Feb 2013 - April 2015
Research school:	Netherlands Institute for Health Sciences (NIHES), Rotterdam, Aug 2006 - Aug 2010
PhD Period:	March 2010 - Feb 2013, Aug 2015 - Jan 2016
Promotors:	Prof. Dr. V.W.V. Jaddoe, Prof. Dr. E.A.P. Steegers
Co-promotor:	Dr. F. Rivadeneira

	Year	Workload (ECTS)
1. PhD Training		
Master of Science in Clinical Research, NIHES, Rotterdam, the Netherlands	2006-2010	
General courses		
Principles of Research in Medicine and Epidemiology	2006	0.7
Methods of Clinical Research	2006	0.7
Clinical Trials	2006	0.7
Pharmaco-epidemiology	2006	0.7
Topics in Evidence-based Medicine	2006	0.7
Introduction to Decision-making in Medicine	2006	0.7
Study Design	2006	4.3
Introduction to Data-analysis	2007	1.0
Introduction to Clinical Research	2007	0.9
Regression analysis	2007	1.9
Topics in Meta-analysis	2007	0.7
Modern Statistical Methods	2008	4.3
Survival Analysis	2009	1.9
Genome Wide Association Analysis	2009	1.4
Advanced courses		
Advanced Topics in Decision-making in Medicine	2007	1.9
Intervention Research and Clinical Trial	2007	0.9
Diagnostic Research	2007	0.9
Prognosis Research	2007	0.9
Research Themes and Methodologies	2007	1.0
Maternal and Child Health	2009	0.9
Pharmaco-epidemiology and Drug Safety	2010	1.9
Advanced Topics in Clinical Trials	2010	1.9
Advanced Analysis of Prognosis Studies	2010	0.9
Principles of Epidemiologic Data-analysis	2010	0.7

Skills courses		
Working with SPSS for Windows	2008	0.3
Scientific English Writing for Publication	2009	2.0
Summer School, Harvard School of Public Health, Boston, USA		
Fundamentals of Epidemiology	2008	2.0
Society & Health	2008	2.0
General academic courses		
Radiation hygiene and protection, Level 5R, Erasmus MC	2009	0.7
Research Integrity	2012	2.0
Seminars and workshops		
Seminars, Epidemiology	2009-2013	1.0
Research Meetings The Generation R Study	2009-2013	1.0
Research Meetings Nutritional Epidemiology (SIGN-E)	2012-2013	0.5
Research Meetings GUSTO Study & Saw Swee Hock School of Public Health, NUS, Singapore	2012	1.0
Symposium Stichting Kind & Groei, Rotterdam	2009	0.3
Pediatrics Research Day, Erasmus MC	2010	0.6
Unilever Nutrition Symposium, Vlaardingen	2010	0.6
Generation R Symposium "Genetics in Child Cohort Studies"	2010	0.3
NVK Dag van de Jonge Onderzoeker, Veldhoven	2011	0.6
Lof der Geneeskunst "Jong geleerd, oud gezond", Rotterdam	2011	0.3
PhD Day, Erasmus MC	2011	0.6
Danone Research Symposium "Bringing Science to Early Life Nutrition", Utrecht	2011	0.6
Symposium "Blik op de Verloskunde", Erasmus MC	2011	0.3
Workshop Nutritional Epidemiology, ErasmusAGE	2012	0.6
VENA Workshop "Networking", Erasmus MC	2012	0.3
VENA Workshop "Vaardiger Onderhandelen", Erasmus MC	2012	0.3
ABCD symposium "Diversiteit in groei en ontwikkeling van jonge kinderen", Amsterdam	2012	0.6
Postdoc Network Workshop "Networking: Social skills and attitude", Erasmus MC	2012	0.3
SICS Nutrition Seminar "Vitamin D, current strategies and future challenges", Singapore	2012	0.6
Workshop "Identity Branding", Erasmus MC	2012	0.3
VENA Workshop "Loskomen van de plakkende vloer", Erasmus MC	2013	0.3
VENA Workshop "Situationeel leiding geven", Erasmus MC	2013	0.3

(Inter)national Conferences		
CELSE Conference of Epidemiological Studies in Europe, Paphos, Cyprus - *Poster presentation*	2010	1.4
N&G International Conference on Nutrition and Growth - *Poster presentation*	2012	1.4
DOHaD Developmental Origins of Health and Disease, Rotterdam - *Oral and poster presentation*	2012	1.4
Pediatrics Research Day, Erasmus MC - *Poster presentation*	2013	0.7
ASBMR American Society for Bone and Mineral Research, Baltimore, USA - *Oral presentation*	2013	1.4
NVCB Nederlandse Vereniging voor Calcium en Botstofwisseling - *Oral presentation*	2013	1.4
Awards		
ASBMR Young Investigator Award, Baltimore, USA	2013	
International Research projects		
Saw Swee Hock School of Public Health, National University of Singapore, Singapore	2012	
Project title: Maternal vitamin D status and infant birth outcomes in the Growing Up in Singapore Towards healthy Outcomes (GUSTO) study.		
2. Teaching Activities		
Yvonne Godei, B thesis Nutrition and Dietetics, The Hague University of Applied Sciences	2011-2012	2.0
Guest lecturer Erasmus Junior College, Wetenschapsknooppunt "Van piep tot stok"	2011-2014	2.0
Mounira Gharsalli, MSc thesis Clinical Epidemiology, NIHES	2012-2013	2.0
Justin van der Tas, MSc thesis Clinical Research, NIHES	2012-2015	1.5
3. Other Activities		
Peer review of articles for scientific journals: European Journal of Epidemiology (2x), Osteoporosis International, Hormone Research in Pediatrics, BMC Public Health	2011-2013	2.0

DANKWOORD

Het is zover! Ik kan het bijna niet geloven, maar het moment is aangebroken om mijn dankwoord te schrijven! Het is alweer een tijd geleden dat ik aan dit proefschrift begon te werken, het studentenleven lag nog kort achter me. De jaren in het onderzoek en de coschappen zijn voorbijgevlogen en wat is er veel gebeurd! Ik wil iedereen die mij op deze weg heeft begeleid, vergezeld en bijgestaan waanzinnig bedanken. Jullie hebben me gebracht tot waar ik nu ben!

Allereerst en in het bijzonder wil ik mijn promotoren en copromotor bedanken, prof. dr. Jaddoe, prof. dr. Steegers en dr. Rivadeneira. Beste Vincent, dankzij jou mocht ik aan dit project beginnen. Ik bewonder je enorme enthousiasme voor epidemiologisch onderzoek, het werkt aanstekelijk en wakkerde ook mijn enthousiasme voor onderzoek aan. Ik heb je ervaren als een strategische, kritische en snelle denker. Volgens mij bezit je een ongelimiteerde stroom van onderzoek ideeën. Waar ik ook tegen aan liep, je had voor alles direct een oplossing. Je leerde me resultaatgericht werken, hetgeen voor mij (als perfectionist) soms een uitdaging was. Er waren verschillen, maar juist daardoor heb ik veel geleerd van onze samenwerking. Bedankt daarvoor!

Beste Eric Steegers, ik wil u bedanken voor uw betrokkenheid bij Generation R en mijn proefschrift. Ik ervaarde u als een belangrijke kracht in onze research meetings. U hield ons scherp op hypothese gebaseerd onderzoek en de vertaalbaarheid daarvan naar de kliniek. Dat is van onmisbare waarde in een groot epidemiologisch onderzoek als het onze! Ik bewonder uw schat aan kennis en uw toewijding aan het vak Verloskunde & Gynaecologie. Ik ben trots dat u mijn tweede promotor wilt zijn!

Beste Fernando, jij kreeg pas in een late fase een rol in mijn promotietraject. Achteraf gezien, vind ik dat eigenlijk heel jammer, want je bleek een hele fijne begeleider! Ik vond het super dat je me meenam naar het NVCB en de ASBMR in Baltimore, het was een geweldige ervaring! Volgens mij werk je heel hard en heb je daardoor een overvolle agenda. Maar als ik wel eens langs je kantoor liep, nam je toch alle tijd voor mij en wilde je mijn vraag tot op de bodem uitzoeken. Je stopte niet voordat ook ik het antwoord begreep. Ik wens je toe dat je verdere wetenschappelijke carrière voorspoedig zal verlopen, met jouw inzet en toewijding verdien je dat zeker!

Professor Hofman, u was de eerste die mij inspireerde voor wetenschappelijk onderzoek. Ik zal de manier waarop u uw liefde voor het vak op zeer enthousiaste wijze overbracht in de introductie cursus van NIHES nooit vergeten! Aan u heb ik ook het bestaan van het Generation R cohort te danken. Daarnaast gaf u mij enkele keren persoonlijk advies, zo

ondersteunde u mijn idee plaats te nemen in het verenigingsbestuur en enthousiasmeerde u mij voor het groeionderzoek binnen Generation R. Ik ben u daar nog steeds dankbaar voor.

Prof.dr. Reiss, prof. dr. de Jongste en prof. dr. Roseboom, ik wil u graag bedanken voor uw bereidheid zitting te nemen in de kleine promotie commissie en het lezen en beoordelen van mijn proefschrift. Prof.dr. Reiss, bedankt dat u als secretaris plaats wilde nemen in de leescommissie. Prof. dr. de Groot en dr. van Rossum, ik wil u graag bedanken voor uw bereidheid met mij van gedachte te wisselen over de inhoud van dit proefschrift. Prof. Cooper, I am very pleased you are a member of my PhD committee. Thank you for your willingness to come all the way to the Netherlands to take part in this ceremony.

Ook wil ik alle coauteurs bedanken voor hun bijdrage aan de artikelen waaruit dit proefschrift bestaat!

Graag wil ik ook dr. van Dam bedanken voor de kans onderzoek te doen aan de National University of Singapore. Beste Rob, ik ben u erg dankbaar voor uw warme onthaal op de NUS. Het zat een beetje tegen met de onderzoeksresultaten, maar ik ben blij dat het artikel nu toch af is! Het was erg leerzaam voor mij eens een kijkje in een andere keuken te krijgen. En van de Singaporese keuken heb ik ook ontzettend genoten! Dear Mary, thanks for guiding and supervising me at the NUS, it has been a great learning experience! Dear Salome, it was a blessing to have you as a roommate! We discussed research, cultural differences, global issues, … what not? It was great to get to know you! Dear Joy, thanks for being a colleague and a friend at the NUS! It was so nice we got to meet a second time in Baltimore and a third time in Singapore! Good luck in Boston! I'm sure there will be a fourth time… I wish you all the best for your careers and personal life!

Lieve Generation R collega's het was fijn jullie aan mijn zijde te hebben afgelopen jaren! Lieve Ralf, Romy, Jessica, Layla, Rob, Rachel, Edith, Busra, Michelle, Sanne, Mònica, Nina, Marieke, Nienke, Rolieke, Claudia, Ilse, Esther, Ankie, Ank, Gerard, Hanneke, Jolien, Sabine, Selma, Anne, Agnes, Irene, Lea, Ryan, Martijn, Kozeta, Claire en Zoë, ik heb goede herinneringen aan alle kamergesprekken, koffiemomentjes, lunches, diners, borrels, promotiefeestjes en/of pizza-parties! Dankzij jullie was onderzoek doen (gelukkig) ook een sociale bezigheid! Lieve Ralf, jij was toch wel de sfeermaker met je legendarische pizza-parties! Ik heb genoten van het enthousiasme waarmee jij over je grote passies praat en zeker ook van de diners bij jou thuis waar ik je passie voor goede wijn en lekker eten mocht meebeleven. Ik mis onze goede gesprekken! Lieve Jessica, ik

kon bij jou altijd terecht voor een vraag of een luisterend oor. Ik wil je bedanken voor al je goede adviezen. Ik vind je een geweldige onderzoeker en docent, en vind het leuk te zien dat je deze kwaliteiten nu weet te combineren! Lieve Marieke, wat was het heerlijk om een dispuutsgenootje in de kamer naast me te hebben! Ik heb genoten van onze koffiebreaks, onze gesprekken en je spontaniteit! Clau, de doppio zal ons gemist hebben, bedankt voor de gezellige momenten! Hopelijk komen we elkaar nog eens tegen! Mijn kamergenootjes, Rachel, Edith, Romy en Gerard, bedankt voor jullie gezelligheid! Lieve Edith, bedankt voor de fijne gesprekken en samenwerking! Ik hoop dat je het heel erg naar je zin zult hebben bij Danone! Lieve Romy, wat was het fijn om met jou op de kamer over alles te kunnen praten, ik kon bij jou m'n hart luchten en samen lachten we om al onze belevenissen. Bedankt ook voor je steun op de momenten dat het even tegenzat. Ik zal onze trip naar Noord-Nederland nooit meer vergeten… wat wij niet over hadden voor extra deelnemers voor het Generation R onderzoek?! Lieve Layla, Rob, Edith, Agnes en Romy, mijn eerste congres met jullie in Paphos, ik krijg nog steeds een glimlach op mijn gezicht als ik terugdenk aan het Roman Hotel… Deze trip was echt een hoogtepunt! Lieve Geeske, samen begonnen als student op Generation R, ik waardeer je nuchtere en positieve instelling en hoop dat we elkaar blijven tegenkomen! Lieve Ester, het was heel leuk om je beter te leren kennen op het NVCB congres, succes met het afronden van je promotie! Dear Caro, our trip to Baltimore was a great success! Your smile and enthusiasm always make me happy! You work so hard, I hope you will be rewarded soon, you definitely deserve that!

Dames van het secretariaat, Patricia, Karin, Rose, bedankt voor jullie inzet! Ronald, Claudia en Marjolein, bedankt voor jullie ondersteuning bij de data (verzameling) en voor de momenten dat ik jullie kamergenoot mocht zijn in de laatste fase! Clau, wat moet Generation R zonder jou? Fijn dat we af en toe even samen konden klagen en bedankt voor je ondersteunende woorden! Doe voorzichtig!

Ook wil ik alle 'focus-dames' ontzettend bedanken voor het verzamelen van de data en natuurlijk de gezelligheid op het onderzoekscentrum! Dankzij jullie harde werk kon ik aan de slag met een prachtige dataset! En zeker niet te vergeten, de kinderen en hun ouders! Ik wil hen bedanken voor hun deelname aan het Generation R onderzoek. Zonder jullie eindeloze inzet zou dit proefschrift niet bestaan!

Lieve Yvonne, ik ben blij dat je nog steeds mijn vriendin bent. Je bent zo zorgzaam en attent. Ik heb de afgelopen jaren graag gebruik gemaakt van je luisterend oor, je gastvrijheid en de kookkunsten van Simon. We delen een liefde voor mode, een goede kop koffie, leuke lunch-plekjes, en ook één eigenschap; al lijkt ons schilletje misschien stevig, van binnen zijn we een zacht ei. Ik hoef jou dan ook nooit iets uit te leggen, je

begrijpt me al voordat ik een verhaal verteld heb. Ik hoop dat we ook in de toekomst nog vele uren zullen kletsen en lachen!

Lieve Maasstad Collega's, bedankt voor een warme ontvangst in een leuk team! Ik weet zeker dat ik komend jaar veel van jullie ga leren. Bedankt voor jullie support en begrip in de laatste fase van het afronden van mijn proefschrift! Ik kijk uit naar de gezamenlijke wintersport!

Lieve co-groepgenootjes, Ralf, Michel, Steph, Marlies, Tijn, Sel, Anne-Joy, Sergei, Ruben en natuurlijk Mien. De co-groep met de meeste promovendi ooit. Meer dan de helft van ons was bezig met het afronden van een promotietraject toen wij elkaar leerden kennen. Het was een verademing om met jullie samen de coschappen te beleven. Ik heb genoten van de borrels en etentjes! Met Michel zijn schaterlach was zelfs reflecteren een feestje! Tijn, topper, jij bent nu mijn collega in een ander ziekenhuis, ik hoop dat er nog veel vrimibo's zullen volgen!

Lieve Harvard homies, na ons tripje Boston 7 jaar geleden zijn we elkaar nooit uit het oog verloren. Es, als we eenmaal een plekje hebben in jouw drukke agenda is het altijd lachen! Ik waardeer je gevoel voor humor! De weekenden bij jou, Lot, in Groningen voelden altijd als een mini-vakantie. Gelukkig woon je nu dichterbij en kunnen we elkaar vaker zien! Ik bewonder hoe je je opleiding, promotie en het moederschap combineert! Ik kijk uit naar jullie promotie(feestje)s!

Lieve Kenya-gangers, bij jullie vergat ik al mijn promotie-zorgen… Ik voelde mij bij jullie weer bijna 20 in plaats van bijna 30 (behalve in de ochtend). Gelukkig hebben wij, de co-tjes, jullie kunnen laten zien dat het leven niet ophoudt naar je 25e… Samen hebben wij veel meegemaakt, daardoor zal ik jullie nooit vergeten! Ik hoop dat er nog vele reünies zullen volgen! Lieve Sel, roomie, ik heb je tijdens de reis veel beter leren kennen, onze belevenissen in Iten en omgeving zijn onvergetelijk! Ik heb genoten van je nuchterheid, grappen en positiviteit! Ik hoop dat we nog lang vriendinnen blijven!

Lief bestuur, jullie zijn als broers en zus voor me! (behalve Geert dan haha) Na al die jaren kijk ik nog altijd met veel plezier terug op ons jaar op de sociëteit. Het heeft ons voor altijd verbonden. Een avond met jullie is altijd feest. Onze grappen zullen maar weinig mensen begrijpen. Al woont een deel nu ver weg, Singapore, London, San Francisco of Aalsmeer, ik hoop dat we elkaar regelmatig zullen blijven zien! Volgend jaar een gezamenlijke vakantie?

Lieve meiden van IZISS, al is ons allermooiste dispuutje ondergegaan en heeft onze naam tegenwoordig een heel andere bijsmaak, onze vriendschap is blijven bestaan. Ik ben blij dat ik altijd bij jullie terecht kan voor een glaasje wijn! High-tea, kerstdiner, sauna, weekendjes weg, fijn dat iemand steeds weer het initiatief neemt! Volgend jaar gaan we gewoon weer op zeilweekend, al staan er wellicht een paar van onze tentjes op het moederkloekveld :)

Lieve Annemarie, jij bent een heel speciale vriendin voor mij. Jij zet je in voor de strijd tegen vrouwenbesnijdenis, ondersteunt slachtoffers van oorlogsgeweld, geen probleem is je te groot. Ik bewonder je om je gedrevenheid die nooit ten koste gaat van iemand anders (dan jezelf?) Ik vind het heel tof dat je (samen met Erik) naar het buitenland vertrekt, ik weet zeker dat het jullie veel zal brengen. Afgelopen jaren stond je altijd voor me klaar, je bent een geweldig luisterend oor en begrijpt me zo goed. Ik zal je erg missen op mijn verdediging, maar ik wens jullie ontzettend veel plezier op de grote reis, dat hebben jullie meer dan verdiend!

Lieve Sanne, jij bent een beetje Generation R en heel veel vriendin! Ik ben je dankbaar voor je lieve kaartjes en alle uren waarin we hebben gekletst, je naar mijn verhalen geluisterd hebt. Je hebt een grote strijd gestreden en soms strijd je stilletjes nog steeds. Desalniettemin sta je altijd voor mij en anderen klaar en ben je bereid met wat dan ook te helpen of advies te geven, daar ben ik je heel dankbaar voor. Ik bewonder je doorzettingsvermogen en ben ontzettend trots dat jij op mijn verdediging naast me staat!

Lieve clubgenootjes, wij zijn al sinds 2004 bij elkaar. Er is een hoop gebeurd en veranderd sinds het moment dat wij ons moesten invechten. Ik ben blij dat we nog steeds bij elkaar zijn! Samen met jullie groeide ik op van tiener naar student naar … de vrouw? (ai) die ik nu ben. Lieve Nad, over jou vertel ik altijd heel trots dat jij met staatshoofden onderhandelde. Je bent een sterk persoon, ik weet zeker dat jij het nog ver zult schoppen in binnen- of buitenland. Als ik de komende tijd iets voor je kan doen (m'n boekje is af), laat het me weten! Lieve Joyce, als eerste de brug over van het moederschap, dat valt wellicht niet altijd mee, maar ik weet zeker dat je het geweldig doet! Bedankt dat ik je vriendinnengroep mocht overnemen tijdens mijn onderzoek in Singapore! Lieve Lein, ik vind het knap hoe je je nieuwe (gezins)leven vorm geeft! Lieve Iris, fijn dat je altijd in bent voor een wijntje! We verschilden wel eens van mening, maar ik ben blij dat je altijd eerlijk bent. Lieve Kar, de stabiele factor in de club, een lieve vriendin, ik kijk uit naar je bruiloft, het wordt vast een prachtige dag! En dan natuurlijk mijn promotie-lot/ clubgenootjes… Lieve Es, jij doet je promotie er als een soort hobby bij… het is werkelijk ongelofelijk! Ik bewonder (en soms benijd) je discipline. Jouw promotie gaat er zeker komen en die GZ-opleiding ook! Ik ken je als een goede en lieve vriendin, die me in de

gaten houdt als ik het druk heb of me zorgen maak. Bedankt voor alle fijne gesprekken en voor het feit dat ik altijd bij je terecht kan! We moeten snel weer eens een dagje naar de sauna! Lieve Mies, harde werker, als laatste begonnen en als eerste klaar! Wat er ook op jouw pad komt, jij klaart de klus… Je bent de stiekem de beste in alles wat je doet, maar dat vertel je niemand. Voor mij ben je een lieve, trouwe vriendin. In de afgelopen jaren heb ik dankzij jou, jou en mijzelf beter leren kennen. Ik vind het supertof dat je nu in London zit en kom je zeker een keer opzoeken! Als laatste, lieve Mien, wat ben ik blij dat ik jou heb! Sinds 2004 doen we alles samen, studeren, studentenvereniging, onderzoek, promoveren, coschappen lopen, Kenya… Of we nu koffie drinken bij Doppio, wijntjes bij LÓuest, mojitos in New York, Smirnoffs in Kenya, GT's in Ballroom, of biertjes op onze vast plek in het Schaap, met jou is het altijd feest! Ik heb zo genoten van al onze momenten samen, onze slechte grappen verlichtten iedere dag. Ook op de minder mooie momenten kon ik op je bouwen. Ik ben je heel dankbaar voor je steun op de momenten dat het tegenzat. Je was de spiegel die ik nodig had en waar ik veel van heb geleerd. Bedankt voor alles!

Lieve pap en mam, jullie hebben mij altijd gesteund. Als oudste van de drie, nam ik jullie een aantal keer mee in een onbekende wereld. Al was het soms even wennen, jullie vertrouwden me in alle keuzes die ik maakte. Jullie luisteren bijna dagelijks naar mijn verhalen, de goede en de slechte. Ik kan jullie altijd bellen voor hulp, een klus, eten of een auto… Wat er ook gebeurt, jullie staan altijd voor ons drie klaar. Bedankt voor alles wat jullie ons hebben meegegeven en voor ons doen!

Lieve zusjes, samen zijn wij een soort synergie met onze eigen Heppe-humor. Ik weet dat jullie altijd voor me klaar staan en de obstakels van een Heppe snappen. Ik vind het heel leuk dat we ons alle drie in een ander vak gestort hebben. Lieve Lynet, ik ben heel trots op wat je doet en weet zeker dat je rechter wordt! Bedankt dat je voor me kwam koken tijdens de laatste loodjes! Lieve Eline, ik vind het heel stoer dat jij ook bent gaan promoveren! Het zat jou vorig jaar even niet mee, maar ik bewonder alle stappen die je daarna gemaakt hebt! Ik vind dat je onwijs gegroeid bent afgelopen jaren. Het is heel leuk om, als grote zus, nu ook van jou te leren!

Lieve familie Heppe en Koppert, al zien we elkaar niet vaak, het is fijn om uit zulke grote families te komen! Als we elkaar weer zien, zijn jullie geïnteresseerd in mijn vorderingen in het ziekenhuis. Het Heppe weekend was een goede afwisseling op mijn laatste onderzoeks-loodjes. In het bijzonder wil ik mijn tante Marlene bedanken die er op alle verjaardagen en uitreikingen steevast bij is. Je zorgt voor voortreffelijk eten en een hoop gezelligheid! Daarnaast ben je de onmisbare tolk op onze vijf-jaarlijkse Argentinië reis. De reünies van de Argentinië groep zijn altijd een succes, ik kijk uit naar de volgende reis!

Dear Viki, my Argentinian sister, it was very special to have you here last year! I enjoyed our Berlin trip a lot! I look forward to visiting you next year! Tante Anne, bedankt voor een dagje coaching! Lieve Ingrid, afgelopen jaar leerde ik jou onverwachts beter kennen. Al viel de aanleiding te betreuren, ik vind het leuk om je nu vaker te spreken! Nu m'n boekje af is, kom ik Amber zeker bewonderen in jullie nieuwe huis! Bedankt voor je grote hulp bij het zoeken en vinden van sponsoring!

Lieve familie van Geert, tijdens mijn jaren in het onderzoek kreeg ik jullie er als mijn Limburgse familie bij. Ik ervaar jullie als een hecht en warm gezin. Bedankt dat jullie mij daarin opnemen als een eigen kind, en natuurlijk bedankt voor Geert! Lieve Jolanda, ik wil je bedanken voor onze fijne gesprekken! Je helpt me naar mijn gevoelens te luisteren en je adviezen geven me kracht.

Geert, mijn allerliefste! Zonder jou weet ik niet of dit gelukt was. Jij helpt me bij het maken van keuzes, je leerde me ontspannen, je bent de interne klok die ik niet heb, je helpt me problemen relativeren, loslaten en mijn gedachten te ordenen. Je hebt eindeloos geduld (voor mij) en vertrouwt in mijn kunnen als mij dat niet lukt. Samen hebben we de mooiste avonturen beleefd in Singapore en op reis. Helaas ben je nu in Saudi Arabië en mis ik je heel erg. Eén voordeel, ik realiseer me des te meer hoe belangrijk je voor me bent. Ik ben blij dat je me op afstand probeert te helpen. In het vliegtuig van Dubai naar Riyad pas jij, mijn computer-nerd, de figuren van mijn proefschrift aan. Maar ik hoop dat we de volgende reis weer samen maken, wat voor reis dan ook, met jou erbij is alles zoveel leuker! Ik houd van je.

ABOUT THE AUTHOR

Denise Helena Maria Heppe was born on the 23rd of November 1986 in Vlaardingen, the Netherlands. She grew up in Hoek van Holland as the eldest of three children. In 2004 she graduated from secondary school (Gymnasium) at the ISW in 's-Gravenzande. In the same year she started her medical education at the Erasmus University of Rotterdam. During her second year she was selected, as one of 30 students, to participate in the Master of Science program Clinical Research at the Netherlands Institute of Health Sciences (NIHES). She combined this program with her medical education during which she attended a summer course at the Harvard school of Public Health, Boston, MA, United States of America. As she was always looking for a new challenge, Denise decided to suspend her studies in 2007 for one year to accept a full-time position as Secretary of the Board of S.S.R.-Rotterdam, one of the largest student associations in Rotterdam. In 2008, she resumed both her studies and was elected into the Student Council of the Erasmus University. For her MSc thesis, she joined 'Generation R' in 2009 and studied the validity of a DXA scan for determining bone age in a pediatric population. In 2010, she obtained both a bachelor degree in Medicine and a MSc degree in Clinical Research.

Denise started the PhD project presented in this dissertation under supervision of dr. F. Rivadeneira (Department of Internal Medicine), prof. dr. V.W.V. Jaddoe (Department of Epidemiology and Pediatrics) and prof. dr. E.A.P. Steegers (Department of Obstetrics and Gynecology) at the Erasmus University Medical Center in Rotterdam. Her work was awarded with the "Young Investigator Award" by the American Society for Bone and Mineral Research in Baltimore, MD, United States of America. Nearing the end of her PhD, she temporarily exchanged living in Rotterdam for the vibrant city of Singapore. For three months she worked as a research fellow at the Saw Swee Hock School of Public Health of the National University of Singapore under supervision of prof. dr. R.M. van Dam.

Early 2013 she started her clinical internships. After an internship in Kenya in 2014, she graduated from medical school in 2015 and returned to the 'Generation R Study' to finalize her PhD research. As from October 2015 she started working as a resident (ANIOS) at the Pediatric Department of the Maasstad Ziekenhuis in Rotterdam under supervision of dr. M. Groeneweg.