Infant Body Composition and Other Early Life Determinants of Obesity and Adult Diseases

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INFANT BODY COMPOSITION AND OTHER EARLY LIFE DETERMINANTS OF OBESITY AND ADULT DISEASES

LICHAAMSSAMENSTELLING OP DE ZUIGELINGENLEEFTIJD EN ANDERE VROEGE DETERMINANTEN VAN OBESITAS EN ZIEKTEN OP DE VOLWASSEN LEEFTIJD

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Chapter 1

General Introduction

GENERAL INTRODUCTION

1. Influence of infant growth on adult health

1.1 Hypotheses

There are several epidemiological studies showing associations between low birth weight, and the risk for obesity, type 2 diabetes and cardiovascular diseases in later life.(1-3) Several hypotheses have been postulated over time to explain the underlying mechanism.

Fetal origin hypothesis

Barker et al. were the first to formulate a hypothesis in the early 1990s, based on the inverse association between birth weight and adult disease. They suggested that events during pregnancy leading to fetal malnutrition could result in permanent endocrine and metabolic changes, so-called re-programming of the fetus (Figure 1). At first the fetus would benefit from these adaptations, as it would remain alive during fetal life, but this re-programming increases the risk for adult diseases in later life.(4)

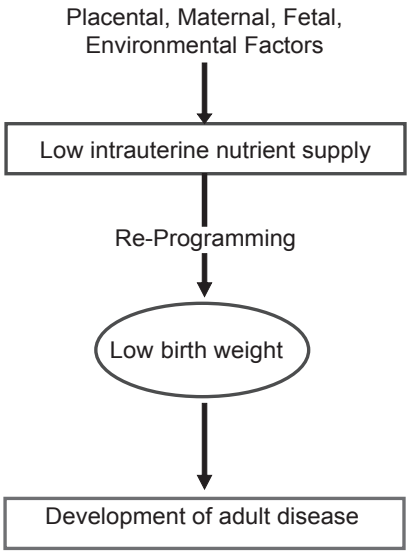

Figure 1. Representation of the fetal origins hypothesis. Adapted from Barker et al.(3)

Fetal insulin hypothesis

This hypothesis was generated in 1999 and states that the inverse association between birth weight and adult insulin resistance is in principal genetically mediated (Figure 2).(5) Fetal genes leading to insulin resistance could result in low fetal growth and to type 2 diabetes in later life.

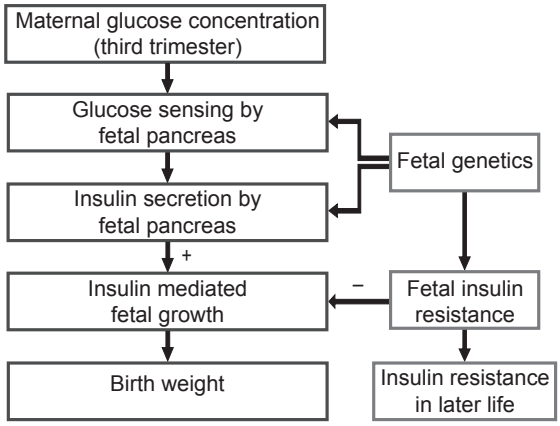

Figure 2. Simplified representation of the fetal insulin hypothesis. Adapted from Hattersley et al.(5)

Growth acceleration hypothesis

Singhal and Lucas postulated in 2004 that not low birth weight per se, but growth acceleration during childhood is responsible for the increased risk for adult diseases (Figure 3).(6, 7)

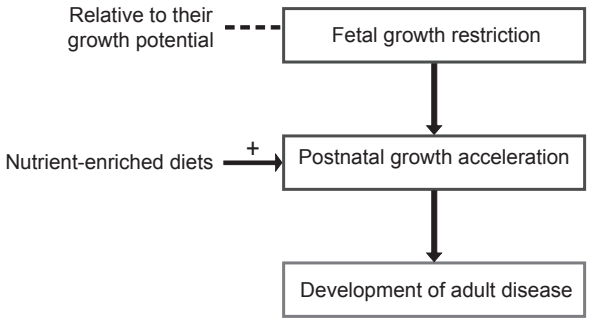

Figure 3. Representation of the growth acceleration hypothesis. Adapted from Singhal et al.(7)

Fat accumulation hypothesis

In the PROGRAM-study, started in 2002, our research group showed that not growth acceleration as such but fat accumulation during childhood was associated with determinants of adult diseases, such as a higher body fat percentage, increased serum levels of total cholesterol, triglycerides, low density lipoprotein cholesterol (LDL) and higher systolic and diastolic blood pressure in early adulthood.(8) This study also showed that the first 3 months of life are a critical window in which increased weight for length should be avoided to reduce the risk for obesity, type 2 diabetes and cardiovascular diseases in later life (Figure 4).(8-10)

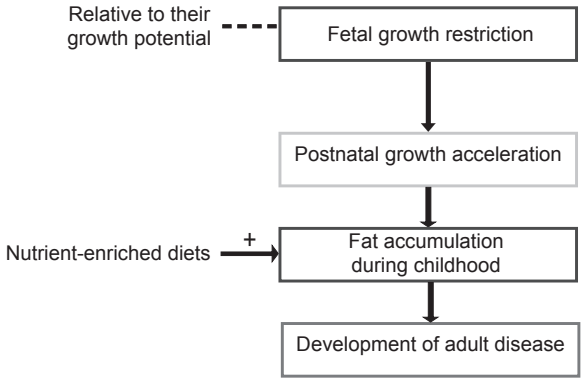

Figure 4. Fat accumulation model. Adapted from Leunissen et al.(8)

1.2 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a condition that resembles that of alcohol induced liver injury, but occurs in patients who do not abuse alcohol.(11) NAFLD has the potential to progress to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. (11) Furthermore, NAFLD is considered as the hepatic manifestation of the metabolic syndrome, and has been associated with several risk factors for cardiovascular disease (CVD) and type 2 diabetes, such as adverse lipid profile and body composition, and insulin resistance.(11, 12) Given the strong association of NAFLD with obesity, the prevalence of NAFLD is rapidly increasing.(13) Some studies demonstrated an association between small size at birth and NAFLD.(14) Given that NAFLD is considered the hepatic metabolic syndrome,(15) it might well be that the previously found association between small size at birth and NAFLD can also be ascribed to accelerated weight gain in early life.

Preterms

Nowadays, 8-10% of all newborns in developed countries is born preterm and due to advances in neonatal intensive care, most of them survive and reach adulthood.(16) Preterm infants are frequently exposed to fetal growth restriction, glucocorticoid treatment and stressful events, which may contribute to the development of organ dysfunction and adverse vascular outcome later in life. In recent years, long-term outcomes for young adults born preterm were found to be less favorable than for those born term. Adults born preterm are at increased risk for developing cardiovascular diseases (CVD) (17-19), and have increased cardiovascular mortality.(20, 21) Because many preterm infants are prone for accelerated weight gain after an initial period of weight loss (22), it might be that adults born preterm with accelerated weight gain after term age are at increased risk for NAFLD compared with those without accelerated weight gain during infancy. Like in term infants, alterations in growth during this highly dynamic developmental time window might have programming effects on risks for non-alcoholic fatty liver disease.

1.2.1 Fatty liver index (FLI)

The FLI is a measure to predict hepatic steatosis in the general population (23), and is determined using an algorithm based on BMI, waist circumference, triglycerides, and gamma-glutamyltransferase (γ-GT), with an accuracy of 0.84 (95%CI 0.81-0.87) in detecting fatty liver.(24, 25) The FLI has been widely used in NAFLD research, and has recently been validated for identifying NAFLD in a large population-based study.(24-28)

2. Early life determinants of obesity, type 2 diabetes and cardiovascular disease

Over the last 20 years, the prevalence of overweight in Dutch children aged 4 to 12 years, increased from 7% to 11%.(29) Childhood obesity is associated with short-term morbidity, such as asthma and psychological problems, and with an increased risk for obesity, chronic morbidity and mortality in adulthood.(30-32)

2.1 Accelerated catch-up in weight for length

Accelerated catch-up in weight for length in the first months of life is associated with higher risk for obesity, type 2 diabetes and cardiovascular diseases in adulthood. Besides this, it is associated with a higher fat mass percentage and a worse cardiovascular and metabolic profile in young adults.(8, 9, 33, 34) Also associations between weight gain in early life and childhood obesity have been described.(35-37) It is, however, unclear how accelerated catch-up in weight in the first months of life determines body composition in early life.

2.2 Infant body composition

There is increasing evidence that fat mass in early life influences fat mass in later life. Adiposity at the age of 6 months of life is a major risk factor for adult diseases, because it tracks into adulthood.(38-40) Unfortunately there were very limited data on the development of the body composition in the first 6 months of life. It is crucial to accurately measure body composition in these months and to generate reference data for infant boys and girls. Differentiation in gender is important because gender differences in body composition might be present due to different sex hormones levels in boys and girls in early life. It was known that newborn girls have higher fat mass and lower fat free mass compared to newborn boys, but there were very limited data on gender differences later in infancy.(41-43)

Relationships between weight gain and later health was mainly studied retrospectively or during follow-up of intervention trials.(9, 10) Due to the lack of available methods for infants below 6 months of age, most studies focused on weight, length, and skinfolds as a proxy for adiposity in infancy, instead of accurately measured body composition. (44, 45)

2.2.1 Methods for measuring infant body composition

Neonatal body composition is traditionally evaluated by measuring birth weight and birth length and then calculating the ponderal index, but these measures do not reflect true body composition.

Peapod

Nowadays, whole-body composition can be assessed by air-displacement plethysmography (Peapod, Infant Body Composition System, COSMED) (Figure 5). This ADP system assesses fat mass and fat free mass by direct measurements of body volume and body mass, based on the whole-body densitometric principle. Details of the principle and operating procedure of the Peapod have been described.(46, 47). These studies have shown that this method is valid for measurement of body composition in neonates.(43-44)

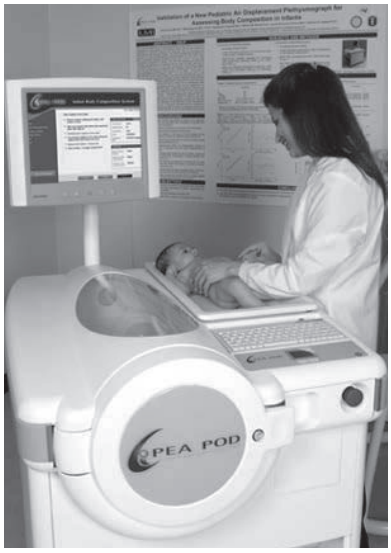

Figure 5. A picture of the Peapod

Ultrasound

Not only the total amount of fat, but also the location of fat is important. Increased visceral fat is a major risk factor for future cardiovascular diseases (CVD).(48) In obese children, greater abdominal adipose tissue is associated with an unfavorable metabolic profile.(49) Ultrasound (US) is a non-invasive method to estimate abdominal adipose tissue distribution in infancy. US-visceral fat thickness and abdominal subcutaneous fat thickness are reliable and reproducible estimates of respectively internal-abdominal (visceral) adipose tissue and abdominal subcutaneous adipose tissue.(50)

2.3 Infant feeding

Nutrition will very likely affect adiposity rate and body composition in early life. Breastfeeding is known as the optimal infant feeding, because it is associated with a lower incidence of childhood obesity and type 1 and type 2 diabetes in later life.(51) Studies about the influence of breastfeeding on body composition at 6 months showed, however, conflicting results.(52-56).

2.4 Maternal characteristics

Maternal pre-pregnancy weight and gestational weight gain are important determinants of birth weight. Pre-pregnancy weight and maternal weight gain during pregnancy influence fetal growth, birth weight and fat mass percentage at birth (57-59), but it is unclear if they also influence adiposity development in the infants in the first months of life. This is of particular importance as the prevalence of overweight in adult women has increased from 29.8% to 43%.(29) Thus, many pregnant women are nowadays overweight or obese.

2.5 Appetite regulating hormones

Growth during early infancy may influence the appetite regulatory system by programming appetite regulating hormones, such as orexigenic (ghrelin) and anorectic (peptide YY) hormones, as shown in animal studies.(60) Stimulating hormones, such as ghrelin, are important in the initiation, cessation and frequency of eating. Other hormones, such as leptin and peptide YY (PYY), decrease food intake and increase metabolic rate. The glucose-dependent insulinotropic peptide (GIP) stimulates pancreatic beta-cells in response to the ingestion of meals or glucose.(61, 62) All these signals act at several central nervous system (CNS) sites but the pathways converge to the hypothalamus, which contains a large number of peptides and other neurotransmitters that influence food intake. Adipose tissue influences appetite regulation by producing appetite regulating hormones, which play a role in the regulation of food intake and body composition by signaling satiety and energy reserves through hypothalamic receptors. However, little

is known about the relation of type of feeding and body composition with appetite regulating hormones in early life.(63)

2.6 Microbiome

It has been suggested that several other factors contribute to the development of obesity, such as (epi)genetic factors, hormones, stress, underlying illnesses, medications, infections and also the gut microbiome.(64)

The human gut is colonized with 100 trillion microbe cells, which are part of the intestinal microbiome. The composition of the microbiome varies between individuals. It is well-known that gut microbiota play an important role in human health by providing a barrier for colonization of pathogens, by stimulating the development of the immune system, but also by exerting important metabolic functions.(65) Because the human gut microbiota are involved in many aspects of human health, it is essential to understand how the composition of the microbiome is established. In early life, there are major changes in the composition of the intestinal microbiome.(66-71) It has been proposed that obesity in adults may be related to the composition of the gut microbiota.(72, 73) Obese adults have different microbiota compared to non-obese individuals and it has been shown that adults who lost weight develop different stool microbiota, but there are limited data available about the gut microbiota in infants, particularly during periods of losing or gaining weight.(74) Differences in growth and body composition could play a role in the establishment of the microbiome or vice versa. Longitudinal data about the development of the microbiome in parallel with data on type of feeding and body composition (ea. fat mass percentage (FM%) and fat-free mass (FFM) are essential to unravel the interactions between early life factors and the composition of gut microbiota.

3. Hypotheses

We hypothesized that accelerated gain in weight for length in the first months of life leads to the development of an unfavorable body composition with higher FM% and more visceral fat in infancy and a higher risk for non-alcoholic fatty liver disease (NAFLD) in young adulthood, in those born term as well as those born preterm.

We also hypothesized that maternal data, type of feeding, levels of appetite regulating hormones will influence the development of infant body composition. Besides this, we hypothesized that an unfavorable gut microbiome will be associated with an unfavorable infant body composition.

4. The Program and PREMS study cohorts and Sophia Pluto Study birth cohort

To investigate the relationship between childhood growth and determinants of adult disease in early adulthood, the PROgramming factors for Growth and Metabolism (PROGRAM) study and PREMaturity and Small for gestational age (PREMS) study were initiated. The inclusion and exclusion criteria are described in Appendix A. The PROGRAM study consists of 323 healthy young adults born term and the PREMS study of 169 healthy young adults, born preterm (gestational age < 36 weeks).

Based on the outcomes of the PROGRAM study, the Sophia Pluto Study birth cohort was initiated to prospectively identify determinants of adult diseases in early life. The inclusion and exclusion criteria are described in Appendix B. The inclusion into the Sophia Pluto Study is ongoing.

5. Aims of the studies

This thesis describes the results of 2 studies performed in young adults, aged 18-24 years, who participated in either the PROGRAM or PREMS study. These studies were started to investigate if size at birth, preterm birth, and different growth patterns during childhood influence determinants of adult diseases, in this thesis non-alcoholic fatty liver disease. Study designs are described in Appendix A.

Based on the outcomes of the PROGRAM study, the Sophia Pluto Study birth cohort was started in 2012. This study was started to examine the postnatal determinants of body composition development during infancy. Study design is described in Appendix B. In this thesis we describe the results of 5 studies in healthy infants, born term, who participate in the Sophia Pluto Study.

5.1 Growth in early life and risk for non-alcoholic fatty liver disease

First year growth data were analyzed to investigate if gain in weight relative to length during a specific time period in the first year had an influence on determinants of non-alcoholic fatty liver disease in young adults born term. Subsequently, we investigated if adults born preterm with accelerated weight gain after term age are at an increased risk for NAFLD compared with those without accelerated weight gain during infancy.

5.2 Body composition during first months of life

To investigate the development of body composition during early infancy, including total fat mass (FM) and total fat mass percentage (FM%), measured by Peapod and abdominal subcutaneous fat and visceral fat, measured by ultrasound and to generate reference values for healthy newborns and infants.

5.3 Determinants influencing body composition at birth and in the first months of life

To investigate the influence of maternal, fetal, feeding and growth determinants on body composition at birth and during the first 6 months of life.

5.4 Body composition and appetite regulating hormones

To investigate (fasting) serum ghrelin, leptin, insulin, GIP, PP and PYY at 3 months and 6 months and associations with type of feeding and body composition (including FM% and visceral and subcutaneous fat) at 1, 3 and 6 months of age.

5.5 Body composition and microbiome

To study the composition of the gut microbiome, including secretory immunoglobulin A (sIgA), short-chain fatty acids and bacteria, at the first and third month of life and subsequently identify correlations of microbiota with type of feeding, early weight gain and infant's body composition in the first months of life.

6. Outline of the thesis

Chapter 1 gives an introduction in the topics described in this thesis.
Chapter 2 reports the association of gain in weight relative to length during different periods with determinants of non-alcoholic fatty liver disease in young adults born term.
Chapter 3 reports the association of gain in weight relative to length during different periods with determinants of non-alcoholic fatty liver disease in young adults born preterm.
Chapter 4 presents associations of fetal growth, maternal data and neonatal anthropometrics with infant's body composition at birth.
Chapter 5 presents longitudinal reference data of body composition, including total fat mass and subcutaneous and visceral fat.
Chapter 6 reports determinants of (changes in) body composition during infancy, including infant characteristics, type of feeding and maternal determinants.
Chapter 7 presents levels of appetite regulating hormones in association with type of feeding and body composition at 3 and 6 months of age.
Chapter 8 describes the composition of the gut microbiome at the first and third month of life and present correlations of sIgA, short-chain fatty acids and microbiota with type of feeding, early weight gain and infant's body composition in the first months of life.
Chapter 9 discusses the results of the studies in relation to the current literature and gives conclusions, clinical implications and directions for future research.
Chapter 10 summarizes the findings of the studies in English and Dutch.

Appendix A

The PROGRAM and PREMS study cohorts

To investigate the relationship between childhood growth and determinants of adult disease in early adulthood, the PROgramming factors for Growth and Metabolism (PROGRAM) and PREMaturity and Small for gestational age (PREMS) were initiated.

Subjects

The PROGRAM study consists of 323 healthy young adults born term, the PREMS study of 169 healthy young adults born preterm (gestational age <36 weeks). Participants were recruited from hospitals in The Netherlands, where they had been registered because of being small at birth (SGA with a birth length below - 2 SDS) and/or showing short stature (with an adult height below - 2 SDS after being born SGA or appropriate for gestational age). In addition, healthy subjects (neither small at birth nor having short stature) from schools with different educational levels were randomly asked to participate.

Inclusion criteria

- Chronological age at inclusion: 18.00-23.99 years
- Neonatal period without signs of severe asphyxia (defined as an Apgar score below three after five minutes), no serious diseases such as long-term artificial ventilation and oxygen supply, bronch-pulmonary dysplasia or other chronic lung disease
- Well documented growth data
- Caucasian
- Born singleton
- Signed informed consent
- PROGRAM study: gestational age of 36 weeks or more
- PREMS study: gestational age of less than 36 weeks

Exclusion criteria

- Chromosomal disorders, known syndromes and serious dysmorphic symptoms suggestive for a yet unknown syndrome, except Silver-Russell Syndrome
- Any disease, endocrine or metabolic disorder that could interfere with growth during childhood (such as diabetes, growth hormone deficiency, malignancies, severe chronic disease)
- Treatment that could have interfered with growth (such as radiotherapy or growth hormone treatment)

Study design

The PROGRAM and PREMS study were cross-sectional studies. The wide range in birth size and childhood growth patterns increased the contrast in the study population, which increased the statistical power to detect relationships between various factors.

Appendix B

The Sophia Pluto study cohort

The Sophia Pluto Study birth cohort was initiated based on the outcomes of the PROGRAM study, to prospectively identify determinants of adult disease in early life.

Subjects

At the time of the studies, 300 healthy infants were included in the Sophia Pluto Study and were included in this thesis. The inclusion into this study is still ongoing.

Inclusion criteria

- Gestational age of 37 weeks or more
- Age < 28 days
- Uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score below three after five minutes), sepsis or long-term complication of respiratory ventilation

Exclusion criteria

- Known congenital or postnatal diseases that could interfere with body composition development
- Confirmed intra-uterine infection
- Maternal use of corticosteroids or significant maternal medical condition that could interfere with infant's body composition development (e.g. diabetes)

Study design

The Sophia Pluto Study is a prospective observational follow-up study of a birth cohort. The infants were included before 28 days and visited the outpatient clinic at 1, 3, 6 and 9 months and 1 year, 18 months and 2 years. During the visits, anthropometrics, body composition and various other parameters were measured. Before the age of 9 months, total FM% was measured by air-displacement plethysmography (Peapod) and after this age by DXA-scan. Visceral and subcutaneous fat were measured by ultrasound.

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2

Chapter 2

Accelerated infant weight gain and risk for non-alcoholic fatty liver disease in early adulthood

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ABSTRACT

Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic metabolic syndrome. Some studies demonstrated an association between small size at birth and NAFLD. Rapid catch-up in weight often follows small birth size and has been associated with metabolic syndrome, but its association with NAFLD remained unknown.

Patients and Methods

In 268 adults aged 18-24 yrs, BMI, waist circumference, triglyceride, gamma-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), alanine aminotranserase (ALT), and aspartate aminotransferase (AS) levels were determined. Fatty liver index (FLI, 0-100) was calculated. Associations of birth weight SDS and first year gain in weight- and length SDS were determined with FLI and other liver markers. Comparisons were performed between subjects with and without rapid catch-up in weight in the first year of life. Furthermore, a FLI-score (low, intermediate, high risk for NAFLD) was assigned to each participant to determine clinical relevance, and ordinal regression analyses were performed.

Results

Gain in weight in the first three months of life was associated with FLI as a continuous variable, whereas low birth weight was not. There were no significant associations with γ-GT, ALT, or AST.

Of the subjects with rapid catch-up in weight for length, 27.8% had an intermediate or high FLI at the age of 21 years, compared with 5.3% of subjects with slow catch up. Rapid catch-up was also associated with a higher FLI-score after adjustments (odds ratio:11.7, p-value:0.016).

Conclusion

Accelerated gain in weight for length in the first three months of life is associated with a higher risk for NAFLD in early adulthood, whereas small size at birth is not.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition that resembles that of alcohol induced liver injury, but it occurs in patients who do not abuse alcohol.(1) NAFLD has the potential to progress to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. (1) Furthermore, NAFLD is considered being the hepatic manifestation of the metabolic syndrome, and has been associated with several risk factors for cardiovascular disease (CVD) and type 2 diabetes, such as adverse lipid profile and body composition, and insulin resistance.(1,2) Given the strong association of NAFLD with obesity, the prevalence of NAFLD is rapidly increasing.(3)

Although some findings point to an association of low birth weight with liver diseases (4), only few studies investigated associations between early life factors and NAFLD in adulthood. Fraser et al. reported an association between low birth weight and increased markers of liver damage in a large group of women aged 60-79 year.(5,6) Furthermore, Nobili et al. reported an association between pediatric NAFLD and being born small for gestational age (SGA).(7) However, the role of growth during the first year of life in these associations remained unknown. The majority of children born SGA show catch-up growth within 2yrs after birth, resulting in a normal stature.(8) Accelerated gain in weight for length in early life has been associated with prevalence of the metabolic syndrome at age 21 years, independently from birth weight (9). Given that NAFLD is considered the hepatic metabolic syndrome,(10) it might well be that the previously found association between small size at birth and NAFLD can also be ascribed to accelerated early weight gain. Based on our previous work,(11,12) we hypothesized that rapid gain in weight for length in the first three months of life, rather than low birth weight, is associated with increased risk for developing NAFLD. We therefore investigated associations of birth weight, first-year gain in weight for length, and rapid catch-up in weight with the fatty liver index (FLI) at 21 years.(13) The FLI is a measure to predict hepatic steatosis in the general population (14), and is developed using an algorithm based on BMI, waist circumference, triglycerides, and gamma-glutamyltransferase (γ-GT), with an accuracy of 0.84 (95%CI 0.81-0.87) in detecting fatty liver.(13,15) The FLI has been widely used in NAFLD research,(16-20) and has been recently validated for identifying NAFLD in a large population-based study.(15) Additionally, we investigated associations with individual biochemical markers of the FLI, and with other markers indicating liver damage (alanine aminotranserase (ALT), aspartate aminotransferase (AST), γ-GT, and alkaline phosphatase (ALP, mainly associated with cholestasis)).(2,6,21,22)

STUDY DESIGN

Subjects

The PROGRAM study cohort consists of 323 healthy participants with an age between 18 and 24 years. Participants were recruited from hospitals in the Netherlands, where they had been registered because of being small at birth (SGA with a birth length below -2 SDS, n=108) (23) and/or showing short stature (with an adult height below -2 SDS after being born SGA or appropriate for gestational age n=77).(24) In addition, healthy subjects (neither small at birth nor having short stature) from schools with different educational levels were randomly asked to participate (n=83).

This design was purposely chosen to increase the contrast within the study population regarding birth size and adult stature. All participants fulfilled the same inclusion criteria: 1) age 18-24 years, 2) born singleton, 3) born at term (≥ 36 weeks of gestational age), 4) Caucasian, 5) uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score below 3 after 5 minutes), without sepsis or long-term complications of respiratory ventilation, such as broncho-pulmonary dysplasia, 6) maximum duration of respiratory ventilation and/or oxygen supply in the neonatal period of 2 weeks. Subjects were excluded if they had been suffering from any serious complication or condition (including necrotizing enterocolitis, intra-ventricular hemorrhage with a degree of 3 or more, spastic hemiplegia or quadriplegia), from any disease or had received any treatment known to interfere with growth (e.g. growth hormone deficiency, severe chronic illness, emotional deprivation, growth hormone treatment, treatment with glucocorticosteroids, radiotherapy) or if they had endocrine or metabolic disorders, chromosomal defects, syndromes or serious dysmorphic symptoms suggestive for a yet unknown syndrome.

Birth data were taken from records of hospitals, community health services and general practitioners. Information regarding socio-economic status (SES), alcohol use,and oral contraceptive use was obtained using questionnaires. Educational level of the participant was used as socio-economic indicator to determine SES.(25) None of the participants had alcoholic abuse. In the Netherlands, periodical measurements of weight and length are performed for each child. Thus, weight and length at 3, 6, 9, and 12 months after birth had been measured prospectively at primary health care centers or hospitals. These data were collected from the records of the health care centers and hospitals during the study period March 2006-September 2007.

The Medical Ethics Committee of Erasmus Medical Centre, Rotterdam the Netherlands, approved the study. Written informed consent was obtained from all participants. Of 323 subjects, data on biochemical markers of NAFLD, liver damage and liver function were available for 268 subjects (of whom n=80 were born SGA). Data on first-3 month's growth were available for 182 of 268 subjects.

Measurements

All participants visited the Erasmus Medical Centre in Rotterdam and were reimbursed for travel expenses. Prior to the visit, participants fasted for at least 12 hours and abstained from smoking and drinking alcohol for at least 16 hours. All anthropometric measurements were performed twice; the mean value was used for analyses. Height was measured to the nearest 0.1cm by a Harpenden stadiometer and weight to the nearest 0.1kg by a scale (Servo Balance KA-20-150S).

All fasting blood samples were drawn between 0800-13.00 h, centrifuged after clotting, and were kept frozen until assayed (-80°C). All serum parameters were determined in the same laboratory. Triglycerides were measured using the GPO-PAP reagent kit (Roche Diagnostics). γ-GT, ALP, ALT, and AST levels were determined using standard laboratory routines.

Statistical analysis

SD-scores for birth length, birth weight, and first year weight and length were calculated to correct for gestational age and sex.(23) SD-scores for adult height and weight were calculated to correct for sex and age.(24) The Fatty Liver Index (FLI) was used to determine the risk for fatty liver disease.(13) FLI is calculated as: $FLI = (e^{0.953 * \ln(\text{triglycerides}) + 0.139 * BMI + 0.718 * \ln(\gamma\text{-GT}) + 0.053 * \text{waist circumference} - 15.745}) / (1 / e^{0.953 * \ln(\text{triglycerides}) + 0.139 * BMI + 0.718 * \ln(\gamma\text{-GT}) + 0.053 * \text{waist circumference} - 15.745}) \times 100$, and varies between 0 and 100. To identify associations of birth weight SDS, birth length SDS, and gain in weight for length in the first three months of life with markers of NAFLD, and liver damage, we performed linear regression analyses with the dependent variables FLI, triglycerides, BMI, waist circumference, γ-GT, AF, ALT, and AST. Non-parametric correlations were performed to study associations of gain in length SDS and gain in weight SDS in first three months of life with FLI. All regression analyses were adjusted for age, gender, gestational age, and SES, and in additional analyses for adult weight SDS. All weight gain analyses were additionally adjusted for gain in length SDS in the same period. The interaction term of birth weight SDS and infant weight gain was not significant (p=0.534), and was therefore not added to the model. When residuals deviated from homogeneity, outcome variables were log transformed. Next, a FLI-score was assigned to the participants as follows: a FLI below 30 resembling a low risk (FLI-score 1, sensitivity=87%(13)), from 30 up to 60 an intermediate risk (FLI-score 2), and above 60 a high risk (FLI-score 3, specificity=86%(13)) for fatty liver. Ordinal regression analyses were performed to determine associations of FLI-score with birth weight SDS, birth length SDS, and weight gain during the four three-month periods in the first year of life.

To study the generalizability of the results in the total group separate analyses were performed using only subjects born appropriate for gestational age.

Additionally, the total study group was divided into two groups, irrespective of birth length or birth weight, one with catch-up in weight during the first year of life and one without it. Catch-up in weight was defined as an SDS of more than 0.67 of weight gain in the first year of life.(26) Of the group with weight catch-up, two subgroups were formed based on rapid (SD>0.5) or slow (SD<0.5) weight gain during the first three months. (11,12) Of the group with rapid weight gain (n=54), 35 subjects were born SGA, and of the group with slow weight gain (n=19), 12 subjects were born SGA. The unadjusted differences between subjects with rapid and slow catch-up with regard to FLI-score were determined with Pearson Chi-Square, and adjusted differences with ordinal regression. Adjusted differences with regard to FLI as a continuous variable were determined using estimated marginal means. All subgroup-analyses were adjusted for age, gender, gestational age, SES, and gain in length in the first year of life.

Statistical package SPSS version 20.0 (SPSS, Inc., Chicago, IL) was used for analyses. Results were regarded statistically significant if p was <0.05 (two-sided).

RESULTS

Clinical characteristics, the Fatty Liver Index (FLI) and its components, and other liver markers are shown in Table 1. Mean(SD) FLI was 13.0(17.1). A high FLI score was present in 3% of the total study group.

Table 1. Clinical characteristics, components of the FLI, and other markers for liver damage

	Total study group		Subgroups with catch-up in first year				p-value
			Rapid catch-up		Slow catch-up		
	N	Mean(SD)	N	Mean(SD)	N	Mean(SD)	
Age	268	20.9(1.66)	54	20.7(1.68)	19	21.0(1.94)	0.557
Gender (M/F)	268	107/161	54	17/37	19	11/8	0.042
Gestational age	268	39.2(1.59)	54	38.6(1.46)	19	39.2(2.00)	0.145
Birth weight SDS	268	-1.12(1.36)	54	-2.19(0.69)	19	-2.09(0.90)	0.620
3 months weight SDS	182	-0.91(1.10)	54	-0.70(0.87)	19	-2.13(0.95)	<0.001
6 months weight SDS	181	-0.83(1.02)	54	-0.40(0.80)	19	-1.51(0.81)	<0.001
9 months weight SDS	178	-0.80(1.07)	54	-0.36(0.73)	19	-1.00(0.82)	0.002
12 months weight SDS	173	-0.72(1.01)	54	-0.28(0.74)	19	-0.77(0.78)	0.017
Birth length SDS	268	-1.39(1.41)	54	-2.13(1.15)	19	-2.07(1.55)	0.859
3 months length SDS	182	-1.02(1.21)	54	-1.04(1.01)	19	-1.71(1.14)	0.018
6 months length SDS	180	-0.94(1.19)	54	-0.61(0.99)	19	-1.60(1.08)	<0.001
9 months length SDS	178	-0.91(1.26)	54	-0.45(0.93)	19	-1.27(1.03)	0.002
12 months length SDS	176	-0.92(1.20)	54	-0.42(0.94)	19	-1.16(1.02)	0.005

Table 1. Clinical characteristics, components of the FLI, and other markers for liver damage (continued)

		Total study group		Subgroups with catch-up in first year				
				Rapid catch-up		Slow catch-up		p-value
		N	Mean(SD)	N	Mean(SD)	N	Mean(SD)	
Adult height SDS		268	-1.00(1.36)	54	-0.63(0.91)	19	-1.01(0.91)	0.118
Adult weight SDS		268	-0.63(1.43)	54	0.04(1.12)	19	-0.72(1.03)	0.011
TG (mg/dl)		265	91.0(44.2)	54	103.9(46.3)	19	85.1(29.2)	0.102
BMI (kg/m)		268	22.4(3.51)	54	23.5(3.59)	19	21.8(2.45)	0.060
Waist (cm)		268	76.9(9.98)	54	91.4(11.2)	19	75.7(8.65)	0.047
γ-GT (U/l)		266	17.3(13.5)	54	22.4(24.6)	19	16.9(8.40)	0.342
FLI		264	13.0(17.1)	54	21.6(23.4)	19	10.2(12.1)	0.046
FLI-score%	Low	233	88.3	39	72.2	18	94.7	
	Intermediate	23	8.7	11	20.4	1	5.3	
	High	8	3.0	4	7.4	0	0	
ALT (U/l)		266	20.3(15.3)	54	23.1(24.2)	19	19.4(11.2)	0.527
AST (U/l)		266	25.8(30.2)	54	25.7(13.2)	19	25.1(10.0)	0.928
ALP (U/l)		267	69.0(22.1)	54	70.5(19.6)	19	70.9(24.1)	0.931

SDS: Standard deviation score, waist: waist circumference, BMI: body mass index, TG: triglycerides, γ-GT: glutamyltransferase, FLI: fatt liver index (0-100), ALT: alanine aminotransferase, AST: aspartate aminotransferase. ALP: alkaline phosphatase. An FLI-score below 30 resembling a low risk (FLI-score 1), from 30 up to 60 an intermediate risk (FLI-score 2), and above 60 a high risk (FLI-score 3) for fatty liver.
Rapid catch-up= weight gain >0.67 SDS in first year of life and weight gain >0.5 SDS in first three months, compared with slow catch-up (weight gain >0.67 SDS in first year of life and weight gain <0.5 SDS in first three months)

Fatty liver index

Total study population

In Table 2, results from linear regression analyses are shown, with FLI (0-100) and individual components of the FLI (TG, BMI, waist circumference, and serum levels of γ-GT) as dependent variables. Gain in weight SDS for length SDS in the first three months of life was significantly associated with FLI at age 21 years (FLI increased with 27.4% per SDS increase in weight gain, p=0.003, Model 2). Gain in weight SDS for length SDS in the first three months of life showed also significant associations with higher serum TG level, BMI, and waist circumference. Furthermore, lower birth weight SDS was initially associated with a higher TG level and waist circumference at age 21 years (Model 1), but this association disappeared after additional adjustment for adult weight SDS (data not shown). There were no significant associations of gain in weight SDS (p=0.227) without correction for length or gain in length SDS (p=0.144) in the first three months of life with FLI. To study the generalizability of our results for all infants we performed separate analyses using only the subjects born appropriate for gestational age. This also showed

Table 2. Associations of birth size and early weight gain with components of the FLI, and other markers for liver damage and liver function at 21 years

					FLI components											
		Fatty liver index			TG*			BMI*			Waist*			γ-GT*		
		Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	P	Adj. R^2
Model 1[1]	Birth weight SDS	-10.24	0.151	0.025	**-6.274**	**0.043**	0.060	-0.421	0.698	-0.005	**-1.816**	**0.035**	0.099	1.021	0.794	0.024
Model 2[2]	Gain in weight 0-3mo	**27.35**	**0.003**	0.096	**9.386**	**0.009**	0.077	**2.358**	**0.038**	0.047	**2.243**	**0.010**	0.144	4.847	0.267	0.067

*:log transformed (natural logarithm) for regression analyses

Beta: regression coefficient in %, p: p-value

All analyses are adjusted for gender, age, SES, and gestational age

[1](getal in supersript)Adjusted for gender, age, SES, gestational age and birth length SDS

[2](getal in superscript)Adjusted for gender, age, SES, gestational age and gain in length 0-3mo

a significant association of gain in weight SDS for length SDS in the first three months with FLI (p=0.032).

There were no significant associations of birth weight SDS or infant weight gain with ALT, AST, and ALP, also not after additional correction for adult weight SDS.

Catch-up subgroups

To assess if tempo of weight gain was associated with determinants of NAFLD in young adults, those with catch-up in weight were divided in two subgroups with either rapid or slow catch-up in weight. Of all young adults with a clinically relevant catch-up in weight of at least 0.67 SDS in the first year of life (n=73), 54 had a weight gain of more than 0.5 SDS in the first 3 months (rapid catch-up), while 19 had a weight gain of less than 0.5 SDS in the first 3 months (slow catch-up). The clinical characteristics of these two subgroups and unadjusted differences are shown in Table 1. Besides the differences in early growth, the rapid weight catch-up group had higher unadjusted FLI (p=0.046), adult weight SDS (p=0.011), and waist circumference (p=0.047) at age 21yr. Furthermore, subjects with rapid catch-up had a higher mean FLI than subjects with slow catch-up, adjusted for gender, SES, gestational age, and gain in length during the first year of life (p=0.019, Figure 1). This difference remained also significant (p=0.016) after additional adjustment for birth weight SDS. Because the rapid catch-up group comprised of relatively more female than male subjects, and 78% of the female subjects in the present study population used oral contraceptives, we additionally adjusted for oral contraceptive use, which did not change the results (data not shown).

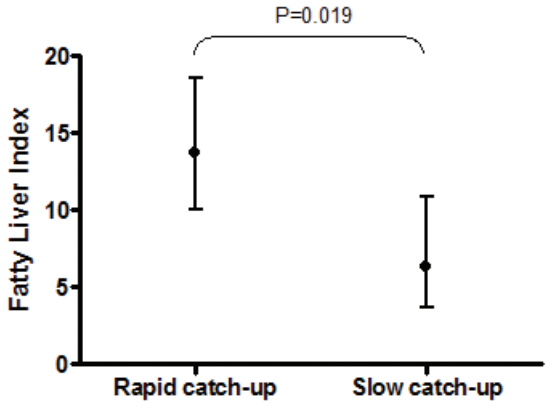

Figure 1. Adjusted Fatty Liver Index of subjects with rapid compared to slow catch-up
Estimated marginal means (95% confidence interval)
Rapid catch-up = weight gain >0.67 SDS in first year of life and weight gain >0.5 SDS in the first three months. Slow catch-up = weight gain >0.67 SDS in first year of life and weight gain <0.5 SDS in first three months. Adjusted for gender, SES, gestational age and gain in length in first year of life. P=0.017 after additional adjustment for birth weight SDS.

Fatty liver index score

A FLI-score was assigned to each participant in order to obtain a NAFLD-risk score (1: low, 2: intermediate, or 3: high). This NAFLD-risk score is used to determine the clinical relevance of findings with a FLI <30 resembling a low risk for fatty liver, a FLI from 30 up to 60 resembling an intermediate risk, and a FLI >60 a high risk for fatty liver.(13)

Influence of early catch-up in weight

Of all participants, 3% (n=8) had a high FLI-score, and 8.7% (n=23) had an intermediate FLI-score (Table 1). Table 3 shows the results of the ordinal regression analyses with FLI-score as dependent variable. Gain in weight SDS in the first three months of life was associated with a higher FLI-score at the age of 21 years (OR: 1.83, CI: 1.15-2.92), adjusted for age, gender, gestational age, SES, and gain in length in the same period. Thus, per one SDS increase in early weight gain, the risk of having a higher FLI-score at 21 years increases with 83%. There were no significant associations between weight gain during the other three-month periods in the first year of life and the FLI-score. Lower birth weight SDS was also not associated with a higher FLI-score at 21 years.

Table 3. Associations of birth size and first year weight gain with FLI-score at 21 years

	FLI-score		
	Odds ratio	95% CI	p-value
Birth weight SDS[1]	0.64	0.39-1.07	0.077
Gain in weight SDS			
0-3 months[2]	**1.83**	**1.15-2.92**	**0.011**
3-6 months[2]	1.08	0.45-2.61	0.859
6-9 months[2]	1.52	0.45-5.11	0.498
9-12 months[2]	0.87	0.23-3.25	0.830
Rapid versus slow catch-up[3]	**11.7**	**1.12-113.2**	**0.016**

FLI-score determined as follows: an FLI<30 equals a low risk (score 1), an FLI of 30-60 an intermediate risk (score 2), and an FLI >60 a high risk (score 3) for fatty liver

Rapid catch-up= weight gain >0.67 SDS in first year of life and weight gain >0.5 SDS in first three months, compared with slow catch-up (weight gain >0.67 SDS in first year of life and weight gain <0.5 SDS in first three months)

Significant odds ratios are indicated in bold

CI: confidence interval

[1]Adjusted for age, gender, gestational age, SES, and birth length SDS

[2]Adjusted for age, gender, gestational age, SES, and gain in length SDS in the same period

[3]Adjusted for age, gender, gestational age, SES, and gain in length SDS in the first year of life

Rapid versus slow catch-up in weight

Of the subjects with rapid catch-up in weight for length, 27.8% had an intermediate or high FLI-score compared with 5.3% of the subjects with slow catch-up in weight for length. Ordinal regression analyses showed that rapid catch-up was significantly associated with a higher FLI-score (OR: 11.7, CI: 1.12-113.2), adjusted for age, gender, gestational age, SES, and gain in length during the first year of life (Table 3). The association remained significant after additional adjustment for birth weight SDS (data not shown).

DISCUSSION

In the present study we investigated whether low birth weight and/or accelerated infant weight gain are associated with increased risk for developing non-alcoholic fatty liver disease (NAFLD) in early adulthood. We showed that gain in weight SDS in the first three months of life was associated with a higher fatty liver index (FLI) at 21 years, a validated measure to predict hepatic steatosis in the general population, whereas low birth weight SDS was not. This was also found in subjects born appropriate for gestational age.

We also found that subjects with rapid catch-up in weight for length have a significantly higher FLI in early adulthood than subjects with slow catch-up in weight, also after adjustment for birth weight SDS. Furthermore, when a FLI-score (low, intermediate, or high risk for NAFLD) was assigned to each participant, and ordinal regression analyses was performed, it was shown that higher gain in weight for length in the first three months of life associates with more risk of having a higher FLI-score in early adulthood (odds ratio:11.7, p-value:0.016), whereas low birth weight does not.

Not only subjects born small for gestational age were at risk for having a higher fatty liver index after accelerated weight gain in weight SDS for length SDS in the first 3 months, but also subjects born appropriate for gestational age. We, therefore, conclude that the our findings are not specific for children born SGA but can be generalized to the whole population of infants.

The FLI refers to a clustering of risk factors, being a condition underlying hepatic steatosis.(13) As our study population consists of healthy young adults, it was not possible to study hard endpoints such as NAFLD, steatohepatitis, and fibrosis. We therefore used FLI as a proxy. A limitation of this approach is the arbitrary character of the FLI definition and the subsequent possibility to miss critical information. We, therefore, decided to also study the components of the FLI separately, and in addition to investigate determinants of liver damage (ALT, AST, ALP). Although the participants of the present study were relatively young, a high FLI was already found in 8 participants. A high FLI-score (above 60) has a positive predictive value of 72.2% for fatty liver.(14) Furthermore, it was striking that 27.8% of the subjects with rapid catch-up in weight for length had an intermediate

or high FLI at the age of 21 years, compared with 5.3% of the subjects with slow catch-up in weight for length.

Components of the Fatty Liver Index overlap the components of metabolic syndrome, but the FLI give additional information. It is possible to have adiposity without an higher risk for NAFLD and also it is possible to have a Non-Alcoholic fatty liver disease as a lean person.(27,28)

To our knowledge, this is the first study investigating the relationship of both small size at birth and accelerated weight gain during infancy with NAFLD risk in early adulthood. One recent paper reported that small body size at birth and at age two was associated with a higher likelihood of NAFLD in adulthood.(29) Furthermore, the authors showed that the most pronounced results were among the subjects who were smallest at age two and subsequently obese in adulthood. Unfortunately, these results cannot be compared with the results of the present study because gain in weight during the first year of life was not studied. We previously showed that accelerated weight gain during infancy is associated with risk factors for cardiovascular disease, insulin resistance, obesity, and the metabolic syndrome in adulthood,(9,11,12,30,31) outcomes which are closely related to NAFLD.(1,2,32) The present results show that subjects with higher gain in weight for length in the first three months of life are also at higher risk of developing NAFLD in early adulthood. We did, however, not find an association of accelerated infant weight gain with all the components of the FLI. More specifically, no significant association was found with γ-GT, and other markers of liver damage, such as ALT and AST. Furthermore, some of the components used to determine NAFLD risk in the present study overlap with the components of the metabolic syndrome.(13,33) Our findings warrant further research into more specific diagnostic markers of NAFLD to confirm our hypothesis that subjects with rapid catch-up in weight for length during infancy have higher risk to develop NAFLD in adulthood. Examples of more specific diagnostic markers of NAFLD are liver ultrasound measurements and the gold standard liver histology. The latter is, however, not feasible because it is unethical to perform liver biopsy in healthy subjects.

Other studies showed that low birth weight was associated with higher NAFLD prevalence.(5-7) These studies, however, did not investigate or adjust for the effect of early weight gain, which appears to be highly associated with low birth weight. In the present study we did not find a significant association between a low birth weight SDS and FLI. Furthermore, we showed that the difference between subjects with and without rapid catch-up in weight during infancy is not due to small size at birth, as the difference remained significant after adjustment for birth weight SDS.

Formula-fed infants have a higher risk of being overweight in childhood and later in life.(34,35) Infant feeding might thus influence later risk for NAFLD. Studies in rats showed that the liver might act as a systemic buffer, largely increasing its lipid content in the early stage of high-fat feeding.(36) It has also been suggested that breast feed-

ing protects against the development non-alcoholic steatohepatitis (NASH) in children with NAFLD.(37) Our study did not have nutritional data to investigate the relationship between early nutrition, growth in infancy, and NAFLD risk factors in later life, but our findings suggest that the use of nutrient-enriched formulas, which induce rapid weight gain in early life, might increase the risk for NAFLD later in life.

In conclusion, our study indicates that accelerated gain in weight compared to length in the first three months of life is associated with a higher risk to develop NAFLD in adulthood. Long-term prospective studies are warranted to study the effect of early gain in weight for length on more specific markers of NAFLD, and the role of infant feeding.

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SUPPLEMENT

Supplemental table 1.

		Subgroups with catch-up in first year				
		SGA		AGA		p-value
Clinical characteristics		N	Mean(SD)	N	Mean(SD)	
Age		108	21.0(1.6)	160	20.8(1.7)	0.349
Gender (M/F)		108	44/64	160	63/97	0.823
Gestational age		108	39.0(1.5)	160	39.4(1.6)	**0.023**
Birth weight SDS		108	-2.14(0.7)	160	-0.43(1.3)	**<0.001**
3 months weight SDS		77	-1.47(1.0)	105	-0.49(1.0)	**<0.001**
Birth length SDS		108	-2.79(0.7)	160	-0.45(0.9)	**<0.001**
3 months length SDS		76	-1.74(1.0)	106	-0.51(1.1)	**<0.001**
Adult height SDS		108	-1.24(1.2)	160	-0.84(1.5)	**0.017**
Adult weight SDS		108	-0.55(1.4)	160	-0.68(1.4)	0.460
TG (mg/dl)		108	96.4(47.5)	157	87.3(41.6)	0.100
BMI (kg/m^2)		108	23.1(3.9)	160	21.9(3.1)	**0.006**
Waist (cm)		108	78.6(10.9)	160	75.8(9.2)	**0.023**
γ-GT (U/l)		108	19.0(18.1)	158	16.1(8.9)	0.078
FLI		108	16.4(20.6)	156	10.7(13.8)	**0.007**
	Low	91	84.3	142	91.0	
FLI-score%	Intermediate	12	11.1	11	7.05	0.471
	High	5	4.63	3	1.92	
ALT (U/l)		108	22.0(19.1)	158	19.2(11.9)	0.139
AST (U/l)		107	25.3(14.7)	159	26.2(37.3)	0.823
ALP (U/l)		108	71.7(22.5)	159	67.1(21.6)	0.097

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3

Chapter 3

Risk for non-alcoholic fatty liver disease in young adults born preterm

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ABSTRACT

Background

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of metabolic syndrome. Accelerated catch-up in weight during infancy in subjects born term has been associated with increased risk for NAFLD in adulthood, but this association has not been studied in subjects born preterm.

Methods

In 162 young adults, gestational age<36 weeks, we assessed associations between fatty liver index (FLI, 0-100) and birth weight SDS and first year gain in weight. We performed comparisons between subjects with and without accelerated catch-up in weight in the first year after term age. A FLI-score was assigned to each participant to determine clinical relevance, and regression analyses were performed.

Results

Accelerated gain in weight in the first three months after term age was associated with FLI as a continuous variable, whereas gestational age and low birth weight were not. Of the subjects with accelerated catch-up in weight for length after term age, 7.3 % had a high FLI at the age of 21 years, whereas none of the subjects without accelerated catch-up in weight had a high FLI.

Conclusion

Our study shows that accelerated weight gain after term age is associated with increased risk for developing NAFLD in young adults born preterm.

INTRODUCTION

Nowadays, 8-10% of all newborns in developed countries are born preterm and due to advances in neonatal intensive care, most of them reach adulthood.(1) Preterm infants are frequently exposed to fetal growth restriction, glucocorticoid treatment and stressful events, which may contribute to the development of organ dysfunction and adverse vascular outcome later in life. In recent years, long-term outcomes for young adults born preterm were found to be less favorable than for those born term. Adults born preterm are at increased risk for developing cardiovascular diseases (CVD)(2-4), and have increased cardiovascular mortality.(5, 6)

Non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic manifestation of metabolic syndrome, and has been associated with several risk factors for CVD.(7) As NAFLD is strongly associated with obesity, its prevalence is rapidly increasing.(8)

Recently, our group showed that young adults, who were born term and underwent accelerated weight gain during infancy had an increased fatty liver index, a measure of NAFLD in early adulthood.(9) While the gold standard for determining NAFLD is liver biopsy, the Fatty Liver Index (FLI) is widely used and has recently been validated for identifying NAFLD in a large population-based study.(10-14) The FLI is a measure to predict hepatic steatosis in the general population, using an algorithm based on BMI, waist circumference, triglycerides, and gamma-glutamyltransferase (γ-GT), with in case of a high FLI-score (above 60) a positive predictive value of 72.2% in detecting fatty liver.

Because many preterm infants are prone for accelerated weight gain after an initial period of weight loss (15), we hypothesized that adults born preterm with accelerated weight gain after term age are at increased risk for NAFLD compared with those without accelerated weight gain during infancy. We also hypothesized that adults born preterm have a higher FLI than adults born term.(9, 16, 17) We, therefore, investigated associations of birth weight, gain in weight for length and accelerated catch-up in weight in the first year after term age with the FLI in general and with individual biochemical markers of FLI as well as with other markers indicating liver damage in a cohort of young adults born preterm.(7, 18-21)

MATERIALS AND METHODS

Study design

Subjects

The study population consisted of 162 healthy young adults (PREMS study) who were registered because of being born preterm (gestational age<36 weeks). Data of partici-

pants were compared with data of 268 young adults born term (PROGRAM study) (9) Both cohorts had similar in- and exclusion criteria, research center, and measurements, except of a different gestational age. All included subjects were aged 18-24 years, Caucasian, born singleton and had an uncomplicated neonatal period without severe asphyxia (defined as an Apgar score below three after five minutes), sepsis, or long-term complications of respiratory ventilation and/or oxygen supply. Birth data were obtained from medical records, primary health care records, and general practitioner records. Data on educational level, socioeconomic status (SES), use of medication and alcohol use were obtained using questionnaires.(22) None of the participants had alcohol abuse, defined as a maladaptive pattern of alcohol use leading to clinically significant impairment or distress. Weight and length at 3, 6, 9, and 12 months after birth had been measured prospectively at primary health care centers or hospitals. The Medical Ethics Committee of Erasmus Medical Centre, Rotterdam, The Netherlands, approved the study. Written informed consent was obtained from all participants.

Measurements

All participants visited the Erasmus Medical Centre in Rotterdam and were reimbursed for travel expenses. Prior to the visit, participants fasted for at least 12 hours. All anthropometric measurements were performed twice; the mean value was used for analyses. Height was measured to the nearest 0.1cm by a Harpenden stadiometer and weight to the nearest 0.1kg by a scale (Servo Balance KA-20-150S).Waist circumference was measured to the nearest 0.1 cm using plastic flexible measuring tape. It was measured at the midway between the lower margin of the lowest rib and the upper margin of the iliac crest, at the end of a normal expiration.

All fasting blood samples were drawn between 08.00-13.00 h, centrifuged after clotting, and were kept frozen until assayed (-80°C). All serum parameters were determined in the same laboratory. Triglycerides were measured using the GPO-PAP reagent kit (Roche Diagnostics). γ-GT, ALP, ALT, and AST levels were determined using standard laboratory routines.

Statistical analysis

SD-scores for birth length, birth weight, and first year weight and length were calculated to correct for gestational age and sex.(23) SD-scores for adult height and weight were calculated to correct for sex and age.(24) The FLI was used to determine the risk for fatty liver disease.(18) FLI is calculated as: $FLI = (e^{0.953 * \ln(\text{triglycerides}) + 0.139 * BMI + 0.718 * \ln(\gamma\text{-GT}) + 0.053 * \text{waist circumference} - 15.745}) / (1/ e^{0.953 * \ln(\text{triglycerides}) + 0.139 * BMI + 0.718 * \ln(\gamma\text{-GT}) + 0.053 * \text{waist circumference} - 15.745}) \times 100$, and varies between 0 and 100. A FLI-score was assigned to the participants as follows: a FLI below 30 resembling a low risk (FLI-score 1, specificity=87%), from 30 up to 60 an

intermediate risk (FLI-score 2), and above 60 a high risk (FLI-score 3, specificity=86% for fatty liver).(18)

Multiple linear regression analyses were performed to investigate the association between weight gain per each three months during one year after term age, and several markers of NAFLD and liver damage in young adults born preterm. The four periods were analyzed separately from each other. In addition, we investigated effect of weight gain from birth to term age at the FLI at 21 years. We performed a multiple regression analysis with FLI at 21 years as dependent variable and as independent variables gestational age, gender, socio-economic status, adult age and as dummy a loss of weight ≥ 1 SDS between birth and term age and no loss in weight.

Ordinal regression analyses were performed to determine associations of FLI- scores with birth weight SDS, birth length SDS, and weight gain during the five periods in the first year of life.

All regression analyses were adjusted for age, gender, gestational age, and SES. To investigate the association between weight and outcome variables independently of length, as a measure for adiposity, adjustments were made for length growth during the same period.

Catch-up in weight in the first year after term age was defined as a weight gain SD-score of more than 0.67, because this represents the width of each percentile band on standard growth curves (second to ninth percentile, ninth to 25th percentile, etc.).(25)

Of the group with catch-up in weight, one subgroup was formed based on accelerated (SD≥0.5) weight gain during the first three months after term age. Additionally, young adults born preterm with accelerated catch-up and without accelerated catch-up in weight were compared to those born term. Differences in clinical characteristics were determined by an independent t-test.

Statistical package SPSS version 20.0 (SPSS, Inc., Chicago, IL) was used for analyses. Results were regarded statistically significant if p was <0.05 (two-sided).

RESULTS

Clinical characteristics, the FLI and its components, and other liver markers are shown in Tables 1 and 2. Mean(SD) FLI was 12.7 (16.4). A high FLI score was present in 5% of the total preterm study group.

Table 1. Clinical characteristics of the total preterm group and subjects with accelerated versus non-accelerated catch-up growth in weight in the first year after term age.

	Preterm				Term				Preterm vs. term	
	Total study group (n=162)	Accelerated catch-up (n=55)	Non-accelerated catch-up (n=36)	p-value*	Total study group (n=268)	Accelerated catch-up (n=54)	Non-accelerated catch-up (n=19)	p-value*	Accelerated p-value	Non-accelerated p-value
Male(%)	50	41.8	50	0.443	107	31.5	57.9	0.042	0.267	0.585
Age	20.8(1.68)	20.5(1.81)	20.8(1.76)	0.595	20.9(1.66)	20.7(1.68)	21.0 (1.94)	0.557	0.683	0.688
Gestationalage	32.0(2.22)	32.7(2.07)	32.1(2.07)	0.146	39.2(1.59)	38.6(1.46)	39.2(2.00)	0.145	<0.001	<0.001
Weight SDS										
Birth	-0.51(1.80)	-0.88(1.75)	-1.16(1.56)	0.439	-1.12(1.36)	-2.19(0.69)	-2.09(0.90)	0.620	**<0.001**	**0.007**
Term age	-1.42(1.17)	-1.58(1.18)	-1.99(0.98)	0.088		-	-	-	-	-
3 months post term	-1.01(1.38)	-0.34(1.38)	-1.99(1.07)	**<0.001**	-0.91(1.10)	-0.70(0.87)	-2.13(0.95)	**<0.001**	0.114	0.625
6 months post term	-0.77(1.23)	-0.06(1.16)	-1.28(0.94)	**<0.001**	-0.83(1.02)	-0.40(0.80)	-1.51(0.81)	**<0.001**	0.072	0.388
9 months post term	-0.68(1.14)	0.01(1.10)	-0.91(0.93)	**<0.001**	-0.80(1.07)	-0.36(0.73)	-1.00(0.82)	**0.002**	0.045	0.740
12 months post term	-0.66(1.17)	0.06(1.12)	-0.69(0.91)	**0.001**	-0.72(1.01)	-0.28(0.74)	-0.77(0.78)	**0.017**	0.065	0.767
Length SDS										
Length	-1.27(1.88)	-1.37(1.96	-1.95(1.72)	0.178	-1.39(1.41)	-2.13(1.15)	-2.07(1.55)	0.859	**0.016**	0.800
Term age	-1.82(1.51)	-1.74(1.51)	-2.56(1.34)	**0.013**		-	-	-	-	-
3 months post term	-1.30(1.22)	-0.98(1.18)	-1.95(1.11)	**<0.001**	-1.02(1.21)	-1.04(1.01)	-1.71(1.14)	**0.018**	0.787	0.464
6 months post term	-0.98(1.09)	-0.56(0.97)	-1.45(0.88)	**<0.001**	-0.94(1.19)	-0.61(0.99)	-1.60(1.08)	**<0.001**	0.789	0.568
9 months post term	-0.68(1.14)	-0.18(0.88)	-0.94(0.80)	**<0.001**	-0.91(1.26)	-0.45(0.93)	-1.27(1.03)	**0.002**	0.122	0.190
12 months post term	-0.66(1.17)	0.02(0.91)	-0.72(0.81)	**<0.001**	-0.92(1.20)	-0.42(0.94)	-1.16(1.02)	**0.005**	**0.015**	0.084
Adult height SDS	-0.42(0.95)	-0.18(0.97)	-0.30(0.91)	0.553	-1.00(1.36)	-0.63(0.91)	-1.01(0.91)	0.118	**0.015**	**0.008**
Adult weight SDS	-0.28(1.22)	0.19(1.09)	-0.57(1.02)	**0.001**	-0.63(1.43)	0.04(1.12)	-0.72(1.03)	**0.011**	0.469	0.618

Data are given as mean(SD). SDS: Standard deviation score. Accelerated catch-up= weight gain >0.67 SDS in first year of life and weight gain >0.5 SDS in first three months, compared with non-accelerated catch-up (weight gain >0.67 SDS in first year of life and weight gain <0.5 SDS in first three months)

*: accelerated vs. non-accelerated

Table 2. Determinants of non-alcoholic fatty liver disease of the preterm group and term group, divided in subjects with accelerated versus non-accelerated catch-up growth in weight in the first year after term age

	Preterm				Term				Preterm vs. term	
	Total study group (n=162)	Accelerated catch-up (n=55)	Non-accelerated catch-up (n=36)	p-value*	Total study group (n=268)	Accelerated catch-up (n=54)	Non-accelerated catch-up (n=19)	p-value*	Accelerated p-value	Non-accelerated p-value
TG (mmol/L)	1.12(0.52)	1.04(0.57)	0.85(0.35)	0.071	1.03(0.50)	1.170.52)	0.96(0.33)	0.102	0.216	0.256
BMI (kg/m^2)	22.3(3.57)	23.2(3.84)	21.1(2.30)	**0.004**	22.4(3.51)	23.5(3.59)	21.8(2.45)	0.060	0.665	0.305
Waist (cm)	77.2(9.21)	79.8(11.0)	74.4(6.51)	**0.010**	76.9(9.98)	91.4(11.2)	75.7(8.65)	**0.047**	0.457	0.545
γ-GT (U/l)	16.7(8.13)	16.7(7.85)	14.5(5.55)	0.164	17.3(13.5)	22.4(24.6)	16.9(8.40)	0.342	0.101	0.216
FLI	12.7(17.4)	16.0(20.8)	6.64(5.95)	**0.012**	13.0(17.1)	21.6(23.4)	10.2(12.1)	**0.046**	0.187	0.157
FLI-score (%)										
Low	89.9	89.1	97.1	0.255	88.3	72.2	94.7	0.121	0.026	0.662
Intermediate	5.0	3.6	2.9	0.255	8.7	20.4	5.3	0.121	0.007	0.662
High	5.0	7.3	0	0.255	3.0	7.4	0	0.121	0.979	-
ALT(U/l)	15.1(9.80)	16.3(14.5)	14.5(5.25)	0.479	20.3(15.3)	23.1(24.2)	19.4(11.2)	0.527	0.076	**0.031**
AST (U/l)	21.5(6.54)	21.6(8.68)	21.8(3.64)	0.884	25.8(30.2)	25.7(13.2)	25.1(10.0)	0.928	0.054	0.058
ALP (U/l)	72.0(24.1)	71.1(25.7)	73.8(25.4)	0.626	69.0(22.1)	70.5(19.6)	70.9(24.1)	0.931	0.889	0.692

Data are given as mean(SD). SDS: Standard deviation score. Waist:waist circumference, BMI: body mass index, TG: triglyceriden, γ-GT: glutamyltransferase, FLI: fatty liver index (0-100), ALT= alanine aminotransferase, AST: aspartate aminotransferase. A FLI-score below 30 resembling a low risk (FLI-score 1), from 30 up to 60 an intermediate risk (FLI-score 2), and above 60 a high risk (FLI-score 3) for fatty liver. Accelerated catch-up= weight gain >0.67 SDS in first year of life and weight gain >0.5 SDS in first three months, compared with non-accelerated catch-up (weight gain >0.67 SDS in first year of life and weight gain <0.5 SDS in first three months)
*: accelerated vs. non-accelerated

Table 3. Associations of birth size and early weight gain with the FLI and the components of the FLI at 21 years in young adults born preterm

		Fattyliver index			FLI components										
					TG*			BMI*			Waist*			γ-GT*		
		Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	p	Adj. R^2	Beta (%)	P	Adj. R^2
Model 1[1]	Birthweight SDS	-6.32	0.446	0.042	0.10	0.989	-0.044	-2.12	0.107	0.040	-1.55	0.090	0.140	5.87	0.103	0.054
Model 2[2]	Gain in weight SDS during 3 months after term age	29.1	**0.034**	0.054	4.92	0.388	-0.026	3.54	0.055	0.046	3.63	**0.004**	0.165	3.73	0.447	0.044

*:log transformed (natural logarithm) for regression analyses

Beta: regression coefficient in %, p: p-value

[1]Adjusted for gender, age, SES, gestational age and birth length SDS

[2]Adjusted for gender, age, SES, gestational age and gain in length during 3 months after term age

Fatty liver index

Total study population of adults born preterm

In Table 3 results from linear regression analyses are shown, with FLI (0-100) and individual components of the FLI (TG, BMI, waist circumference, and serum levels of γ-GT) as dependent variables. Gain in weight SDS adjusted for length SDS in the first three months after term age was significantly associated with FLI at age 21 years (FLI increased with 29.1% per 1 SDS increase in weight gain, p=0.034, model 2). In addition, the gain in weight SDS for length SDS in the first three months after term age showed a significant association with waist circumference (p=0.004). Birth weight SDS and gestational age were not significantly associated with the FLI or with the individual components of the FLI (Model 1).

In addition, subjects with a loss in weight ≥ 1 SDS between birth and term age had a higher FLI at the age of 21 years than those without any loss in weight SDS (p=0.01).

Adults born preterm with catch-up in weight after term age

To determine if tempo of weight gain after term age was associated with determinants of NAFLD in young adults born preterm, those with catch-up in weight in the first year after term age were divided in two subgroups with either accelerated or without accelerated catch-up in weight. Of all young adults with a clinically relevant catch-up in weight of at least 0.67 SDS in the first year after term age (n=91), 55 had a weight gain of more than 0.5 SDS in the first 3 months after term age (accelerated catch-up), while 36 had no accelerated catch-up, defined as a weight gain of less than 0.5 SDS in the first 3 months after term age. The clinical characteristics of these two subgroups are shown in Table 1.

Gestational age, sex, age, birth length SDS and birth weight SDS were not different between the accelerated and non-accelerated catch-up group. The accelerated catch-up group had a higher FLI (p=0.012), adult weight SDS (p=0.001), BMI (p=0.004), and waist circumference (p=0.010) at age 21 year than the non-accelerated catch-up group, even after adjustment for gender, SES, gestational age, and gain in length during the first year of life (p=0.003, Figure 1).

To investigate whether the effect of accelerated catch-up in weight in subjects born preterm was similar to that in subjects born term, we compared the tempo of catch-up in young adults born preterm versus term (Table 2). Our data showed no difference between subjects born term versus preterm.

Fatty liver index score

A FLI-score was assigned to each participant in order to obtain an NAFLD-risk score (1:low, 2:intermediate, or 3:high). Of all adults born preterm, 5% (n=8) had a high FLI-score, and

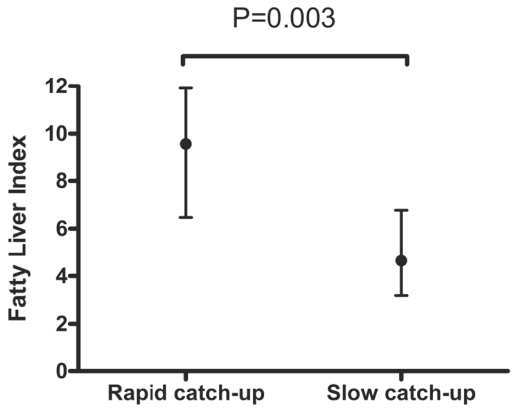

Figure 1. Adjusted Fatty Liver Index of subjects with accelerated compared to non-accelerated catch-up after term age

Estimated marginal means (95% confidence interval) Accelerated catch-up= weight gain >0.67 SDS in first year after term age and weight gain >0.5 SDS in first three months after term age. Non-accelerated catch-up= weight gain >0.67 SDS in first year after term age and weight gain <0.5 SDS in first three months after term age. Adjusted for gender, SES, gestational age and gain in length in first year of life after term age

5% (n=8) an intermediate FLI-score (Table 2). In the accelerated catch-up group, 7.3% had a high FLI-score and 3.6% an intermediate FLI-score, while in the non-accelerated catch-up group, 0% had a high FLI-score and 2.9% an intermediate score. Lower birth weight and gestational age were not associated with a higher FLI-score at 21 years.

DISCUSSION

Our study in 162 young adults born preterm shows that accelerated infant weight gain after term age is associated with an increased risk for developing NAFLD in early adulthood. We also demonstrate that subjects born preterm with accelerated weight gain after term age have a significantly higher FLI in early adulthood than subjects without accelerated catch-up in weight, independent of size at birth and gestational age. Those findings are similar to those found in young adult born term.

We found an association between accelerated weight gain during infancy in preterm born subjects and a higher NAFLD risk in early adulthood. Previous studies reported associations between small body size at birth and risk factors for NAFLD in adulthood (26, 27), but associations with preterm birth were not investigated. Some studies showed an association between preterm birth and waist circumference, a component of the Fatty Liver Index (FLI), but none of these studies investigated associations with the FLI.(28, 29)

Because our study population consisted of young adults, hard endpoints such as NAFLD and steatohepatitis could not be studied. The FLI was used as a proxy for a

clustering of risk factors, a condition which precedes hepatic steatosis. A high FLI-score (above 60) has a predictive value of 72.2% for a fatty liver(18) and although our study population was relatively young and healthy, with a relatively low BMI, a high FLI-score was found in 5% of subjects born preterm.

In addition, it was striking that 10.9% of subjects born preterm with accelerated catch-up in weight for length after term age had an high or intermediate FLI at the age of 21 years, compared with only 2.9% of the subjects without accelerated catch-up in weight for length. We previously showed a similar association between accelerated infant weight gain and increased risk for NAFLD in young adults born term.(9) Our present study shows that this association is also apparent in young adults born preterm. Besides this, our study shows that preterm subjects with a loss in weight ≥ 1 SDS between birth and term age had a higher FLI at 21 years than those without any decrease in weight. These both findings indicate that a balance in neonatal gain in weight compared to length is also important for preterm infants to reduce the risk for developing NAFLD. This may be achieved by modifying nutritional intervention according to weight for length trajectories, particularly in the first months after term age. These data should not be interpreted that we recommend slow weight gain in preterm babies, but discourage accelerated catch-up in weight for length after term age. It also points out that not only weight but also length should be routinely measured during infancy. Earlier studies showed that accelerated weight gain, mediated by nutrition-enriched diets, have adverse effects on cardiovascular risk factors in later life.(6, 30) Our findings warrant informing parents about the long-term effects of accelerated weight gain, as the first months after discharge of preterm infants, usually around term age seem to be a critical window for the programming of later body composition.

We acknowledge that when investigating differences between subjects born preterm and term, differences in early nutritional intake should be taken into account. The prevalence and duration of breastfeeding is less in infants born preterm.(31) Formula-fed infants have a higher risk of being overweight in childhood and in later life.(32, 33) Infant feeding might thus influence later risk for NAFLD. Studies in rats showed that the liver might act as a systemic buffer, largely increasing its lipid content in the early stage of high-fat feeding.(34) It has been suggested that breastfeeding protects against the development non-alcoholic steatohepatitis (NASH) in children.(35) We did not have sufficient nutritional data to investigate differences in nutritional intake in early life between adults born preterm or term, and its relation with growth in infancy and the risk for NAFLD, but our results warrant further investigations.

In conclusion, our study shows that accelerated gain in weight compared to length in the first three months after term age in subjects born preterm is associated with a higher risk to develop NAFLD in adulthood. Long-term prospective studies are warranted to study the effects of various infant feeding practices on the risk for NAFLD.

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Chapter 4

Maternal and fetal determinants of neonatal body composition

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ABSTRACT

Background

Body composition in early life influences the development of obesity during childhood and beyond. It is, therefore, important to adequately determine neonatal body composition. Fetal growth and maternal factors might influence neonatal fat mass percentage (FM%), independent of birth weight.

Methods

In 194 healthy neonates, we investigated neonatal body composition, measured by air-displacement plethysmography, and its associations with estimated fetal weight (EFW), neonatal anthropometric data and maternal preconceptional BMI and maternal weight gain during pregnancy.

Results

There was a large variation in neonatal FM%, even in case of a similar birth weight, corrected for gender and gestational age. Neonatal FM% was associated with EFW at 30 and 36 weeks of gestation and with catch-up in weight between 30 and 36 weeks of gestation, but not with EFW at 20 weeks ($p<0.01$, $p<0.01$, $p=0.64$, resp.). Neonatal FM% was also associated with preconceptional BMI of the mother ($p<0.01$). There was no correlation with maternal weight gain.

Conclusion

Our study shows that term neonates have a large variation in fat mass percentage. Neonatal FM% is associated with EFW at 30 and 36 weeks, catch-up in weight between 30 and 36 weeks of gestation and preconceptional BMI of the mother.

INTRODUCTION

Over the last 20 years, the prevalence of overweight in Dutch children aged 4 to 12 years, increased from 7% to 11%.(1) Childhood obesity is associated with short-term morbidity, such as asthma and psychological problems, and with an increased risk for obesity, chronic morbidity and mortality in adulthood.(2-4) Accelerated catch-up in weight in the first months of life is associated with a higher fat mass percentage and a worse cardiovascular and metabolic profile in young adults.(5-7) There is increasing evidence that fat mass in early life tends to track into adulthood.(8-10)

The prevalence of overweight in adult women has increased from 29.8% to 43%.(1) As a result, many pregnant women are nowadays overweight or obese. Preconceptional maternal weight and maternal weight gain during pregnancy influence fetal growth and birth weight.(11, 12) Only few population-based studies investigated infant fat mass in relation with maternal weight and observed associations between preconceptional body mass index (BMI) and weight gain during pregnancy and infant body composition. (13-18)

Most studies have focused on weight, length, and other anthropometric measures as a proxy for neonatal adiposity, but given the well-known relationship between adiposity and health outcomes, it is reasonable to question whether body composition, rather than just body weight, might better explain or predict health risk. Due to superior data collection, enhanced feasibility and increasing availability; Peapod is increasingly used to measure neonatal body composition as a marker of health, however, fetal and maternal factors that may affect newborn findings are still not fully recognized.(19-21)

We hypothesized that neonates have different fat mass percentages due to differences in fetal growth, gestational age at delivery, gender, preconceptional maternal BMI and weight gain of the mother during pregnancy.

We, therefore, investigated associations between these fetal and maternal determinants and FM% of the neonate, measured by Peapod. For clinical practice, we assessed whether measurement of skinfolds could be a proxy for FM% measured by Peapod.

MATERIAL AND METHODS

Subjects

The study population comprised 194 healthy neonates, born between February 2012 and July 2013 at the department of Obstetrics of the Erasmus University Medical Center, Rotterdam, The Netherlands. The recruitment took place directly after birth.

Inclusion criteria were a gestational age between 36 and 42 weeks, and an uncomplicated neonatal period without severe asphyxia (Apgar score <3 at 5 minutes), sepsis, respiratory ventilation and/or oxygen supply. Written informed consent was obtained from both parents unless mother was single.

The Medical Ethics Committee of Erasmus Medical Center approved the study.

Fetal growth

Fetal ultrasound examinations were carried out at standard time points, at a median gestational age (IQR) of 20.4 weeks (20.1-20.7), 30.3 weeks (29.9-30.6) and 36.1 weeks (35.6-36.4). Fetal ultrasound examinations were carried out by well-trained staff at the department of Obstetrics and Gynaecology using standardized ultrasound procedures. Ultrasound examinations were performed using the Aloka model SSD-1700 (Tokyo, Japan) or the ATL-Philips Model HDI 5000 (Seattle, WA, USA). Estimated fetal weight (EFW) was calculated using the formula by Hadlock.(22) Longitudinally measured EFW can be used as proxy for fetal growth.(23, 24) The fetal growth charts generated in the Dutch Generation R study were used to calculate standard deviation scores.(25)

Maternal and pregnancy characteristics

Maternal data, i.e. preconceptional weight and weight just before delivery, height, parity, smoking, ethnicity and complications during pregnancy (gestational hypertension, pre-eclampsia, gestational diabetes), were obtained from the medical records. Height and weight were measured and smoking/alcohol intake were asked at the beginning and during their pregnancy. Ethnicity was classified as previously described.(26) Women, who reported any or no smoking during pregnancy were respectively classified as 'smokers' and 'non-smokers'.

Data on gestational age was extracted from medical records.

Maternal underweight was defined as a BMI <18.5 kg/m^2, overweight as a BMI ≥25 kg/m^2, and obesity as a BMI ≥30 kg/m^2. Weight gain during pregnancy was classified as low, appropriate and high, according to the Institute of Medicine guideline, a guideline with commendations for total weight gain during pregnancy, by preconceptional BMI.(27)

Anthropometrics of the neonate

Measurements took place within 24 hours after birth. Neonatal weight was measured to the nearest 0.1 g on the integrated electronic scale of the Peapod and neonatal length and head circumference to the nearest 0.1 cm using measuring tape (SECA, circumeter). Skinfolds (subscapular, biceps, triceps and suprailiacal) were measured to the nearest mm using a skinfold caliper (slimguide C-120, Creative Health). Central skinfolds were calculated as the sum of subscapular and suprailiacal skinfolds and peripheral skinfolds as the sum of biceps and triceps skinfolds.(8)

Body composition

Whole-body composition was assessed using air-displacement plethysmography (Peapod, Infant Body Composition System, COSMED). This air-displacement plethysmography system assesses fat mass and fat free mass by direct measurements of body volume and body mass, based on the whole-body densitometric principle. Details of the principle and operating procedure of the Peapod have been described.(19, 21) These studies have shown that this method is valid for measurement of body composition in neonates. All measurements were obtained by experienced personnel, using standardized protocol. The Peapod was calibrated every day, according to the instructions of COSMED.

Statistical analysis

SD-scores for birth length and birth weight were calculated to correct for gestational age and gender (28). All SD-scores were calculated using Growth Analyser software (http://www.growthanalyser.org).

All variables were normally distributed. Differences between boys and girls were examined with Student's t-tests, chi square tests or ANOVA analysis. The associations of maternal anthropometrics with FM% of the neonate were assessed using linear regression models. Similar models were used to examine associations of FM% with EFW at a median gestational age (IQR) of 20.4 weeks (20.1-20.7), 30.3 weeks (29.9-30.6) and 36.1 weeks (35.6-36.4), gestational age, gender, birth weight, neonatal BMI, Ponderal Index and skinfolds measurements. Multiple linear regression analysis was performed to determine associations with the dependent variable FM%. In model A we entered gestational age at delivery, gender, birth weight and birth length standard deviation score (SDS). In model B, we investigate the association of EFW on body composition. EFW at 30 weeks was, therefore, added, and we removed birth weight SDS and birth length SDS because of multicollinearity. Additionally we added EFW at 36 weeks in model C, instead of EFW at 30 weeks. In model D, we investigated the role of preconceptional BMI of the mother and weight gain during pregnancy on neonatal body composition.

In addition, we examined the effect of catch-up in EFW on FM% at birth. We used the change in SD-scores of EFW from 20 to 30 and 30 to 36 weeks of gestation. Catch-up in EFW was defined as a gain in weight-SD-score >0.67, indicating clinically significant catch-up growth.(29)

SPSS statistical package version 20.0 (SPSS Inc. Chicago, Illinois) was used. All statistical tests were performed two-sided and results were regarded statistically significant if the p-value was <0.05.

RESULTS

Clinical characteristics

Clinical characteristics of the study population are shown in Table 1. Mean (SD) gestational age was 39.3 (1.4) weeks and birth weight SDS was 0.07 (1.2). Mean (SD) age of the mothers was 31.0 (5.4) years and weight gain during pregnancy was 14.6 (6.1) kg. Fifty percent of the mothers had a Dutch Caucasian ethnicity.

Table 1. Clinical characteristics

Total population N=194	*Mean (SD)*
Neonatal characteristics	
Gestational age at birth *	39.3 (38.4-40.6)
Male (%)	54
Birth weight (kg)	3.384(0.5)
Birth weight SDS	0.07 (1.2)
Birth length (cm)	49.8(2.5)
Birth length SDS	-0.24 (1.2)
Maternal characteristics	
Age at child's birth (yr)	31.0 (5.4)
Height (cm)	168 (0.08)
Preconceptional weight(kg)	71.5 (16.4)
Preconceptional BMI (kg/m^2)	25.5 (5.6)
Highest weight in pregnancy (kg)	86.6 (17.2)
Weight gain during pregnancy (kg)	14.6 (6.1)
Primiparity (%)	50.5
Ethnicity (%)	
Dutch Caucasian	50
Surinamese and Dutch Antillean	14.5
Moroccan and Turkish	13.5
Others	22
Smoking during pregnancy (%)	7
Complications of pregnancy (%)	25
Gestational hypertension	3.7
Pre-eclampsia	2.8
Gestational diabetes	4.9
Others	13.8

*median (interquartile range)

Body composition

Table 2 shows the mean (SD) parameters of body composition measured in the neonates. Fat mass percentage (FM%) of the neonates was 10.3 (4.0) % (Table 2). Mean (SD) fat free mass percentage was 89.8 (3.9) %. Mean(SD) sum of all skinfolds was 19.1 (3.3) mm, central skinfolds 9.1(1.9) mm and peripheral skinfolds 9.9 (1.7) mm.

Variation in fat mass percentage (FM%) was high, even in neonates with a similar weight SDS or Ponderal Index (Figure 1 and 2). For instance, in neonates with a birth weight of 3.5 kg, the difference in fat mass amounted to 0.5 kg, while the variation in FM% was 15%.

Table 2. Body composition of neonates

	Total	*Boys*	*Girls*	*p-value*
Weight (kg)	3.259 (0.49)	3.332 (0.52)	3.171 (0.44)	0.022
Weight SDS	0.07 (1.2)	0.06 (1.2)	0.07(1.1)	0.947
Length (cm)	49.8 (2.5)	50.1 (2.6)	49.6 (2.4)	0.25
Length SDS	-0.02 (1.2)	-0.16 (1.2)	0.14 (1.1)	0.08
Head circumference (cm)	34.7 (1.5)	34.9 (1.5)	34.5 (1.5)	0.06
Head circumference SDS	0.08 (1.3)	-0.01 (1.4)	0.21 (1.2)	0.24
BMI (kg/m^2)	13.0 (1.4)	13.2 (1.5)	12.8 (1.2)	**0.01**
BMI SDS	-0.30 (1.1)	-0.16 (1.2)	-0.47 (1.0)	0.06
Ponderal Index	26.2 (3.1)	26.6 (3.3)	25.8 (2.8)	0.08
Fat mass (kg)	0.345 (0.17)	0.3430 (0.17)	0.3469 (0.16)	0.87
Fat mass percentage (%)	10.3 (4.0)	10.0 (3.9)	10.6 (4.0)	0.31
Fat-free mass (kg)	2.916 (0.4)	2.990 (0.4)	2.826 (0.3)	**0.003**
Fat-free mass percentage (%)	89.8 (3.9)	90.0 (3.9)	89.4 (3.9)	0.27
Triceps skinfolds (mm)	5.1 (1.0)	5.1 (1.0)	5.2 (1.0)	0.89
Biceps skinfolds (mm)	4.7 (1.0)	4.7 (1.0)	4.9 (1.0)	0.17
Subscapular skinfolds (mm)	5.0 (1.3)	4.9 (1.3)	5.1 (1.3)	0.66
Suprailiacal skinfolds (mm)	4.1 (1.1)	4.1 (1.2)	4.3 (1.0)	0.20
Sum of all skinfolds (mm)	19.1 (3.3)	18.9 (3.3)	19.5 (3.4)	0.26
Sum of central skinfolds (mm)	9.1 (1.9)	9.0 (2.0)	9.4 (1.8)	0.22
Sum of peripheral skinfolds (mm)	9.9 (1.7)	9.8 (1.6)	10.1 (1.8)	0.30

Data expressed as mean (SD)
Significant P values ($p<0.05$) are indicated in boldface

Relation-ship between fetal growth and FM%

EFW at gestational ages of 30 and 36 weeks was significantly associated with FM% at birth ($p<0.01$, $p<0.01$, resp.) (Table 3). After adjustment for gender and gestational age, EFW at 30 and 36 weeks was still significantly associated with FM% (Model B and C, Table 4). EFW at 20 weeks was not associated.

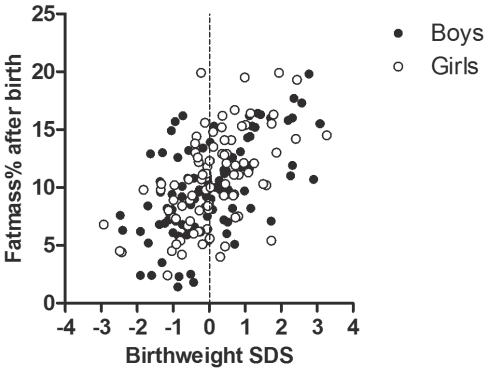

Figure 1. Relationship between birth weight SDS and fat mass percentage after birth
Birth weight SDS is corrected for gestational age and gender.
Correlation coefficient= 0.57, p<0.001

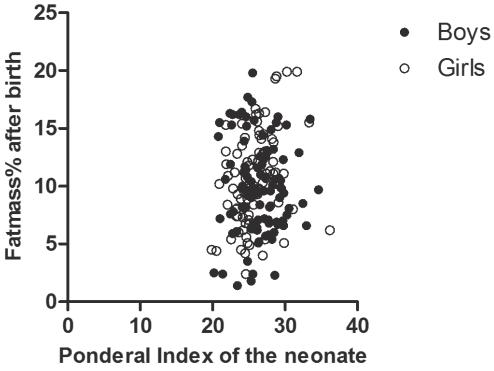

Figure 2. Relationship between Ponderal Index and fat mass percentage after birth
Correlation coefficient= 0.28, p<0.001

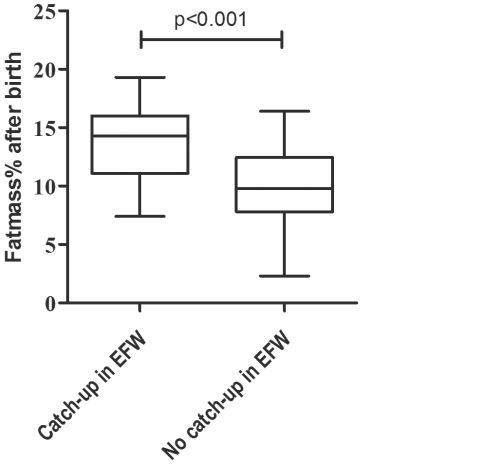

Figure 3. Difference in FM% between catch-up and non catch-up in estimated fetal weight between 30 and 36 weeks of gestation
Catch-up = estimated fetal weight gain >0.67 SDS in period between 30 and 36 weeks of gestation

No association was found in the gain in EFW between 20 and 30 weeks and neonatal FM%, but the gain between 30 and 36 weeks was associated with FM% (r=0.46 p<0.01).

Neonates with catch-up in fetal weight (gain in weight-SD-scores > 0.67) between 30 and 36 weeks of gestation had a higher FM% compared to those without catch-up (p<0.001) (Figure 3).

Relationship between maternal variables and FM%

Preconceptional BMI of the mother was associated with neonatal FM%, even after correction for gestational age, gender, birth weight SDS, birth length SDS and weight gain of the mother during pregnancy (Tables 3 and 4).

Neonates had a significantly higher FM% as well as fat mass in kg when mothers were overweight before pregnancy, compared with those of preconceptional underweight mothers (p=0.05, p=0.03 resp.). Weight gain of the mother during pregnancy was, however, not associated with neonatal FM%. FM% tended to be higher in mothers with a high weight gain during pregnancy, according to the guideline, versus those with low weight gain, but the difference was not statistically significant (p=0.09). The higher the preconceptional BMI of the mothers, the lower the weight gain during pregnancy (r=-0.17, p=0.03). In the same line, mothers with preconceptional obesity, defined as a BMI>30, tended to show less weight gain than mothers with a preconceptional normal BMI between 18 and 25 (p=0.10).

There were no significant differences in neonatal FM% between ethnic groups and between mothers with and without complications.

Relationship between neonatal characteristics and FM%

Regression analyses showed that neonatal FM% and fat mass were associated with birth weight, also after adjustment for gestational age and gender (R^2= 0.32 and R^2= 0.51 resp.).

Neonatal FM% and fat mass(kg) were also associated with the BMI (β=1.52 p<0.01, β=0.08 p<0.01, resp.) and the Ponderal Index of the infant (β=0.45 p<0.01, β=0.02 p<0.01, resp.). BMI was significantly different between boys and girls (p=0.01). However, there was no significant difference in FM% between neonate boys and girls, as the median (IQR) FM% was 9.8 (7.0-12.6) in boys and 10.3 (7.4-13.0) in girls (p=0.31). Mean (SD) fat free mass was significantly higher in boys than in girls, being respectively 3.0 (0.4) and 2.8 (0.3)kg (p<0.01). The fat free mass % was, however, not significantly different.

FM% was associated with gestational age, after adjustment for birth weight SDS, birth length SDS and gender (model A, Table 4).

Table 3. Univariate linear regression analysis for FM% in neonates and pre- and postnatal determinants

Fatmass percentage(FM%)	β	95% CI	p-value
Fetal growth			
EFW at 20 weeks of gestation (kg)	2.38	-7.72, 12.48	0.64
EFW at 30 weeks of gestation (kg)	5.51	3.93, 7.08	**<0.01**
EFW at 36 weeks of gestation (kg)	4.70	3.91, 5.49	**<0.01**
Maternal characteristics			
Age of mother (yrs)	0.03	-0.32, 0.91	0.34
Height (m)	3.42	-0.63, 7,48	0.10
BMI before pregnancy (kg/m^2)	0.11	0.05, 0.16	**<0.01**
Weight gain during pregnancy (kg)	0.01	-0.42, 0.07	0.66
Neonatal characteristics			
Gestational age (wks)	0.13	-0.97, 0.36	0.26
Gender	0.25	-0.40, 0.88	0.45
Birth weight (kg) *	0.005	0.005, 0.006	**<0.01**
BMI of newborn *	1.52	1.32, 1.73	**<0.01**
Ponderal Index of newborn *	0.45	0.35, 0.56	**<0.01**
Sum of skinfolds (mm)	0.65	0.57, 0.73	**<0.01**
Sum of central skinfolds (mm)	1.05	0.91, 1.20	**<0.01**
Sum of peripheral skinfolds (mm)	1.05	0.91, 1.20	**<0.01**

EFW= estimated fetal weight, BMI=body mass index, weight gain during pregnancy= difference between weight at 36 weeks of gestation – pre-pregnancy weight, FM% = fat mass (grams)/ weight at birth(grams) x 100, sum of central skinfolds=suprailiacal skinfolds + subscapular skinfolds, sum of peripheral skinfolds=biceps skinfolds + triceps skinfolds

β= unstandardized regression coefficients and reflecting the difference in fat mass percentage for maternal, fetal and postnatal anthropometrics. Gender: male=0, female=1

*: adjusted for gestational age

Significant p-values (p<0.05) are indicated in boldface.

Relationship between skinfolds thickness and FM%

Skinfold measurements were associated with FM% (Table 3). The correlation with biceps skinfolds was 0.35 (p<0.001), triceps 0.54 (p<0.001), subscapular 0.48 (p<0.001) and suprailiacal 0.40 (p<0.001). The correlation between FM% and central skinfolds was 0.52 (p<0.001) and between FM% and peripheral skinfolds 0.51 (p<0.001). The correlation between FM% and total sum of skinfolds was 0.56 (p<0.001).

Table 4. Multiple regression for determinants of neonatal FM%

	Model A		Model B		Model C		Model D	
	β	P value	β	P value	β	P value	β	P value
Gestational age (wk)	0.013	**0.031**	-0.021	0.946	-0.141	0.586	0.400	**0.008**
Gender	0.001	0.976	-0.130	0.871	0.588	0.375	0.895	0.868
Birth weight SDS	0.099	**<0.001**					0.098	<0.001
Birth length SDS	0.009	0.124					0.152	0.127
Estimated fetal weight at 30 wks			0.005	**0.003**				
Estimated fetal weight at 36 wks					1.238	**<0.001**		
Preconceptional BMI of the mother							0.004	**0.016**
Weight gain mother during pregnancy							0.001	0.961
Overall p-value	**<0.001**		**0.027**		**<0.001**		**<0.001**	
Adjusted R2	0.518		0.073		0.177		0.557	

Significant p-values (P<0.05) are indicated in boldface. β=unstandardized regression coefficients. Gender: male=0, female=1

DISCUSSION

Our study evaluated the differences in neonatal body composition in 194 healthy neonates and the possible explanations of these differences. It shows that term neonates have a large variation in fat mass percentage, ranging from 1.4 to 19.9%, also after correction for gender and gestational age, indicating that is difficult to predict fat mass based on weight or ponderal index at birth. EFW at 30 and 36 weeks and catch-up in weight between 30 and 36 weeks of gestation as well as preconceptional BMI of the mother were positively associated with more FM% of the neonate. In addition, skinfolds were moderately correlated with FM% measured by Peapod.

In this study, a large variation in FM% in neonates was observed, even in neonates with a similar weight. The average FM% was 10.3%, which is in line with reported FM% in term neonates.(15, 30) However, the large variation in FM% in neonates with a similar birth weight has not yet been reported.

We showed associations of FM% with EFW at 30 and 36 weeks of gestation. Catch-up in weight between 30 and 36 weeks of gestation was positively associated with neonatal FM%, but catch-up between 20 and 30 weeks was not. This implicates that only gain in fetal weight in this last period is associated with FM% at birth.

Besides associations of FM% in neonates with fetal growth, we also showed associations with the preconceptional BMI of the mother. However, no differences in FM% could be found in neonates of mothers with a high weight gain during pregnancy compared to those with a low weight gain. It has been reported that neonates of women who gained excessive weight in the first part of pregnancy have higher FM% compared to

neonates of women who gained weight later in pregnancy.(31-33) As it is common in the Netherlands to gain more weight in the last trimester of pregnancy (34), this might explain why we found no differences in FM% in neonates of mothers with a high weight gain compared to those without. Our findings show that mothers with a higher preconceptional BMI tend to have less weight gain during pregnancy than those with a normal preconceptional BMI. This might indicate that the international guideline with recommendations for total weight gain during pregnancy based on preconceptional BMI is followed successfully.(27) This could also be an explanation why no differences in FM% can be found in neonates of mothers with a high weight gain compared to those without. In mothers with a high preconceptional BMI caretakers will try to control the weight gain during pregnancy.

Despite a significant difference in BMI between boys and girls, we found no significant difference in FM%. It is known that female children and adult women have more fat mass and less lean body mass than males, but there are less data about differences in fat mass between neonatal boys and girls. Possibly the difference in FM% is still very small at birth, but becomes larger later in childhood.(30, 35) Fat-free mass was indeed higher in neonatal males than females. This gender difference could be attributed to the higher intrauterine androgen levels in boys in the last trimester, which increase lean body mass. (36)

In our study, 96 of the 194 mothers did not have a Dutch origin, which is common in Dutch cities like Rotterdam. We observed no significant difference in FM% between neonates of Dutch origin and other ethnicities.

The linear regression analyses showed that gestational age, birth weight, EFW at 30 weeks and 36 weeks, and preconceptional BMI of the mother are significant contributors to the variance of FM% at birth. A model including all these variables is not possible, because several ones are closely related. For that reason, we present different models. The highest explained variance of these models was 56% in model D. In this model, the variables gestational age, birth weight SDS and preconceptional BMI are significant contributors to the variance in FM%.

In clinical practice, skinfold measurements are used to estimate fat mass in infants above 3 months of age.(37) Reference charts from birth up to 3 months of age are, however, not available. In the present study, we measured skinfolds in neonates and demonstrated that skinfold measures are reasonable indicators of overall fatness. However, skinfolds give only information about subcutaneous fat, while Peapod measures total FM%. Although skinfolds did correlate with FM%, it cannot reliably predict FM% in individual neonates and it can therefore not replace Peapod measurements.

Our study population consisted of term neonates with a gestational age above 36 weeks and no serious complications, thus our results cannot be generalized to all neo-

nates. Further research is required in preterm neonates and neonates with postnatal complications or medication use.

In conclusion, our study shows that term neonates have a large variation in fat mass percentage. Neonatal FM% is associated with EFW at 30 and 36 weeks, catch-up in weight between 30 and 36 weeks of gestation and preconceptional BMI of the mother.

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Chapter 5

Longitudinal fat mass and visceral fat during the first 6 months after birth in healthy infants: support for a critical window for adiposity in early life

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ABSTRACT

Introduction

Body composition in early life influences the development of obesity during childhood and beyond. It is, therefore, important to adequately determine longitudinal body composition during the first months of life.

Patients and methods

In 203 healthy term infants, we investigated longitudinal body composition, including fat mass percentage (FM%) and fat-free mass (FFM), by air-displacement plethysmography, at 1, 3 and 6 months of age, and abdominal visceral fat and abdominal subcutaneous fat, by ultrasound, at 3 and 6 months.

Results

We found a significant increase in FM% between 1 and 3 months, but not between 3 and 6 months ($p<0.001$, $p=0.098$, resp.). Girls had higher FM% than boys at 1 and 6 months ($p=0.05$, $p<0.001$ resp.) and less FFM than boys at 1, 3 and 6 months ($p=0.02$, $p=0.02$, $p<0.001$ resp.). There was a large variation in FM% at all ages, even between infants with similar weight SDS. Visceral fat and abdominal subcutaneous fat did not change between 3 and 6 months. FM% was highly correlated with abdominal subcutaneous fat, but not with visceral fat.

Conclusion

Changes in FM% occur mainly in the first 3 months of life, and FM%, visceral and abdominal subcutaneous fat do not change between 3 and 6 months, supporting the concept of a critical window for adiposity development in the first three months of life. In addition, our study provides longitudinal reference data of FM%, FFM, visceral fat and abdominal subcutaneous fat during the first 6 months of life.

INTRODUCTION

Childhood obesity is a significant public health problem in developed countries. In Dutch children aged 4 to 12 years, the prevalence increased from 7 to 12% over the last 20 years.(1) Childhood obesity is not only associated with short-term morbidity, but also with long-term morbidity, such as obesity, type 2 diabetes and cardiovascular disease(CVD) in adulthood.(2-4) Increasing adiposity in the first 6 months of life is a major risk factor for these adult diseases, because it tracks into adulthood.(5, 6)

Consequently, it is crucial to accurately measure body composition and generate reference data for infant boys and girls. Differentiation in gender is important because differences in early life body composition in boys and girls will be present due to different sex hormones levels in early life. At birth, there is evidence that girls already have higher fat mass and lower fat free mass compared to boys, but there are very limited data on gender differences later in infancy.(7-9) Accurately measured body composition and reference data are currently scarce.

Relationships between growth and later health have been mainly studied in retrospective cohort studies, or during follow-up of intervention trials.(10, 11) Most studies have focused on weight, length, and other anthropometric measures as a proxy for adiposity in infancy, instead of accurate body composition, due to the lack of available methods.(12, 13) The Peapod, a device based on air-displacement plethysmography, can assess the body composition (i.e. fat and fat-free mass) of infants. This technique allows for safe and accurate body composition measurements, suitable for frequent studies during the first 6 months of life.(14-16) Few Peapod data are available on longitudinal infant body composition (17), as studies either focused on body composition at birth (18), at one time point after birth (19), in a limited number of subjects (20) or in low-income population.(21) No study combined longitudinal Peapod measurements with ultrasound measurements of subcutaneous and visceral fat.

Not only the total amount of fat, but also the location of fat accumulation is important. Increased visceral adipose tissue, is a major risk factor for future cardiovascular diseases. In obese children, greater abdominal adipose tissue is associated with an unfavorable metabolic profile.(22, 23) Ultrasound (US) is a non-invasive method to estimate abdominal adipose tissue distribution in infancy. US-visceral fat thickness and abdominal subcutaneous fat thickness are reliable and reproducible estimates of respectively internal-abdominal (visceral) adipose tissue and abdominal subcutaneous adipose tissue quantities.(24)

We hypothesized that FM% will gradually increase in the first 6 months of life and that girls will have more fat accrual than boys. We also hypothesized that subcutaneous and visceral fat thickness will also gradually increase, and that these measurements will correlate with FM%. We, therefore, longitudinally measured body composition, by Peapod,

in a large group of infants at 1, 3 and 6 months of age, separately in boys and girls. We also measured visceral fat thickness and abdominal subcutaneous fat thickness by ultrasound at 3 and 6 months of age and studied their relationship with body composition, measured by Peapod.

MATERIAL AND METHODS

Subjects

The study population consisted of 203 healthy term infants. The current study is part of a birth cohort study (Sophia Pluto Study) aiming to provide detailed data on body composition and growth in early life. Children born from January 2013 were recruited from several hospitals in and near Rotterdam, a large city in The Netherlands. All participants fulfilled the same inclusion criteria: 1) born term (≥37 weeks of gestation), 2) Age <28 days, 3) uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score below three after five minutes), sepsis or long-term complication of respiratory ventilation. Exclusion criteria were known congenital or postnatal diseases that could interfere with body composition development, confirmed intra-uterine infection, maternal use of corticosteroids or significant maternal medical condition that could interfere with infant's body composition development (e.g. diabetes). The Medical Ethics Committee of Erasmus Medical Center approved the study. Written informed consent was obtained from both parents unless mother was single.

Data collection and measurements

Outpatient clinic visits were scheduled at 1, 3 and 6 months. Birth data were taken from midwife and hospital records. Trained pediatric nurses performed the measurements according to standard procedures.

Anthropometrics

Weight was measured to the nearest gram by using an electronic infant scale (Seca, Hanover, MD). Length was measured twice with the two-person technique to the nearest 0.1 cm using an Infantometer (Seca). Head circumference was measured to the nearest 0.1 cm using measuring tape (Seca, circumeter). Skinfolds (subscapular, biceps, triceps and suprailiacal) were measured to the nearest mm using a skinfold caliper (Slimguide C-120, Creative Health). Central skinfolds were calculated as the sum of subscapular and suprailiacal skinfolds and peripheral skinfolds as the sum of biceps and triceps skinfolds. (5)

Body composition

Whole-body composition was assessed using air-displacement plethysmography (Peapod, Infant Body Composition System, COSMED). A detailed description of the air-displacement plethymogragphy (ADP) system is provided elsewhere.(16) Briefly, this ADP system assesses fat mass (FM), fat mass percentage (FM%) and fat free mass (FFM) and fat-free mass percentage (FFM%) by direct measurements of body volume and body mass, based on the whole-body densitometric principle. Body mass was measured on the integrated electronic scale of the ADP system. Body volume was measured in the test chamber by applying pertinent gas laws that relate pressure changes to volumes of air in the enclosed chamber. Assessment of body volume lasted 2 min. Body density was then computed from the study subject's measured mass and volume and then converted to %fat and fat-free mass using sex-specific equations by Fomon et al.(13) All measurements were obtained by experienced personnel, according to standardized protocol. The Peapod was calibrated every day, according to the protocol recommended by the supplier.

Abdominal fat thickness

Visceral and abdominal subcutaneous fat thickness were measured at 3 and 6 months using a Prosound 2 ultrasound, with a UST-9137 convex ultrasound transducer (both from Hitachi Aloka Medical, Switzerland). For both measures, the transducer was positioned where the xiphoid line intercepted the waist circumference measurement plane. Visceral fat thickness was measured of a longitudinal plane with a probe depth of 9 cm and was defined as the distance between the peritoneal boundary and the corpus of the lumbar vertebra. Subcutaneous abdominal fat thickness was measured at the same location, but on a transverse plane with a probe depth of 4 cm, and was defined as the distance between the cutaneous boundary and the linea alba.(24)

Statistical analysis

Based on literature, a power calculation was performed to calculate the sample size needed for determining a significant difference in percent body fat (95% confidence interval) between male and female infants at 6 months of age. Fields et al (17) showed that body fat percentage of female infants was 28.3% and 25.9% in male infants at 6 months. The reported difference can be determined with a power of 90% if each group consists of 81 infants. To take into account a possible drop-out of 30-40% over time, we included 285 infants. There were 381 parents eligible to participate in the study. Of the 285 infants enrolled in study, 203 infants had complete data on body composition from birth to 6 months of age (Supplemental figure 1). SD-scores for birth length and birth weight were calculated to correct for gestational age and gender, SD-scores for length and weight at

1, 3 and 6 months were calculated to correct for age and gender.(25) All SD-scores were calculated using growth analyser software (http://www.growthanalyser.org).

All variables were normally distributed. Baseline characteristics are expressed as mean(sd). Differences in clinical characteristics and anthropometrics between boys and girls were determined with an independent sample student's t-test. Differences in body composition measured by Peapod were determined using Estimated Marginal Means, with adjustment for length. The longitudinal changes in body composition were analyzed using repeated measures analysis. SPSS statistical package

version 20.0 (SPSS Inc. Chicago, Illinois) was used. All statistical tests were performed two-sided and results were regarded statistically significant if the p-value was <0.05.

RESULTS

Clinical characteristics of the subjects are presented in Table 1. The mean(SD) gestational age was 39.7(1.2) weeks and 59% of the infants were male and 87% were Caucasian. The mean(SD) age of the infants at follow-up was 30.1(3.4) days, 3.0(1.1) months and 6.0(0.2) months.

Age, anthropometrics, BMI, sum of skinfolds, Peapod measurements and ultrasound measurements of boys and girls at 1, 3 and 6 months are shown in Table 1.

Table 1. Body composition of the total group and comparison between boys and girls in first 6 months of life.

	Total group (n= 203)	Boys (n=119)	Girls (n=84)	p-value
Birth				
Gestational age (weeks)	39.7(1.2)	39.7	39.8	0.30
Birthweight (grams)	3.435(1.0)	3.356	3.254	0.27
Birthweight SDS	-0.34(1.1)	-0.36	-0.33	0.85
Birth length (cm)	50.0(2.5)	50.0	49.6	0.98
Birth length SDS	-0.19(1.3)	-0.33	0.02	0.14
Age 1 month				
Age (days)	30.1(3.4)	30.1	30.1	0.97
Weight (kg)	4.27(0.6)	4.39	4.15	**0.02**
Weight SDS	0.36(1.2)	0.36	0.35	0.94
Length (cm)	54.6(2.1)	54.9	54.1	**0.006**
Length SDS	0.11(0.9)	0.13	0.07	0.67
Body mass index (kg/m^2)	14.3(1.3)	14.4	14.1	0.14
Body mass index SDS	0.31(1.1)	0.30	0.33	0.87
Sum of peripheral skinfolds (mm)	12.0(2.0)	11.8	12.0	0.37

Table 1. Body composition of the total group and comparison between boys and girls in first 6 months of life. (continued)

	Total group (n= 203)	Boys (n=119)	Girls (n=84)	p-value
Age 1 month				
Sum of central skinfolds (mm)	11.1(2.4)	10.9	11.3	0.30
Fat mass (kg)	0.76(0.5)	0.71	0.82	0.16#
Fat mass percentage (%)	16.8(4.8)	16.3	17.6	**0.05**
Fat-free mass (kg)	3.55(0.4)	3.64	3.42	**0.02#**
Age 3 months				
Age (months)	3.01(1.1)	2.93	3.14	0.21
Weight (kg)	6.01(0.7)	6.20	5.72	**<0.001**
Weight SDS	0.47(1.2)	0.58	0.31	0.11
Length (cm)	61.4(2.1)	62.1	60.4	**<0.001**
Length SDS	0.37(0.9)	0.48	0.23	0.06
Body mass index (kg/m^2)	15.9(1.4)	16.1	15.6	**0.03**
Body mass index SDS	0.22(1.1)	0.28	0.14	0.39
Sum of peripheral skinfolds (mm)	14.7(2.3)	14.9	14.4	0.23
Sum of central skinfolds (mm)	13.2(3.1)	13.1	13.3	0.63
Fat mass(kg)	1.39(0.8)	1.40	1.36	0.50#
Fat mass percentage (%)	22.8(5.3)	22.4	23.3	0.30
Fat-free mass (kg)	4.64(0.5)	4.80	4.41	**0.02#**
US-visceral fat thickness (cm)	2.51(0.6)	2.56	2.44	0.17
US-abdominal subcutaneous fat thickness (cm)	0.43(0.1)	0.44	0.42	0.54
Age 6 months				
Age (months)	6.03(0.2)	6.03	6.03	0.92
Weight (kg)	7.67(0.8)	7.87	7.37	**<0.001**
Weight SDS	0.47(1.2)	0.25	0.13	0.95
Length (cm)	68.1(2.2)	68.8	67.0	**0.006**
Length SDS	0.23(0.9)	0.30	0.13	0.67
Body mass index (kg/m^2)	16.5(1.4)	16.6	16.4	0.28
Body mass index SDS	0.05(1.1)	0.07	0.01	0.71
Sum of peripheral skinfolds (mm)	16.0(3.2)	15.6	16.7	**0.03**
Sum of central skinfolds (mm)	13.4(3.7)	13.2	13.8	0.23
Fat mass (kg)	1.85(0.5)	1.80	1.92	**0.04#**
Fat mass percentage (%)	24.0(5.3)	22.7	25.8	**<0.001**
Fat-free mass (kg)	5.80(0.6)	6.05	5.47	**<0.001#**
US-visceral fat thickness (cm)	2.48(0.6)	2.50	2.45	0.57
US-abdominal subcutaneous fat thickness (cm)	0.44(0.1)	0.43	0.45	0.35

Data expressed as mean(SD) Significant p-values are indicated in boldface
#=corrected for length

Peapod measurements

Mean(SD) fat mass percentage (FM%), measured by Peapod, was at 1 month 16.8(4.8)%, at 3 months 22.8(5.3)% and at 6 months 24.0(5.3)%.

In the total group, there was a significant increase in FM% between 1 and 3 months, but not between 3 and 6 months ($p<0.001$, $p=0.098$, resp.). When girls and boys were analyzed separately, we observed the same pattern in boys ($p<0.001$, $p=0.55$, resp.), but girls showed significant increases in FM% during both age periods ($p<0.001$, $p<0.001$, resp.) although less in the second period. Girls had a higher FM% than boys at 1 and 6 months ($p=0.05$, $p<0.001$, resp.) (Table 1).

Table 2 shows the reference values for FM%, FFM%, FM and FFM. Figures 1 and 2 show the reference curves of FM%, FFM%, FM in kg and FFM in kg for boys and girls for ages 1 to 6 months. Data are presented as median ± 1 and ± 2 SD- scores.

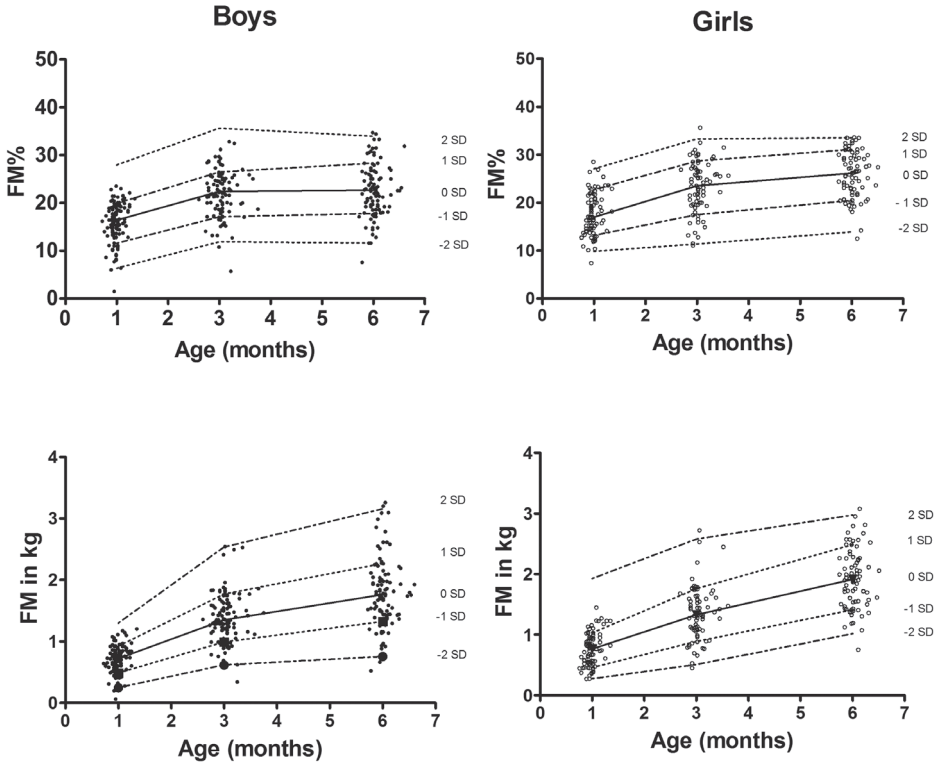

Figure 1. Reference curves for FM% and FM in kg for boys and girls

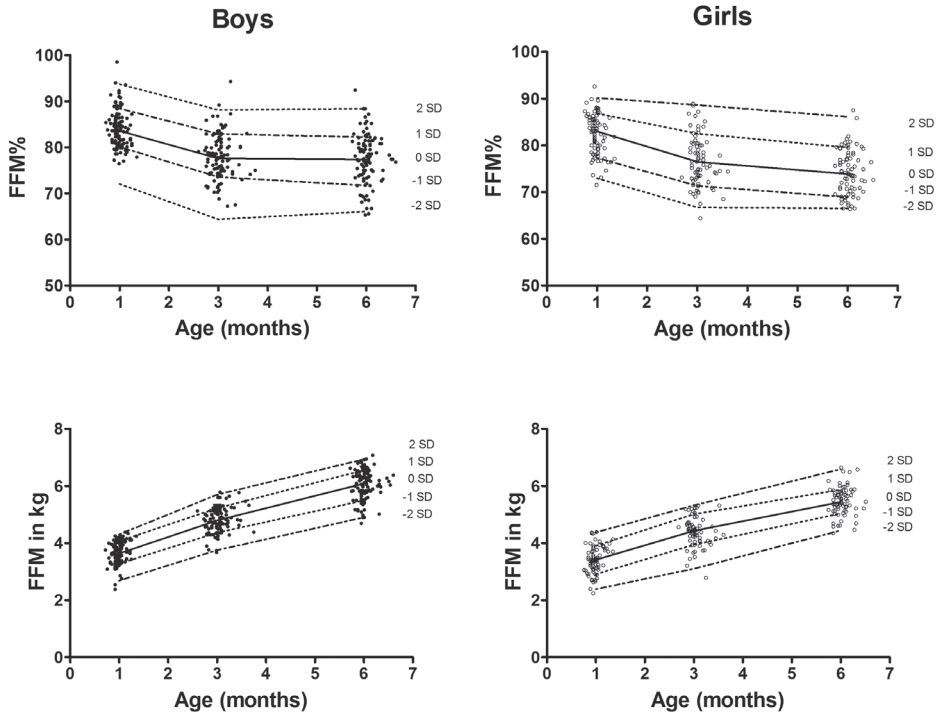

Figure 2. Reference curves for FFM% and FFM in kg for boys and girls

Ultrasound measurements

Mean(SD) visceral fat thickness was 2.51(0.57) cm at 3 months and 2.48(0.64) cm at 6 months of age (p=0.24). Mean(SD) abdominal subcutaneous fat thickness was 0.43(0.12) cm at 3 months and 0.43(0.14) cm at 6 months. There was no difference between abdominal subcutaneous fat thickness at 3 and 6 months. Visceral fat thickness and abdominal subcutaneous fat thickness were weakly correlated at 6 months (r=0.17, p=0.02), but not at 3 months (r=0.01, p=0.94). Reference values are presented in Table 2. Supplemental figure 2 shows the scatterplots of visceral fat thickness and subcutaneous fat thickness at 3 and 6 months.

Relationship between Peapod and ultrasound measurements

At the age of 3 and 6 months, FM% was unrelated to visceral fat thickness, but did correlate positively with abdominal subcutaneous fat thickness (r=0.36, p<0.001, r=0.54, p<0.001, resp.).

Table 2A. References values for FM%, FFM%, FM, FFM and visceral and abdominal subcutaneous fat thickness for boys

Boys					
Age	SDS				
	-2	-1	0	1	2
Peapod					
FM%					
1 month	6.37	11.56	16.40	19.90	27.95
3 months	11.89	17.10	22.40	26.45	35.64
6 months	11.60	17.78	22.65	28.30	33.94
FFM%					
1 month	72.05	80.10	83.60	88.44	93.73
3 months	64.36	73.55	77.60	82.90	88.11
6 months	66.06	71.70	77.35	82.22	88.40
FM (kg)					
1 month	0.2497	0.4885	0.7229	0.924	1.299
3 months	0.6167	0.9910	1.3533	1.771	2.535
6 months	0.7557	1.3241	1.7622	2.266	3.157
FFM (kg)					
1 month	2.6854	3.2753	3.6345	4.0653	4.3050
3 months	3.7541	4.3639	4.7924	5.2230	5.6970
6 months	4.9012	5.4888	6.0881	6.5558	6.9365
Ultrasound					
Visceral fat thickness (cm)					
3 months	1.59	1.92	2.50	3.16	4.04
6 months	1.37	1.74	2.38	3.23	4.16
Subcutaneous fat thickness (cm)					
3 months	0.21	0.32	0.41	0.54	0.76
6 months	0.25	0.32	0.41	0.55	0.67

Table 2B. References values for FM%, FFM%, FM, FFM and visceral and abdominal subcutaneous fat thickness for girls

Girls					
Age	SDS				
	-2	-1	0	1	2
Peapod					
FM%					
1 month	9.85	13.16	17.00	22.69	27.05
3 months	11.40	17.50	23.50	28.67	33.28
6 months	13.89	20.46	26.15	31.10	33.52
FFM%					
1 month	72.95	77.31	83.00	86.84	90.15
3 months	66.72	71.33	76.50	82.50	88.60
6 months	66.48	68.90	73.85	79.54	86.11
FM (kg)					
1 month	0.2746	0.4574	0.7647	1.0299	1.9258
3 months	0.5085	0.8874	1.3295	1.7581	2.5781
6 months	1.0161	1.4090	1.9141	2.4858	2.9735
FFM (kg)					
1 month	2.3864	2.9109	3.4143	3.9085	4.3761
3 months	3.1018	3.9399	4.4336	5.0084	5.3220
6 months	4.4447	5.0346	5.4393	5.8724	6.5913
Ultrasound					
Visceral fat thickness (cm)					
3 months	1.46	1.92	2.41	2.95	3.51
6 months	1.39	1.83	2.39	3.03	3.64
Subcutaneous fat thickness (cm)					
3 months	0.21	0.31	0.42	0.53	0.72
6 months	0.26	0.31	0.45	0.56	0.81

Relationship with other anthropometric measurements

In boys, FM% correlated with weight at the age of 3 and 6 months ($r=0.45$, $p<0.001$, $r=0.50$, $p<0.001$, resp.) (Table 3). In girls, FM% was also correlated with weight at 1, 3 and 6 months of age ($r=0.57$, $p<0.001$, $r=0.49$, $p<0.001$, $r=0.46$, $p<0.001$, resp.). FM% correlated also with BMI at all points in boys and girls (Table 3). Despite the correlation between FM% and weight, the variation in FM% was high, even between infants with a similar weight or BMI. For instance, in infants with a weight SDS of 1 at 1 month, the FM% ranged from 10 to 25% and this range remained wide at 3 and 6 months with a variation in FM% between 12 and 30% and 17 and 33% respectively (data not shown).

Table 3. Linear correlation coefficients between anthropometric parameters, for boys and girls separately

Boys	1 month		3 months				6 months			
Parameter	FM%	FFM	FM%	FFM	US-V	US-S	FM%	FFM	US-V	US-S
Weight	0.28*	**0.85***	**0.45***	**0.62***	0.17	0.22^	**0.50***	**0.69***	0.16	**0.49***
Length	0.17^	**0.69***	0.01	**0.51***	0.10	0.07	0.02	**0.60***	0.14	0.07
BMI	0.29*	**0.53***	**0.55***	**0.38***	0.12	0.22^	**0.58***	**0.37***	0.09	**0.54***
Sum of central skinfolds	0.37*	**0.36***	**0.45***	-0.06	-0.09	**0.33#**	**0.40***	0.18	0.06	0.19
Sum of peripheral skinfolds	0.21^	**0.34***	**0.38***	0.16	0.00	0.24^	**0.47***	0.06	-0.01	0.28#

Girls	1 month		3 months				6 months			
Parameter	FM%	FFM	FM%	FFM	US-V	US-S	FM%	FFM	US-V	US-S
Weight	**0.57***	**0.94***	**0.49#**	**0.80***	-0.20	**0.46***	**0.46***	**0.73***	**0.30#**	0.29#
Length	**0.34^**	**0.82***	0.04	**0.75***	-0.18	0.26^	-0.10	**0.60***	0.06	-0.16
BMI	**0.61***	**0.80***	**0.62***	**0.58***	-0.14	**0.45***	**0.61***	**0.43***	**0.31#**	**0.46***
Sum of central skinfolds	**0.59***	**0.46***	**0.51***	**0.43***	-0.06	0.28^	**0.63***	-0.01	0.14	0.28^
Sum of peripheral skinfolds	**0.55***	**0.54***	0.23	**0.43***	0.06	**0.46***	**0.33#**	0.04	0.08	0.13

Correlation coefficients above 0.30 are indicated in boldface. p-values: *<0.001; #<0.01;^<0.05
FM%: fat mass percentage; FFM: fat free mass in kg; US-V: visceral fat thickness measured by ultrasound; US-S; abdominal subcutaneous fat thickness

In boys, increase in FM% between 1 and 3 months, and between 3 and 6 months were correlated with increase in weight SDS and BMI SDS (r=0.50, p<0.01, r=0.43, p<0.01, r=0.40, p<0.01, r=0.45, p<0.01 resp.). In girls, the correlations between increase in FM% and increase in weight SDS and BMI SDS between 1 and 3 months and between 3 and 6 months were similar (r=0.41, p<0.01, r=0.56, p<0.01, r=0.34, p<0.01, r=0.49, p<0.01, resp.)

The sum of central and peripheral skinfolds at 1, 3 and 6 months correlated significantly with FM%, both in boys and girls (Table 3).

The sum of central or peripheral skinfolds at 3 and 6 months did not correlate with the visceral fat thickness, but did correlate with the abdominal subcutaneous fat thickness by ultrasound, both in boys and girls (Table 3).

DISCUSSION

This paper describes longitudinal reference data on fat mass percentage (FM%) and fat-free mass (FFM) measured by Peapod and subcutaneous and visceral fat thickness measured by ultrasound, in a cohort of 203 healthy term infants during the first 6 months of life. We revealed a significant increment in FM% during mainly the first 3 months of life, without a change between 3 and 6 months. When girls and boys were analyzed

separately, we observed the same pattern in boys but girls showed also a significant increase in FM% between 3 and 6 months. Also visceral fat and abdominal subcutaneous fat thickness remained similar between 3 and 6 months. Girls had a significantly higher FM% and lower FFM at 1 and 6 months than in boys. FM% correlated with abdominal subcutaneous fat thickness, but not with visceral fat thickness.

Previous studies showed that the first months of life are important in the developmental programming of adiposity.(5, 10, 11) Thus data on accurate body composition in early life are essential. Our study is one of the first studies investigating longitudinal FM% and FFM by Peapod in a large group of healthy term infants.(17) Other studies investigated this in a smaller group or in infants in Ethiopia.(19-21, 26)

Our study shows that mainly the first 3 months of life are characterized by a rapid fat deposition followed by a very modest rate, supporting the concept of a critical window for adiposity development in the first 3 months of life.(5, 10, 11) In our study, the girls had a higher FM% at 1 and 6 months of age than the boys. Data on gender differences in body composition in the first months are limited but in studies where body composition was measured at 6 months by dual energy x-ray absorptiometry, girls had also a higher FM% than boys.(27) Our study shows that at every time point, fat-free mass was higher in boys than girls. This gender difference could be attributed to the higher androgen levels in boys in the last trimester of fetal life and during mini-puberty in the first months of life, which increase lean body mass.(28)

Our study also presents normative longitudinal data of abdominal subcutaneous and visceral fat thickness by ultrasound in a large group of infants. We found that both subcutaneous and visceral fat do not significantly increase in the period between 3 and 6 months. Previous studies have consistently shown that particularly visceral obesity, compared to overall obesity, is a key factor in the pathogenesis of metabolic syndrome. (29, 30) Visceral fat is considered more metabolically active compared to subcutaneous fat depot. Through increased release of free fatty acids to the portal circulation, it may be involved in the genesis of insulin resistance.(31) Furthermore, visceral adipocytes are known to produce vasoactive peptides and cytokines which may increase cardiovascular risk via direct effects on the vasculature.(32) Therefore, accurate measurement of visceral fat is highly relevant when assessing infant's risk for developing cardiovascular diseases later in life.

We observed a positive correlation between FM% measured by Peapod and abdominal subcutaneous fat thickness at 3 and 6 months, but not with visceral fat thickness, indicating that overall FM% could not predict the amount of visceral fat and therefore addition of ultrasound measurements is needed.

Although there was a strong correlation between weight SDS and FM%, the variation in FM% was high, even in infants with a similar weight, indicating the importance of accurate measurement of body composition, instead of only weight or weight for length.

Our findings also show that BMI is not a good marker of body composition. BMI is based on an estimate of the infant's body weight and length and does not adequately capture the infant's fatness. For infants, body mass index can be especially misleading because the relationship of lean body mass to height changes as they become older.(33)

In the present study, we measured skinfolds in infants and demonstrated that skinfold measures are reasonable indicators of overall fatness in a study population. However, skinfolds give only information about subcutaneous fat, while Peapod measures total FM%. Although skinfolds did correlate with FM%, it cannot reliably indicate FM% in individual infants, because of the wide variation, and can therefore not replace Peapod measurements.

Our study population was a population born in a hospital, but our data were also generalizable to healthy term infants born at home, because all anthropometric data were within -2 SDS and + 2 SDS and all variables were normally distributed, illustrating a normal distribution in our population. The infants of our study were mostly Caucasian which make our data generalizable for a large part of the developed countries. US measures were not performed at 1 month because visceral and subcutaneous fat thickness is then very low and therefore unreliable to measure. De Lucia Rolfe et al. reported data on abdominal subcutaneous and visceral fat thickness, measured by ultrasound, at 3 and 12 months of age.(24) Our ultrasound measurements at 3 months were comparable with the ultrasound measurements by De Lucia Rolfe et al. There are limited data of abdominal subcutaneous and visceral fat thickness, measured by MRI, but these outcome data are not comparable with ultrasound data, because of different calculation of fat. In this study we did not include nutritional data to investigate the relationship between early nutrition and body composition in early life, which is a limitation of our study. Further studies are needed to investigate this relationship.

In conclusion, FM% increases mainly in the first 3 months of life and stabilizes after 3 months. Also visceral fat thickness remained stable in the period between 3 and 6 months. These data support the concept of a critical window for adiposity development in the first three months of life. Girls had a longer duration of fat mass accrual than boys. Our study provides longitudinal reference data on body composition (FM%, FFM, subcutaneous fat thickness and visceral fat thickness) during the first 6 months of life.

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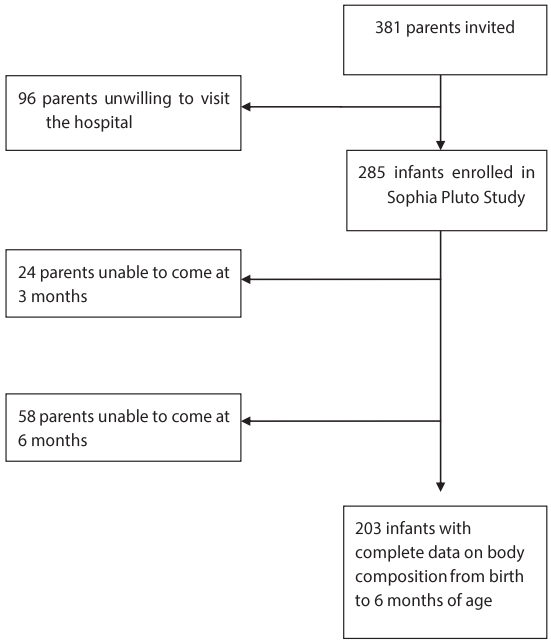
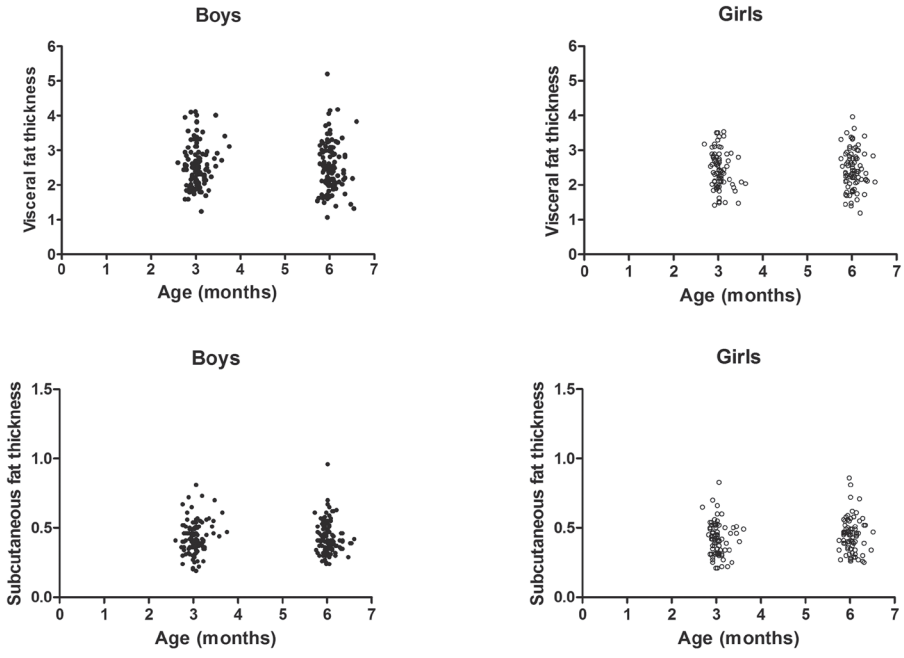


Supplemental figure 1. Flow chart



Supplemental figure 2. Visceral fat and abdominal subcutaneous fat thickness at 3 and 6 months in boys and girls

data interpretation. All authors were involved in writing the manuscript and had final approval of the submitted version.

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6

Chapter 6

Impact of infant growth, type of feeding and maternal factors on total body fat mass and visceral fat at 3 and 6 months of age

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ABSTRACT

Introduction

Accelerated gain in fat mass and particularly in visceral fat in the first months of life might be major risk factors for adult diseases, because fat mass tracks into adulthood. Infant growth, type of feeding and maternal variables might influence the gain in weight, fat mass and visceral fat in early life.

Patients and methods

In 300 healthy term infants, we investigated longitudinal changes in fat mass percentage (FM%), measured by air-displacement plethysmography (Peapod) at 1, 3 and 6 months of age in combination with changes in abdominal visceral and subcutaneous fat, measured by ultrasound, at 3 and 6 months, and their associations with infant growth, type of feeding and maternal variables

Results

Gain in weight or FM% in the first 3 months of life, but not size at birth, was positively associated with FM% at 3 and 6 months of age. Gain in weight or FM% between 1 and 3 months was positively associated with visceral fat at 3 months, and gain in FM% between 3 and 6 months was positively associated with visceral fat at 6 months. Exclusive breastfeeding (EBF) duration was positively associated with subcutaneous fat, but not visceral fat. Maternal characteristics did not associate with infant fat mass or visceral fat at 3 or 6 months.

Conclusion

Higher gain in weight and FM% in the first postnatal months lead not only to higher FM%, but also higher visceral fat at 6 months of age. In contrast, exclusive breastfeeding appears to promote subcutaneous but not visceral fat.

INTRODUCTION

The first three months of life are known to be a critical window for the programming of adiposity and cardiovascular diseases.(1-3) Unravelling the modifiable determinants that influence the adiposity and fat mass development in early life can provide valuable insights to support an optimal infant development.

We have previously shown that there were differences in risk factors for type 2 diabetes and cardiovascular diseases at the age of 21 years between those who had a high versus low gain in weight-for-length during the first months of life, indicating that early growth might be a determinant for later life metabolic health.(4) One of the key elements driving early life growth is the nutrition provided to the young infant. As an example, it is well-known that breastfeeding is the optimal infant feeding, because it is associated not only with less infections and better cognitive development, but also with a lower incidence of childhood obesity and type 1 and type 2 diabetes.(5) However, the influence of breastfeeding on infant's body composition is not straight-forward.(6-10)

Apart from postnatal factors, higher pre-pregnancy maternal weight and more maternal weight gain during pregnancy reportedly result in higher birth weight and fat mass percentage at birth.(11-13) However, it is not known whether these maternal factors continue to influence adiposity development in the first months of life. Given that the prevalence of overweight in adult women has increased from 29.8% to 43%(14) with many pregnant women being overweight or obese, this could potentially contribute to a vicious cycle from mother to offspring.

Although total body fat has adverse consequences on adult disease risks, location of body fat is even more important. Already in childhood, abdominal visceral fat appears to be associated with an unfavorable metabolic profile in later life.(15) Recently, ultrasound has been validated to measure infant visceral fat and abdominal subcutaneous fat, enabling a non-invasive approach to obtain more insights in the development of these fat depots during infancy.(16)

Our aim was to identify the determinants of total fat mass percentage (FM%), as well as visceral and abdominal subcutaneous fat in the first months of life. We hypothesized that, independent of birth weight, early weight gain and gain in FM% between birth and 3 months of age are associated with a higher FM% and more visceral fat at 3 and 6 months. We expected that a longer duration of exclusive breastfeeding would associate with a higher FM% at 6 months due to more subcutaneous fat. We also hypothesized that maternal weight gain during pregnancy and pre-pregnancy BMI would influence infant's FM% and visceral fat at 3 and 6 months.

We, therefore, measured in a cohort of healthy term infants longitudinal total body composition by air-displacement plethysmography at 1, 3 and 6 months of age as well

as visceral and abdominal subcutaneous fat by ultrasound at 3 and 6 months of age and associated these outcomes with infant data, type of feeding and maternal variables.

MATERIAL AND METHODS

Subjects

The study population consisted of 300 healthy term infants, who are part of a birth cohort study (Sophia Pluto Study) examining the postnatal determinants of body composition during infancy. Children were recruited from several hospitals in and near Rotterdam, a large city in The Netherlands. All participants fulfilled the same inclusion criteria: 1) born at term (≥37 weeks of gestation), 2) Age at recruitment <28 days, 3) uncomplicated neonatal period without severe asphyxia (defined as an Apgar score below three after five minutes), sepsis or long-term complication of respiratory ventilation. Exclusion criteria were known congenital or postnatal diseases that could interfere with body composition development, confirmed intra-uterine infection, maternal use of corticosteroids or a maternal medical condition that could interfere with infant's body composition development (e.g. diabetes). The Medical Ethics Committee of Erasmus Medical Center approved the study. Written informed consent was obtained from both parents unless the mother was single.

Data collection and measurements

Parental and pregnancy characteristics

Maternal data, i.e. pre-pregnancy weight and weight just before delivery, height, parity, smoking, ethnicity and complications during pregnancy, were obtained from medical records and questionnaires.

Maternal underweight was defined as a pre-pregnancy BMI <18.5kg/m^2, overweight as a BMI ≥25 kg/m^2 , and obesity as a BMI ≥ 30 kg/m^2.(17) Information regarding socio-economic status, educational levels of the parents was obtained using questionnaires.

The Dutch Standard Classification of Education was used to categorize mothers to one of four levels of education: high (university degree), mid-high (higher vocational training, Bachelor's degree), mid-low (>3 years general secondary school, intermediate vocational training), low (no education, primary school, lower vocational training or 3 years or less general secondary school).(18) Data on gestational age were extracted from medical records.

Infant characteristics

Research clinic visits were scheduled at ages 1, 3 and 6 months. Birth data were taken from midwife- and hospital records. Information on breast- and formula feeding was asked at the clinic visits.

Anthropometrics

Weight was measured to the nearest gram by an electronic infant scale (Seca, Hanover, MD). Length was measured with the two-persons technique to the nearest 0.1 cm by a length meter (Seca). Head circumference was measured to the nearest 0.1 cm using measuring tape (Seca, circumeter). Weight SDS, length SDS and weight for length SDS were calculated with Growth Analyser Research Calculation Tools 4.0 (available at www.growthanalyser.org), according to Dutch age- and gender-matched reference values.(19)

Body composition

Whole-body composition was estimated using air-displacement plethysmography (ADP) using the Peapod, Infant Body Composition System (COSMED).(20-23) Briefly, this ADP system assesses fat mass (FM), fat mass percentage (FM%) and fat free mass (FFM) and fat-free mass percentage (FFM%) by direct measurements of body volume and body mass, based on the whole-body densitometric principle. All measurements were obtained by experienced personnel, according to standardized protocol. The Peapod was calibrated every day, according to the protocol recommended by the supplier.

Abdominal fat

Visceral and abdominal subcutaneous fat were estimated at 3 and 6 months using a Prosound 2 ultrasound, with a UST-9137 convex ultrasound transducer (both from Hitachi Aloka Medical, Switzerland). For both measurements, the transducer was positioned where the xiphoid line intercepted the waist circumference measurement plane. Visceral fat was estimated by measuring visceral depth, which is the distance between the peritoneal boundary and the corpus of the lumbar vertebra, assessed in the longitudinal plane with the ultrasound probe depth set at 9 cm. Subcutaneous abdominal fat was estimated by the distance between the cutaneous boundary and the linea alba at the same location, but on a transverse plane with a probe depth of 4 cm.(16)

Statistical analysis

SPSS statistical package version 20.0 (SPSS Inc. Chicago, Illinois) was used. Variables were normally distributed. Baseline characteristics were expressed as median and interquartile range (IQR). Linear correlations, with adjustments for gender and age, were performed in the total study population to determine associations between birth size SDS, increase in FM% between 1-3 months, duration of exclusive breastfeeding, maternal variables,

such as pre-pregnancy BMI and maternal weight during pregnancy with the dependent variables: FM%, visceral and abdominal subcutaneous fat at ages 3 and 6 months. We also performed multiple regression (MR) analyses to investigate the independent determinants of the above dependent variables. Firstly, we entered age, gender, birth weight SDS, duration of exclusive breastfeeding and W/L_{SDS} at 1 month to the model (model A). Secondly, we entered W/L_{SDS} at 3 months, instead of W/L_{SDS} at 1 month, to the model (model B). Thirdly, we entered delta $W/L_{SDS0\text{-}1mo}$, instead of W/L_{SDS} at 3 month, to the model (model C). Fourthly, we entered delta $W/L_{SDS1\text{-}3mo}$, instead of delta $W/L_{SDS0\text{-}1mo}$, to the model (model D). Finally, we entered delta $FM\%_{1\text{-}3mo}$, instead of delta $W/L_{SDS1\text{-}3mo}$, to the model (model E). In all multiple linear regression models, adjustments were made for gender and age. Models testing maternal variables were adjusted for birth weight to test for their independent effects on postnatal adiposity. All statistical tests were performed two-sided and results were regarded statistically significant if the p-value was ≤0.05.

RESULTS

Clinical characteristics

Maternal and infant demographic characteristics are presented in Table 1. The median (IQR) age of the mothers was 33.0 (29.5-35.7) years and gestational age was 39.7 (38.9-40.7) weeks. Fifty-eight percent of the infants were male and 87% were Caucasian.

Table 1 also summarises the body composition and abdominal fat measurements. Median (IQR) fat mass percentage (FM%) at 1 month was 16.4% (14.1-19.4), at 3 months 23.0% (19.7-25.9) and at 6 months 23.4% (19.9-27.5). Median (IQR) visceral fat was 2.48 cm (2.10-2.89) at 3 months and 2.39 cm (2.06-2.94) at 6 months. The median (IQR) duration of breastfeeding was 99 days (41-178). Median (IQR) maternal pre-pregnancy BMI was 23.4 kg/m^2 (21.2-26.5) and maternal weight gain during pregnancy was 14.0 kg (10-18). Weight gain during pregnancy differed between maternal pre-pregnancy BMI-groups ($p<0.001$).

Table 1. Maternal and infant characteristics

	Median	Interquartile range
Infant characteristics		
Gender (boys) (%)	58	
Mode of delivery (cesarean delivery) (%)	32.3	
Gestational age (weeks)	39.7	38.9-40.7
Birth weight SDS	-0.29	-0.99-0.33
Birth length SDS	-0.36	-1.00-0.69

Table 1. Maternal and infant characteristics (continued)

	Median	Interquartile range
At 1 month		
Weight SDS	0.44	-0.49-1.08
Length SDS	0.03	-0.58-0.68
Fat mass%	16.4	14.1-19.4
At 3 months		
Weight SDS	0.52	-0.22-1.23
Length SDS	0.48	-0.18-0.94
Fat mass%	23.0	19.7-25.9
US-visceral fat (cm)	2.48	2.10-2.89
US-abdominal subcutaneous fat (cm)	0.41	0.35-0.50
At 6 months		
Weight SDS	0.11	-0.35-0.78
Length SDS	0.25	-0.40-0.82
Fat mass%	23.4	19.9-27.5
US-visceral fat (cm)	2.39	2.06-2.94
US-abdominal subcutaneous fat (cm)	0.41	0.35-0.50
Duration of breastfeeding (days)	99	41-178
Maternal characteristics		
Age (y)	33.0	29.5-35.7
Height (cm)	169	165-174
Pre-pregnancy weight (kg)	68	61-75
Pre-pregnancy body mass index (kg/m^2)	23.4	21.2-26.5
Highest weight in pregnancy (kg)	83	74-92
Highest body mass index in pregnancy (kg/m^2)	28.7	25.6-32.8
Weight gain during pregnancy (kg)	14.0	10.0-18.0
In mothers with low pre-pregnancy BMI(<18.5)	14.0	13.0-18.5
In mothers with normal pre-pregnancy BMI (18.5-24.9)	14.0	11.0-17.0
In mothers with high pre-pregnancy BMI (25.0-30.0)	15.0	11.5-22.0
In mothers with obese pre-pregnancy BMI (>30.0)	10.0	4.8-17.0
Smoking during pregnancy (%)	4.7	
Caucasian ethnicity (%)	87	
Educational level (%)		
High	26	
Mid-high	25	
Mid-low	21	
Low	4	
Other or unknown	23	

Data are expressed as median(interquartile range) or percentage. US-visceral fat = visceral fat thickness, measured by ultrasound. US-abdominal subcutaneous fat = abdominal subcutaneous fat, measured by ultrasound.

Associations with FM% at 6 months

Table 2 shows the associations of infant and maternal variables with FM% at 3 and 6 months.

Table 2. Associations of FM% at 1,3 and 6 months with infant and maternal variables

	FM% at 3 months		FM% at 6 months	
	β	p-value	β	p-value
Infant characteristics				
Gestational age (weeks)	-0.67	**0.01**	-0.40	0.18
Birth weight SDS	0.11	0.71	-0.14	0.68
Birth length SDS	-0.10	0.78	-0.01	0.99
At 1 month				
Weight SDS	0.47	0.09	0.13	0.67
W/L_{SDS}	1.58	**<0.001**	0.96	**0.02**
FM%	0.46	**<0.001**	0.32	**<0.001**
Delta $W/L_{SDS0\text{-}1mo}$	1.05	0.08	0.59	0.34
At 3 months				
Weight SDS	2.15	**<0.001**	1.21	**0.001**
W/L_{SDS}	2.94	**<0.001**	2.10	**<0.001**
FM%			0.72	**<0.001**
Delta $W/L_{SDS1\text{-}3mo}$	2.86	**<0.001**	2.53	**<0.001**
Delta $FM\%_{1\text{-}3mo}$	0.53	**<0.001**	0.40	**<0.001**
At 6 months				
Weight SDS			2.51	**<0.001**
W/L_{SDS}			2.87	**<0.001**
Delta $W/L_{SDS3\text{-}6mo}$			1.92	**0.002**
Delta $FM\%_{3\text{-}6mo}$			0.52	**<0.001**
Duration of exclusive breastfeeding (days)	0.01	**0.02**	0.02	**<0.01**
Maternal variables				
Age (y)	0.09	0.23	0.15	0.06
Height of mother (cm)	-0.05	0.44	-0.03	0.61
Maternal pre-pregnancy BMI (kg/m^2)^	-0.14	0.13	-0.10	0.30
Maternal weight gain during pregnancy (kg)^	0.01	0.82	0.01	0.79
Parity mother ^	0.88	**0.04**	0.55	0.23
Smoking mother during pregnancy (yes/no)	0.77	0.62	1.38	0.40
Ethnicity	0.01	0.89	0.02	0.86
Educational level	0.00	0.38	0.00	0.81

Values presented are results of multiple linear regression. β=unstandardized regression coefficient. All models are adjusted for gender and age. ^=Adjusted for birth weight. W/L_{SDS} = weight for length SDS. Delta W/L_{SDS}= gain in weight for length SDS. Delta FM%=gain in FM%. Significant p-values are indicated in boldface

Weight for length SDS (W/L_{SDS}), a measure of adiposity, at 1 and 3 months was positively associated with FM% at 6 months (p=0.02, p<0.001, respectively). Gains in weight for length SDS (delta $W/L_{SDS1-3mo}$) and FM% (delta $FM\%_{1-3mo}$) between 1 and 3 months were both positively associated with FM% at 6 months (all p<0.001). Duration of exclusive breastfeeding was positively associated with FM% at 6 months (p<0.01). We could not identify an association between any of maternal variables (including pre-pregnancy BMI and maternal weight gain during pregnancy) and FM% at 6 months.

Associations with visceral and subcutaneous fat at 3 and 6 months

Table 3 shows the linear associations, corrected for gender and age, of infant and maternal variables with visceral and subcutaneous fat at 3 and 6 months.

Delta $W/L_{SDS1-3mo}$ was positively associated with visceral fat at 3 months (p=0.01), but not at 6 months(p=0.78), while a delta $W/L_{SDS3-6mo}$ showed a positive trend with visceral fat at 6 months (p=0.06).

W/L_{SDS} at 1 month was positively associated with subcutaneous fat at 3 months and 6 months (p=0.04, p=0.05, resp.). W/L_{SDS} at 3 months was also positively associated with subcutaneous fat at 3 and 6 months (both p<0.001) while delta $W/L_{SDS1-3mo}$ and delta $W/L_{SDS3-6mo}$ were positively associated with subcutaneous fat only at 6 months (p<0.001).

Delta $FM\%_{1-3mo}$ was positively associated with visceral fat at 3 months (p=0.02), but not at 6 months(p=0.98), while delta $FM\%_{3-6mo}$ was positively associated with visceral fat at 6 months (p=0.02).

Delta $FM\%_{1-3mo}$ was also positively associated with subcutaneous fat at 3 and 6 months (p=0.01, p<0.001, resp.).

Duration of exclusive breastfeeding was positively associated with subcutaneous fat at 3 and 6 months (p=0.01, p=0.03, resp.), but not visceral fat (Table 2 and 3).

Maternal pre-pregnancy BMI was not associated with infant visceral or subcutaneous fat at 3 and 6 months. Maternal weight gain during pregnancy was positively associated only with subcutaneous fat at 6 months (p=0.03). Other maternal variables were not associated with visceral or subcutaneous fat.

Multiple regression models with dependent variables FM%, visceral and subcutaneous fat

To identify determinants of FM% at 6 months that were independent of age, gender and birth weight, we used multiple regression (MR) models (Table 4). The first model showed that female gender, duration of exclusive breastfeeding and W/L_{SDS} at 1 month were positively associated with FM% at 6 months, after correction for age and birth weight (model R^2=0.166). W/L_{SDS} at 3 months, as well as delta W/L SDS between 1 and 3 months were positively associated with FM% at 6 months, after correction for age, gender, birth

Table 3. Associations of ultrasound measurements at 3 and 6 months with infant and maternal variables

	At 3 months				At 6 months			
	Visceral fat		Subcutaneous fat		Visceral fat		Subcutaneous fat	
	β	p-value	β	p-value	β	p-value	β	p-value
Infant variables								
Gestational age (weeks)	-0.05	0.13	0.01	0.07	0.00	0.74	0.00	0.68
Birth weight SDS	-0.02	0.67	0.00	0.35	0.04	0.83	0.00	0.84
Birth length SDS	-0.03	0.45	0.01	0.15	0.10	0.01	0.00	0.88
At 1 month								
Weight SDS	-0.07	0.05	0.03	**0.001**	0.06	0.08	0.00	0.42
W/L_{SDS}	0.02	0.76	0.02	**0.04**	0.03	0.52	0.02	**0.05**
FM%	-0.01	0.11	0.01	**0.01**	0.00	0.62	0.00	0.30
Delta $W/L_{SDS0\text{-}1mo}$	-0.13	0.07	0.05	**0.002**	0.07	0.31	0.00	0.88
At 3 months								
Weight SDS	0.01	0.86	0.04	**<0.001**	0.07	0.07	0.03	**<0.001**
W/L_{SDS}	0.07	0.20	0.04	**<0.001**	0.04	0.44	0.04	**<0.001**
FM%	0.00	0.63	0.01	**<0.01**	0.00	0.76	0.01	**<0.001**
Delta $W/L_{SDS1\text{-}3mo}$	0.16	0.01	0.02	0.16	0.00	0.95	0.04	**<0.001**
Delta $FM\%_{1\text{-}3mo}$	0.02	0.02	0.01	**0.01**	0.00	0.99	0.01	**<0.001**
At 6 months								
Weight SDS					0.13	0.004	0.05	**<0.001**
W/L_{SDS}					0.10	0.02	0.05	**<0.001**
FM%					0.09	0.09	0.01	**<0.001**
Delta $W/L_{SDS3\text{-}6mo}$					0.14	0.06	0.04	**0.004**
Delta $FM\%_{3\text{-}6mo}$					0.03	0.02	0.01	**<0.001**
Duration of exclusive breastfeeding (days)	0.00	0.49	0.00	**0.01**	0.00	0.74	0.00	**0.03**
Maternal variables								
Age (y)	0.00	0.91	0.00	0.57	0.02	0.13	0.00	0.06
Height of mother (cm)	-0.01	0.08	0.00	0.64	-0.01	0.19	0.00	**0.05**
Maternal pre-pregnancy BMI (kg/m2)	0.01	0.43	0.00	0.13	0.00	0.18	0.00	0.57
Maternal weight gain during pregnancy (kg)	0.01	0.14	0.00	0.46	0.00	0.18	0.00	0.20
Parity mother	-0.02	0.79	0.02	0.15	0.07	0.17	0.02	**0.02**
Smoking mother during pregnancy (yes/no)	0.30	0.11	-0.07	0.10	0.00	1.00	-0.01	0.78
Ethnicity	0.00	0.78	0.00	0.65	0.02	0.09	0.00	0.17
Educational level	0.00	0.47	0.00	0.42	0.00	0.62	0.00	0.42

Values presented are results of multiple linear regression. β=unstandardized regression coefficient. All models are adjusted for gender and age. W/LSDS = weight for length SDS. Delta W/LSDS= gain in weight for length SDS. Delta FM%=gain in FM%Significant p-values are indicated in boldface.

weight and duration of exclusive breastfeeding (β=2.148, p<0.001, β=2.249, p<0.001, β=0.459, p<0.001, resp.).

As weight SDS and W/L_{SDS} at 6 months, and delta $W/L_{SDS3\text{-}6mo}$ and delta $FM\%_{3\text{-}6mo}$ were the only determinants for visceral fat at 6 months, we did not investigate MR models for 6 month of age. MR analyses with visceral fat at 3 months of age as dependent variable showed associations with delta $W/L_{SDS1\text{-}3mo,}$ after adjustment for gender and age (β=0.161, p=0.010) and also further adjustment for duration of breastfeeding (β=0.128, p=0.044).

To investigate which determinants determine subcutaneous fat at 6 months, we used the same models (data not shown). After correction for age, gender, birth weight SDS and duration of breastfeeding, W/L_{SDS} at 3 months, but not at 1 month, was positively associated with subcutaneous fat at 6 months (β=0.042, p<0.001). Also delta W/L_{SDS} between 1 and 3 months was positively associated with subcutaneous fat at 6 months (β=0.047, p=0.001).

Delta FM% between 1 and 3 months was significantly associated with visceral fat at 3 months, after adjustment for gender and age (β=0.007, p=0.022), also after further adjustment for duration of breastfeeding (p=0.029)(data not shown). A higher delta FM% between 1 and 3 months was also associated with subcutaneous fat at 6 months (β=0.007, p=0.002)(data not shown).

DISCUSSION

In this prospective cohort study, we found a strong association of gain in weight SDS and FM% in the first 3 months of life with FM% at 6 months. Gain in weight for length SDS and in FM% between 1 and 3 months was associated with visceral fat at 3 months and a higher gain in FM% between 3 and 6 months was associated with more visceral fat at 6 months. Exclusive breastfeeding duration was positively associated with FM% and subcutaneous fat at 6 months, but not visceral fat.

To our knowledge, this is the first study investigating determinants of infant FM% as well as visceral and subcutaneous fat during the first 6 months of life. Gain in weight for length SDS in the first 3 months of life was associated with higher FM% at 6 months. This is in line with previously reported associations of weight for length SDS in the same period with risk factors for type 2 diabetes and cardiovascular diseases.(24) These data support our previous findings, showing that young adults at the age of 21 years had higher risk factors for type 2 diabetes and cardiovascular diseases when they had a higher gain in weight for length SDS in the first 3 months, while after 3 months no associations between adiposity and risk factors at 21 years could be found. Also other research-groups have confirmed these findings.(1,25)

Table 4. Multiple regression for FM% at 6 months

	Model A		Model B		Model C		Model D		Model E		Model F	
	β	p	β	p	β	p	β	p	β	p	β	p
Age (months)	1.351	0.499	1.407	0.477	-0.164	0.927	1.973	0.328	1.481	0.444	2.354	0.214
Gender*	2.719	**0.001**	2.735	**<0.001**	2.711	**<0.001**	2.673	**0.001**	3.259	**<0.001**	3.025	**<0.001**
Birth weight SDS	-0.791	**0.043**	-0.585	0.097	-0.521	0.104	-0.428	0.233	0.226	0.533	0.220	0.518
Duration of breastfeeding (days)	0.017	**<0.001**	0.018	**<0.001**	0.019	**<0.001**	0.018	**<0.001**	0.019	**<0.001**	0.016	**0.001**
W/L_{SDS} 1 month			1.149	**0.010**								
W/L_{SDS} 3 months					2.148	**<0.001**						
Delta $W/L_{SDS0\text{-}1mo}$							-0.322	0.521				
Delta $W/L_{SDS1\text{-}3mo}$									2.249	**<0.001**		
Delta $FM\%_{1\text{-}3mo}$											0.459	**<0.001**
Overall p-value	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Adjusted R^2	0.166		0.184		0.307		0.139		0.243		0.297	

*gender:0=boys, 1=girls. Ws_{DS}-L_{SDS} = Weight SDS minus length SDS. W/L_{SDS} = weight for length SDS. Delta W/L_{SDS}= gain in weight for length SDS. Delta FM%=gain in FM%. Significant p-values are indicated in boldface.

We show that especially the gain in FM% between 1 and 3 months leads to a higher FM% at 6 months and more visceral fat at 3 months. For example, there is 0.459% higher FM% at 6 months per 1% increase between 1 and 3 months. We have previously shown that infants with a higher FM% at 6 months, measured by DXA scan, tended to keep this higher FM% during childhood (26), while other studies showed that visceral fat in early life tends to track into childhood and adulthood.(27-29) Our results show that this might start in the first 3 months of life. A strength of the current study is its prospective design to investigate whether different growth patterns result indeed in differences in total body FM%.

We found no association between total body FM% and visceral fat, which is in line with the literature showing that in children total body FM% is more associated with subcutaneous fat rather than with visceral fat.(30) Our data show that associations with total FM% cannot be simply extrapolated to visceral fat.

Infants with exclusive breastfeeding at 6 months had a higher FM% at 6 months than those with exclusive formula feeding. This contrasted our expectations, as exclusive breastfeeding during infancy has been associated with a lower risk of childhood obesity.(31) However, we found that longer duration of exclusive breastfeeding was associated with a higher FM%, due to more subcutaneous fat and not to more visceral fat, indicating that the higher FM% in exclusively breastfed infants consists predominantly of a higher amount of subcutaneous fat. Increased subcutaneous fat in childhood has been associated with a more beneficial phenotype for obesity and adult diseases.(32)

While parents play a major role in deciding the mode of nutrition for their infant, other parental and heritable factors may be even more important in determining the body composition and obesity risk in their infants. In contrast to our expectations, maternal variables, like BMI before pregnancy, did not associate with infant FM% at 3 and 6 months. In a previous study, we showed that maternal BMI before pregnancy associates with FM% at birth (2) and also other studies have shown associations between maternal variables and FM% of newborns (13,33), but apparently this early effect disappears after the first month of life. Our findings are in line with another study, showing that maternal BMI and weight gain during pregnancy had no influence on FM%, measured by DXA, in infants at 6 months of age.(34) We now show that there is already a lack of association between maternal variables and FM% of the infants at the age of 3 months.

In conclusion, our study shows that a higher gain in weight for length in the first months of life leads to more visceral fat at 3 months and to more FM% at 3 and 6 months. Similar associations were found with a higher increase in FM% in the first months of life. We consider that these changes in adiposity may underlie the reported long-term associations between weigh for length SDS in early life and risks for obesity and adult diseases in later life. We also show that the consistently reported association between

exclusive breastfeeding and higher total body FM% in mid-infancy appears to be due to higher subcutaneous fat, but not visceral fat.

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7

Chapter 7

Appetite regulating hormones in early life and relationships with type of feeding and body composition in healthy term infants

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Submitted

ABSTRACT

Introduction

Body composition in early life influences the development of obesity during childhood and beyond. Appetite regulating hormones, such as ghrelin and leptin, play a role in the regulation of food intake and energy balance and might thus influence body composition in later life. Studies on associations between appetite regulating hormones and body composition in early life are limited. We, therefore, investigated serum appetite regulating hormones and associated these with type of feeding and body composition, including fat mass percentage (FM%), visceral and subcutaneous fat.

Patients and methods

In 197 healthy term infants, we measured serum fasting levels of ghrelin, leptin, insulin, glucose-dependent insulinotropic peptide (GIP), pancreatic polypeptide (PP) and peptide YY (PYY) at 3 months and in 41 infants also at 6 months. We investigated their associations with type of feeding and longitudinal fat mass percentage (FM%) measured by air-displacement plethysmography at 1, 3 and 6 months and with abdominal visceral and subcutaneous fat, measured by ultrasound, at 3 and 6 months.

Results

Infants with exclusive formula feeding for 3 months had significantly higher serum levels of ghrelin, leptin, insulin, GIP and PP ($p=0.026$, $p=0.018$, $p=0.002$, $p<0.001$, resp.) and lower serum levels of PYY ($p=0.002$) at 3 months than breastfed infants. Leptin and ghrelin were positively correlated with FM% at 3 months and insulin with change in FM% between 1 and 3 months ($r=0.40$, $p<0.001$, $r=0.23$, $p<0.05$, $r=0.22$, $p<0.01$, resp.). Leptin at 3 months correlated with subcutaneous fat at 3 months ($r=0.23$, $p<0.001$), but not with visceral fat. Other appetite regulating hormones did not correlate with body composition.

Conclusion

Formula fed infants had a very different profile of serum appetite regulating hormones than breastfed infants, suggesting that lower levels of ghrelin, leptin and insulin in breastfed infants could contribute to the protective role of breastfeeding against obesity development. Leptin, ghrelin and insulin were associated with fat mass percentage or changes in fat mass percentage.

INTRODUCTION

Childhood obesity is a worldwide problem with an increasing prevalence of 7 to 11% in Dutch children aged 4 to 12 years.(1) It is not only associated with short-term morbidity, but also with long-term morbidity, such as adult obesity, type 2 diabetes and cardiovascular diseases.(2-4) Accelerated weight gain during the first three months of life is associated with accumulation of fat mass during childhood and a worse cardiovascular and metabolic profile in young adulthood.(5-7) Appetite regulating hormones play a role in the regulation of food intake and body composition by signaling satiety and energy reserves through hypothalamic receptors.(8) However, little is known about the relation between body composition and appetite regulating hormones in early life.(9)

Changes in body composition might be influenced by programming of the orexigenic and anorectic appetite regulating hormones.(10) Orexigenic appetite stimulating hormones, such as ghrelin, are important in the initiation, cessation and frequency of eating. Anorectic appetite regulating hormones, such as leptin and peptide YY (PYY), decrease food intake and increase metabolic rate. The glucose-dependent insulinotropic peptide (GIP) stimulates pancreatic beta-cells in response to the ingestion of meals or glucose. (11,12) All these signals act at several sites in the central nervous system (CNS) but the pathways converge to the hypothalamus, which contains a large number of peptides and other neurotransmitters that influence food intake.

The protective role of breastfeeding for obesity could be partly explained by the composition of the human milk, but probably also by different appetite regulating hormones in infants due to breastfeeding. Knowledge about the changes in appetite regulating hormones during infancy is very limited.(13)

We hypothesized that formula fed infants would have a different profile of serum appetite regulating hormones than breastfed infants and that fasting serum ghrelin, leptin and insulin levels would be positively associated with gain in weight and in fat mass during the first 3 months after birth while GIP, PP and PYY levels would be negatively associated. We, therefore, investigated fasting serum levels of ghrelin, leptin and insulin, GIP, PP and PYY at 3 months and associated these with type of feeding and body composition, including FM% and visceral and subcutaneous fat at 3 and 6 months.

MATERIAL AND METHODS

Subjects

The current study is part of a birth cohort study (Sophia Pluto Study) which started in January 2013, aiming to provide detailed data on body composition and growth in early life.

Serum levels of appetite regulating hormones (ghrelin, leptin, insulin, GIP, PYY, PP) were determined in a random subgroup of 197 infants at 3 months of age and in 41 of them also at 6 months of age.

Infants were recruited from several hospitals in and near Rotterdam, a large city in The Netherlands. All participants fulfilled the same inclusion criteria: 1) born term (≥37 weeks of gestation), 2) age <28 days, 3) uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score below three after five minutes), sepsis or long-term complication of respiratory ventilation. Exclusion criteria were known congenital or postnatal diseases that could interfere with body composition development, confirmed intra-uterine infection, maternal use of corticosteroids or significant maternal medical condition that could interfere with infant's body composition development (e.g. diabetes).

The Medical Ethics Committee of Erasmus Medical Center approved the study. Written informed consent was obtained from both parents unless mother was single.

Data collection and measurements

Information about the type of feeding was recorded during research clinic visits.

Infant characteristics

Research clinic visits were scheduled at 1, 3 and 6 months. Birth data were taken from midwife and hospital records. Trained pediatric nurses performed the measurements according to standard procedures.

Anthropometrics

Weight was measured to the nearest gram by an electronic infant scale (Seca, Hanover, MD). Length was measured to the nearest 0.1 cm by a length meter (Seca). Head circumference was measured to the nearest 0.1 cm using measuring tape (Seca, circumeter). Weight SDS, length SDS and weight for length SDS were calculated with Growth Analyser Research Calculation Tools 4.0 (available at www.growthanalyser.org), according to Dutch age- and gender-matched reference values.(14)

Body composition

Whole-body composition was assessed using air-displacement plethysmography (Peapod, Infant Body Composition System, COSMED). A detailed description of the air-displacement plethymogragphy (ADP) system is provided elsewhere.(15-18) Briefly, this ADP system assesses fat mass (FM), fat mass percentage (FM%) and fat free mass (FFM) and fat-free mass percentage (FFM%) by direct measurements of body volume and body mass, based on the whole-body densitometric principle. All measurements were

obtained by experienced personnel, according to standardized protocol. The Peapod was calibrated every day, according to the protocol recommended by the supplier.

Abdominal fat

Visceral and abdominal subcutaneous fat were measured at 3 and 6 months using a Prosound 2 ultrasound, with a UST-9137 convex ultrasound transducer (both from Hitachi Aloka Medical, Switzerland). For both measures, the transducer was positioned where the xiphoid line intercepted the waist circumference measurement plane. Visceral fat was estimated by measuring visceral depth, which is the distance between the peritoneal boundary and the corpus of the lumbar vertebra, assessed in the longitudinal plane with the ultrasound probe depth set at 9 cm. Subcutaneous abdominal fat was estimated by the distance between the cutaneous boundary and the linea alba at the same location, but on a transverse plane with a probe depth of 4 cm.(19)

Collection of blood and assays

At 3 months, blood samples were collected from a heel prick after the infants had fasted for at least 3 hours. To stabilize the appetite regulating hormones, blood samples were collected in EDTA tubes and dipeptidyl peptidase-4 inhibitor (DPP4-inhibitor, Merck Chemicals) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (serine protease inhibitor, Calbiochem) were added at the time of collection. Blood was centrifuged at 4 ºC to prepare plasma, which was quickly frozen. Samples were stored at -80 ºC.

Ghrelin (active), leptin, insulin, GIP, PP and PYY concentrations in serum were determined by the MILLIPLEX MAP-Human Metabolic Hormone Magnetic Bead Panel, catalog number HMHMAG-34K (Millipore Corporation, Billerica, MA) using the commercial protocol.

Statistical analysis

Descriptive results are expressed as median (interquartile range). Differences between groups were examined using Mann-Whitney U tests. We assessed linear correlations between levels of ghrelin, leptin, insulin, GIP, PP and PYY and other parameters using Pearson's correlation coefficient. Correlation coefficient below 0.20 was considered to be a negligible correlation. SPSS statistical package version 20.0 (SPSS Inc. Chicago, Illinois) was used. All statistical tests were performed two-sided and results were regarded statistically significant if the p-value was <0.05.

Table 1. Clinical characteristics

	Total group		Girls	Boys	p-value	Subgroup	p-value #
	(n=197)		(n=93)	(n=104)	♀ vs. ♂	(n=41)	
Birth							
Gestational age (weeks)	39.9	38.9-40.6	39.9	39.7	0.58	39.4	**0.03**
Birth weight SDS	-0.38	-1.12-0.33	-0.42	-0.35	0.60	-0.85	0.08
Birth length SDS	0.13	-1.05-0.80	0.13	-0.38	0.47	-0.97	0.10
Age 1 month							
Age (months)	0.95	0.92-1.05	0.95	0.99	0.38	0.95	0.34
Weight SDS	0.33	-0.51-1.09	0.42	0.33	0.79	-0.04	0.09
Length SDS	0.04	-0.61-0.68	0.04	0.02	0.61	-0.11	**0.03**
Sum of peripheral skinfolds (mm)	11.0	10.0-13.0	11.0	11.0	0.73	11.0	0.33
Sum of central skinfolds (mm)	11.0	9.0-12.0	11.0	11.0	0.67	11.0	0.46
Fat mass (kg)	0.68	0.54-0.86	0.65	0.73	0.62	0.74	0.65
Fat mass percentage (%)	16.4	13.7-19.4	16.5	16.3	0.39	17.7	0.49
Age 3 months							
Age (months)	2.99	2.92-3.06	2.99	2.99	0.90	2.99	0.40
Weight SDS	0.49	-0.24-1.22	0.36	0.57	**0.05**	0.27	0.58
Length SDS	0.43	-0.17-0.90	0.27	0.56	**0.04**	0.18	0.14
Sum of peripheral skinfolds (mm)	15.0	13.0-16.0	14.0	15.0	0.10	15.0	0.28
Sum of central skinfolds (mm)	13.0	11.0-15.0	12.0	13.0	0.53	13.0	**0.05**
Fat mass (kg)	1.33	1.12-1.59	1.29	1.37	0.23	1.32	0.79
Fat mass percentage (%)	22.6	19.7-25.8	23.1	22.4	0.34	23.2	0.92
Visceral fat (cm)	2.53	2.03-2.89	2.39	2.54	0.23	2.54	0.31
Abdominal subcutaneous fat (cm)	0.42	0.35-0.50	0.42	0.43	0.14	0.41	0.42
Age 6 months							
Age (months)	6.01	5.95-6.11	6.01	6.01	0.57	5.98	0.57
Weight SDS	0.14	-0.31-0.71	0.06	0.18	0.33	0.09	0.37
Length SDS	0.29	-0.34-0.78	0.25	0.31	0.64	0.20	0.14
Sum of peripheral skinfolds (mm)	16.0	14.0-17.0	17.0	15.0	0.18	15.0	0.75
Sum of central skinfolds (mm)	12.0	11.0-15.0	13.0	12.0	0.36	13.0	0.85
Fat mass (kg)	1.76	1.44-2.09	1.79	1.75	0.42	1.70	0.31
Fat mass percentage (%)	23.5	20.2-27.3	24.8	22.8	**<0.01**	22.6	0.16
Visceral fat (cm)	2.34	2.00-2.84	2.34	2.45	0.80	2.19	0.91
Abdominal subcutaneous fat (cm)	0.41	0.35-0.51	0.45	0.41	0.63	0.40	0.39

Data expressed as median (interquartile range)
Significant p-values are indicated in boldface # Differences between total group and subgroup

RESULTS

Clinical characteristics of the infants are presented in Table 1. The median (IQR) gestational age was 39.9 (38.9-40.6) weeks and 53% of the infants were boys. Forty-one boys had their appetite regulating hormones longitudinally determined at 3 and 6 months. Fifty-six per cent of the infants received exclusive breastfeeding at 1 month, 32% at 3 months and 16% at 6 months.

Table 2 shows serum levels of ghrelin, leptin, insulin, GIP, PP and PYY at 3 and 6 months. Median fasting time had been 3:00 (2:30-3:40) hours at 3 months and 2:52 (2:13-4:15) hours at 6 months. Ghrelin, GIP and PP levels increased significantly from 3 to 6 months ($p<0.001$, $p=0.016$, $p<0.001$, resp.), while the leptin levels decreased between 3 and 6 months ($p=0.009$).

There were no differences in appetite regulating hormone levels between boys and girls (Table 2).

Table 2. Serum levels of appetite regulating hormones in infants at 3 and 6 months of age

	3 months				6 months		p-value girls vs. boys at 3 months
	Girls		Boys		Boys		
Total group (n=197)							
Hours fasting (h)	2:50	2:22-3:30	3:12	2:30-3:55			0.11
Ghrelin (pg/ml)	44.0	23.4-82.1	48.9	23.4-68.3			0.57
Leptin (pg/ml)	1439.6	875.3-2364.7	1405.2	742.6-2001.4			0.24
Insulin (pg/ml)	369.0	232.3-619.5	430.5	265.7-738.1			0.30
GIP (pg/ml)	244.8	133.0-393.1	237.3	138.5-350.8			0.87
PP (pg/ml)	59.8	31.7-94.2	68.3	46.5-104.1			0.08
PYY (pg/ml)	210.6	159.4-293.8	196.9	156.3-250.8			0.24
							p-value 3 vs. 6 months
Subgroup (n=41)							
Hours fasting (h)			3:25	2:38-4:00	2.52	2.13-4.15	0.252
Ghrelin (pg/ml)			46.2	20.2-67.6	83.1	48.7-102.6	**<0.001**
Leptin (pg/ml)			1560.4	945.4-1991.4	827.2	424.6-1247.2	**<0.001**
Insulin (pg/ml)			464.9	291.6-718.5	483.8	324.4-635.6	0.821
GIP (pg/ml)			263.1	137.1-345.4	310.9	201.5-421.1	**0.008**
PP (pg/ml)			70.9	41.4-127.8	115.7	68.4-241.6	**<0.001**
PYY (pg/ml)			171.6	150.1-231.7	158.7	130.7-205.6	0.065

Data expressed as median (interquartile range)
Significant p-values are indicated in boldface

Associations between appetite regulating hormones and type of feeding

Table 3 shows the serum levels of appetite regulating hormones during exclusively formula feeding vs. exclusively breastfeeding for 3 months. There were no significant differences in weight SDS, FM%, visceral and subcutaneous fat at 1, 3 and 6 months between the formula fed and breastfed infants but all appetite regulating hormones were different between the formula fed and breast fed groups. Serum levels of ghrelin, leptin, insulin, GIP and PP were significantly higher in the formula fed group compared to the breastfed group (p=0.026, p=0.018, p=0.002, p<0.001, resp.), whereas PYY was significantly lower (p=0.002).

At 3 months, a shorter duration of exclusive breastfeeding correlated only with a higher PP level (r=-0.24, p=0.005). At 6 months, we found no significant correlations of duration of breastfeeding with appetite regulating hormones.

Table 3. Serum levels of appetite regulating hormones in 197 infants at 3 months, divided by type of feeding

	Exclusive FF at 3 months	Exclusive BF at 3 months	p-value
Ghrelin (pg/ml)	49.1	33.2	**0.026**
Leptin (pg/ml)	1719.1	1190.1	**0.018**
Insulin (pg/ml)	560.9	287.8	**<0.001**
GIP (pg/ml)	304.1	198.5	**0.002**
PP (pg/ml)	87.7	46.3	**<0.001**
PYY (pg/ml)	180.2	231.0	**0.002**

Data were expressed as medians. Significant p-values are indicated in boldface

Associations between appetite regulating hormones and fat mass percentage

Serum leptin at 3 months correlated with fat mass percentage (FM%) at that age (r=0.37, p<0.001) (Table 4). Similar associations were found between serum leptin at 3 months and FM% at 6 months (r=0.41, p<0.001). Serum insulin at 3 months correlated with the increase in FM% between 1 and 3 months (r=0.22, p<0.01). The other appetite regulating hormones did neither correlate with FM% nor with changes in FM%. We found in the formula fed infants a positive correlation between ghrelin at 3 months and FM% (r=0.23, p<0.05) at that age. In the breastfed infants, we did not find this correlation, but a stronger correlation between leptin and FM% (r=0.61, p<0.001) (data not shown).

Associations between appetite regulating hormones and visceral and subcutaneous fat

Serum leptin, ghrelin and insulin at 3 months did not correlate with visceral and subcutaneous fat at 3 and 6 months. Breastfed infants showed a correlation between leptin and subcutaneous fat at 3 months (r=0.38, p<0.01) (data not shown).

Associations between appetite regulating hormones and anthropometrics

As a proxy for body composition, anthropometrics are often used. We, therefore, also analyzed correlations between appetite regulating hormones and anthropometrics. Serum leptin at 3 months correlated positively with weight for length SDS at 3 months (r=0.48, p<0.001) and 6 months (data not shown) (Table 4). A higher PYY at 3 months correlated with lower weight for length SDS at 3 months (r=-0.23, p<0.01). Serum leptin and insulin at 3 months correlated with the increase in weight for length SDS between 1 and 3 months of life (r=0.45, p<0.001, r=0.20, p<0.01, resp.).

Table 4. Correlations of appetite regulating hormones with body composition and anthropometry at 3 months in 197 infants

	FM%	Delta FM% 1-3mo	Visceral fat	Subcutaneous fat	Weight for length SDS	Delta weight for length SDS 1-3mo
Ghrelin (pg/ml)	0.11	0.01	<0.01	<0.01	0.06	0.04
Leptin (pg/ml)	**0.37****	0.04	0.01	0.16^	**0.48****	**0.45****
Insulin (pg/ml)	<0.01	**0.22***	0.19*	-0.01	0.08	**0.20***
GIP (pg/ml)	0.03	-0.16^	0.11	-0.08	0.04	0.13
PP (pg/ml)	0.04	-0.02	0.04	-0.02	0.03	0.09
PYY (pg/ml)	-0.08	0.03	0.03	-0.12	**-0.23***	-0.12

Significant p-values are indicated in boldface if r>0.20 and p<0.05. ^:p<0.05 *: p<0.01 **: p<0.001

DISCUSSION

In this study, we investigated the fasting serum levels of appetite regulating hormones, such as ghrelin, leptin, insulin, GIP, PP and PYY in infants at 3 and 6 months and their associations with type of feeding, FM%, visceral fat and subcutaneous fat. Interestingly, all appetite regulating hormones were different between infants with formula feeding compared to the breastfed infants. Serum levels of ghrelin, leptin, insulin, GIP and PP were significantly higher in the formula fed group, whereas PYY was significantly lower. Leptin at 3 months correlated positively with FM% at 3 and 6 months, but only with subcutaneous fat and not with visceral fat. Also ghrelin at 3 months correlated with FM% at 3 months, but only in formula fed infants. Serum insulin correlated positively with gain in FM% between 1 and 3 months. Other appetite regulating hormones were not associated with fat mass percentage and visceral fat in the first 3-6 months.

We show that formula fed infants have different levels of appetite regulating hormones than infants with exclusive breastfeeding. For healthy term infants exclusive breastfeeding is considered the reference to which formula feeding must be compared. Serum levels of ghrelin were significantly higher in formula fed than in breastfed infants. These data are in line with a cross-sectional study where the same results were found in

the first year of life in a group of Italian infants.(20) It has been demonstrated in animal studies that neonatal ghrelin is important for normal maturation of hypothalamic neural circuits. The neural developmental activity of ghrelin is essentially restricted to the neonatal period.(21) Proper expression of ghrelin during neonatal life is, therefore, crucial for lifelong metabolic regulation and too high ghrelin levels during the first months of life result in lifelong metabolic disturbances.(22) In the formula fed infants, we did not find very high levels of ghrelin, but their levels were significantly higher than in the breastfed infants. In this critical period, this could have adverse effects on the maturation of the hypothalamic neural circuits resulting in less favorable metabolic regulation in later life.(23) We found also higher leptin levels in formula fed infants. Like ghrelin, leptin is known to promote the development of the arcuate nucleus in the hypothalamus and its developmental action is also restricted to a critical window in the first months of life(24), but higher ghrelin levels could impair leptin signaling in the arcuate nucleus in early life. (21) Our data are in line with another study, reporting higher leptin levels in formula fed infants compared to breastfed newborns.(25)

Infants with formula feeding had lower levels of PYY. One of the actions of PYY is to reduce food intake by reducing the gastro-intestinal tract motility and the gastric emptying, via the vagal-brainstem-hypothalamic pathway.(26) As the breastfed infants had higher PYY levels, that could be a link in the protective role of breastfeeding for obesity. In addition, we found that higher levels of PYY were associated with a lower weight for length SDS at 3 months. These results are in line with the data of Helsinki Birth Cohort Study, where they found higher PYY levels in adulthood when the infants had a lower growth rate during infancy.(27) Our study shows that these differences can already be found in early life. Infants with formula feeding had also higher levels of insulin, GIP and PP. A possible explanation of the higher insulin levels in formula fed infants could be the amount of proteins, which is in general higher in formula feeding than in breastfeeding. (28) Another higher appetite regulating hormone in formula fed infants was GIP, which plays an important role in the regulation of plasma glucose and insulin secretion.(29) It is possible that higher GIP levels could account for an enhanced insulin release.(30) Also PP was higher in formula fed infants. The function of PP is to self-regulate pancreatic secretion activities.(31) However, the mechanism how the type of feeding influences the GIP and PP levels is not fully understood. For further interpretation of the potential relevance of these findings more research is required.

To our knowledge, this is the first large study investigating the relationship between simultaneously measured serum levels of appetite regulating hormones and detailed measurements of infant body composition, by air-displacement plethysmography (Peapod). We found a significant positive correlation between serum ghrelin and FM% at 3 months in the formula fed infants, but not in the breastfed group. Leptin was also correlated with FM% in both formula fed and breastfed groups. The associations of leptin

with FM% could be explained by the known physiology of adipose tissue. During early life, the adipocytes proliferate and differentiate.(32) Leptin levels increase in parallel to the number of adipocytes during the last part of the third trimester during gestation and the first months of life.(33) We demonstrated that leptin levels in male infants were higher at 3 months than at 6 months. This fits with our previous findings that there is an increase in fat mass in the first 3 months of life and a stabilization thereafter in male infants.(34) Serum insulin correlated positively with gain in FM% between 1 and 3 months. Earlier studies showed a positive association of insulin and insulin resistance with fat mass in childhood and adulthood.(35,36) The other appetite regulating hormones such as PP and GIP did not associate with FM% or gain in FM% in the total group, which partly contrasted our expectations. There is a need for further studies to identify the underlying mechanisms and potential implications for possible programming effects of serum appetite hormones on later body composition.

We could not identify differences in appetite regulating hormones between boys and girls, which is in line with studies in older children and in a study with a subset of appetite regulating hormones in infants.(29,37,38)

We did not investigate hormone levels in breastmilk. Some studies investigated levels of ghrelin, leptin or insulin in breastmilk.(39-41) In these studies, associations between appetite regulating hormones in breast milk and anthropometric data of infants were found. An unhealthy maternal diet can lead to an unfavorable appetite regulating hormone profile in breast milk, but it is not clear by which mechanism this unfavorable profile in breastmilk could influence infant growth as hormones will be degraded when passing the stomach. In our study, we only investigated fasting levels of appetite regulating hormones. We realize that several appetite regulating hormones are low in fasting state (29), but our study shows that even in fasting state all hormones were different between the formula fed and breastfed infants. It would be interesting to investigate appetite regulating hormone levels before and after a meal, but in our study it was found unethical to collect blood for research purpose twice in healthy infants.

In conclusion, we found significantly higher serum levels of ghrelin, leptin, insulin, GIP and PP and lower serum levels of PYY in infants who received exclusive formula feeding for at least 3 months compared to breastfeeding. Besides associations of leptin, ghrelin and insulin with body composition in infants of 3 and 6 months of age, we could not identify other associations of serum appetite regulating hormones with body composition. Further studies on appetite regulating hormones and the interactions between these and body composition later in infancy would be helpful to further elucidate whether appetite regulating hormones contribute to the programming of metabolic health in later life.

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8

Chapter 8

Microbiome in early life and relationships with type of feeding, accelerated weight gain and body composition in healthy term infants

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Submitted

ABSTRACT

Introduction

The first three months of life are known to be a critical window for the programming of adiposity and cardiovascular diseases. Studies suggest a relationship between the compositional pattern of the gut microbiota and obesity in adults, but such data are very limited in infants. We, therefore, investigated several fecal parameters in early life, such as secretory immunoglobulin A (sIgA) and short chain fatty acids (SCFA) contents, and the composition of the microbiota at the first and third month of life, also in relation to type of feeding, infant's body composition and accelerated weight gain.

Patients and methods

Fecal samples of 51 infants were obtained at 1 and 3 months after birth. The microbiota composition was characterized by targeted 16S rRNA gene amplicon pyrosequencing. Associations of type of feeding, infant's body composition and accelerated weight gain in combination with the sIgA, SCFAs and microbiota were investigated.

Results

Formula fed (FF) infants had lower levels of sIgA at 1 and 3 months than their breastfed (BF) counterparts (both $p<0.001$), but levels increased over time in contrast to BF infants. In FF infants, we found that a higher change in sIgA between 1 and 3 months correlated with a higher increase in FM% between 1 and 3, and 3 and 6 months ($p=0.02$, $p=0.003$) and that higher sIgA and acetic acid at 3 months correlated with more visceral fat at that age ($p=0.04$, $p=0.02$), while a higher level of propionic acid at 1 month correlated with a lower increase in FM% between 3 and 6 months ($p=0.02$). Besides higher levels of Firmicutes at 3 months, FF infants had no different microbiota compared to BF infants. Infants with accelerated weight gain had higher amounts of sIgA at 3 months and lower levels of butyric acid at 1 month ($p=0.009$, $p=0.02$, resp.). Microbiota were not different between infants with and without accelerated weight gain at month 1, 3, or over time, although infants with accelerated weight gain had a significantly higher increase in *Enterobacter* compared to FF and BF infants without accelerated weight gain ($p=0.048$, $p=0.035$, resp.).

Conclusion

Formula fed infants had lower levels of sIgA and higher SCFAs at 1 and 3 months. Only in FF infants, we found correlations of sIgA and SCFAs with total fat mass, visceral and subcutaneous fat. Besides higher levels of Firmicutes at 3 months, FF infants had no different microbiota compared to BF infants. Infants with accelerated weight gain in the critical window had a higher increase in *Enterobacter* which might contribute to a different metabolic programming and increased risks for adult diseases.

INTRODUCTION

The prevalence of overweight and obesity in children is increasing.(1) Overweight and obesity in childhood increases the risk for obesity, type 2 diabetes and cardiovascular diseases in later life. The first three months of life are known to be a critical window for the programming of adiposity and cardiovascular diseases. Accelerated weight gain and particularly fat accumulation in early life has been associated with a higher risk for these adult diseases.(2-5) Obesity and its associated disorders are characterized by a state of chronic, low-grade inflammation.(6)

Various studies suggest a relationship between the compositional pattern of the gut microbiota and obesity in adults.(7) Especially the levels of short-chain fatty acids (SCFAs) are linked to obesity.(8) These SCFAs, such as acetate, butyrate and propionate are the main fermentation products of the gut bacteria ensuing from fiber breakdown.(9) Although the SCFAs are neither purely obesogenic nor anti-obesogenic and their mechanism of action is still unknown, butyrate and propionate show more anti-obesogenic potential whereas acetate shows more obesogenic potential.(10-12)

Secretory immunoglobulin A (sIgA) protects against infections of the intestinal epithelium and is the first line of defense in protecting epithelium from enteric toxins and pathogenic microorganisms.(13) Elevated levels of sIgA have been associated with an upregulated immune response, but high levels could also be measured in infants with breastfeeding in the first months of life because of sIgA in breast milk.(14) The microbiome of obese patients contains more Firmicutes and less Bacteroidetes, suggesting that specific compositions of the gut microbiota might be linked to weight status of the host.(15-17) Different hypotheses are currently being investigated, with one postulating that an altered gut microbiota composition is related to obesity via low-grade inflammation.(18) Other studies showed that microbiota in humans changed after losing weight, suggesting that body weight can also influence microbiota.(19) However, no data are available about microbiota in infancy and the relation with body composition and weight gain in early life. The latter is unfortunate since published data suggest that some bacteria which colonize the gut during the first months of life become part of the adult microbiome.(20) An adult colon harbors a vast number of microorganisms, which is extremely diverse, yet infants are born without microbes, or at least very few, and experience a huge influx of maternal and environmental bacteria after birth which rapidly colonize the infant gut.(21,22) The microbial composition in the first year of life is typically characterized by high instability and low species diversity.(23) Type of feeding is one of the major determinants of subsequent development of the microbiota.(24-27) Breastfed infants have a higher amount of sIgA and a relative high abundance of bifidobacteria and lactic acid bacteria, such as *Lactobacillus* and *Enterococcus spp*, which are linked to a reduced risk for infections and chronic illness.(28)

Based on the literature, we hypothesized that infants with formula feeding have, compared to those with exclusive breastfeeding, a different composition of sIgA, SCFAs and microbiota in their stools at 1 and 3 months. In addition, we hypothesized that infants with formula feeding have, compared to infants with exclusive breastfeeding, different development of sIgA and SCFAs and microbiota in the first 3 months of life, which are associated with development of more total fat mass and visceral fat.

Thirdly, we hypothesized that accelerated weight gain in the first 3 months of life is of influence on the levels of sIgA, SCFAs and microbiota at 1 and 3 months and their changes.

We, therefore, investigated several fecal parameters, such as sIgAs, SCFAs and the microbiota composition at the first and third month of life and associated the results with type of feeding, infant body composition and accelerated vs. non-accelerated weight gain in the first months of life. We first present the sIgA and SCFA levels and subsequently the microbiota data.

MATERIAL AND METHODS

Subjects

The current study is part of a birth cohort study (Sophia Pluto Study) examining the postnatal determinants of body composition during infancy. Detailed analyses of the stool microbiota at the age of 1 and 3 months were conducted in a subgroup of 51 infants, 30 with exclusive breastfeeding and 21 with exclusive formula feeding for 3 months, with oversampling of infants with accelerated weight gain in the first 3 months in order to increase the statistical power to find differences in body composition development. Accelerated gain in weight was defined as an increase in weight between birth and 3 months of ≥ 0.67 SDS. The study group consisted of 15 infants with exclusive breastfeeding and 6 with exclusive formula feeding with accelerated weight gain and 15 infants with exclusive breastfeeding and 15 with exclusive formula feeding without accelerated weight gain.

Children were recruited from several hospitals in and near Rotterdam, a large city in The Netherlands. All participants fulfilled the same inclusion criteria: 1) born term (≥37 weeks of gestation), 2) Age <28 days, 3) uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score below three after five minutes), sepsis or long-term complication of respiratory ventilation. Exclusion criteria were known congenital or postnatal diseases that could interfere with body composition development, confirmed intra-uterine infection, maternal use of corticosteroids or significant maternal medical condition that could interfere with infant's body composition development (e.g. diabetes). For the present study, infants were excluded when they received antibiotics

in the first 3 months of life. The Medical Ethics Committee of Erasmus Medical Center approved the study. Written informed consent was obtained from both parents unless mother was single.

Data collection and measurements

Birth characteristics

Birth data were taken from midwife and hospital registries.

Feeding information

Information on formula and breastfeeding was recorded at the research clinic visits at 1 and 3 months. Most formula fed infants were fed with prebiotic formula (n=19).

Anthropometrics of the child

Weight was measured to the nearest gram by an electronic infant scale (Seca, Hanover, MD). Length was measured with the two-person technique to the nearest 0.1 cm by a length meter (Seca). Head circumference was measured to the nearest 0.1 cm using measuring tape (Seca, circumeter). Weight SDS, height SDS and BMI SDS were calculated with Growth Analyser Research Calculation Tools 4.0 (available at www.growthanalyser.org), according to Dutch age- and gender-matched reference values.(29)

Body composition

Whole-body composition was assessed using air-displacement plethysmography (Peapod, Infant Body Composition System, COSMED). This ADP system assesses fat mass and fat free mass by direct measurements of body volume and body mass, based on the whole-body densitometric principle.(30-32) All measurements were obtained by experienced personnel, using standardized protocol. The Peapod was calibrated every day, according to the instructions of COSMED.

Fecal samples

Fecal samples were collected at home at 1 and 3 months of age and stored at -18 °C or lower from the day of collection. Samples remained no longer than 4 months in -18 °C and were transported in cool-bags with freezing-elements to our hospital, where the samples were stored at -80 °C.

The following parameters were determined: pH, secretory immunoglobulin A (sIgA), short chain fatty acid (SCFA) levels (i.e. acetate, butyrate, propionate, iso-butyrate, valerate, and iso-valerate), D- and L-lactate, and as previously described.(33,34) In short, a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector was used to determine the different SCFA levels, while using

2-ethylbutyric acid as an internal standard. Lactic acids were determined enzymatically using a D/L-lactic acid detection kit with D- and L-lactate dehydrogenase (EnzyPlus, BioControl Systems, Inc., Bellevue, WA, USA). The following SCFA could be detected in a lower number of samples: Butyric acid at 1 month was detected in 20 samples and at 3 months in 24 samples; Iso-butyric acid at 1 month was detected in 7 samples, at 3 months in only 14 samples; Iso-valeric acid at 1 month in 31 samples and at 3 months in 23 samples.

Fecal DNA extraction and pyrosequencing

DNA was extracted from the fecal samples by combining bead-beating with QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturers protocol except for the addition of two bead-beating steps: to 0.2 - 0.3 g of fecal sample 300 mg of 0.1 mm glass beads together with 1.4 mL of ASL (lysis) buffer and on this suspension the first bead-beating step was applied, after addition of the InhibitEx tablet the second bead-beating step was applied. Each bead-beating step consisted of bead-beating three times for 30 sec (FastPrep-24 instrument program 5.5) and was followed by cooling the sample for 5 min on ice. Extracted DNA purity was checked using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc.), whereas DNA quality and concentration was measured using the Quant-iTTM 193 dsDNA BR Assay kit (Invitrogen™). DNA aliquots were stored at -20°C until use. On the purified fecal DNA extracts primers 357F (5'-CCTACGGGAG-GCAGCAG-3') and 926Rb (5'-CCGTCAATTYMTTTRAGT-3') were used to amplify the V3-V5 regions of the bacterial 16S rRNA gene and the generated amplicons were subsequently pyrosequenced with a 454 FLX Sequencer (454 Life Sciences, Branford, CT, USA) as described previously.(35)

Bioinformatic analysis

Sequencing data was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) v.1.9.0 pipeline.(36) Sequences with mismatched primers were discarded. Quality control filters were set to retain sequences with: a length between 200 and 1000 bases; a mean sequence quality score >25; no ambiguous bases; no homopolymers stretches of >6 bases. The filtered sequences were grouped into Operational Taxonomic Units (OTUs) by *de novo* OTU picking using the USEARCH algorithm (37) at 97% sequence identity. Subsequently, the Ribosomal Database Project Classifier (RDP) (38) was applied to assign taxonomy to the representative sequence (i.e. the most abundant sequence) of each OTU by alignment to the SILVA ribosomal RNA database (release version 1.0.8).(39) ChimeraSlayer was applied, as part of QIIME, to filter for chimeric sequences. Rarefaction was applied to the OTUs by QIIME to ensure identical number of reads per sample in order to perform α-diversity calculations using the following metrics: Chao-1 and Observed species.

Statistical analysis

SD-scores for birth length and birth weight were calculated to correct for gestational age and gender.(40) All SD-scores were calculated using growth analyser software (http://www.growthanalyser.org).

Continuous variables are presented using medians with inter-quartile ranges (IQR). Differences in fecal parameters between 2 groups were assessed with Mann-Whitney test, as the data were not normally distributed. Differences in the fecal parameters between the 4 subgroups were assessed with Kruskal-Wallis, as the data were not normally distributed. All statistical tests were performed two-sided and results were regarded statistically significant if the p-value was <0.05.

Differences between the infant groups at the different phylogenetic levels were calculated on the relative abundances of each taxonomic category and compared between two infants groups by dependent 2-group Wilcoxon signed rank tests. No statistical comparisons were performed within the formula fed infants with accelerated weight gain due to low number. For all statistical tests performed on multiple parameters, the false discovery rate was estimated by calculating q-values. Results with both p-values and q-values <0.05 were regarded as significant. For taxa that occurred in >50% of the samples in only one of the investigated groups a Fisher's exact test was performed. Spearman correlations were applied for the correlations of fecal parameters at 1 and 3 months with growth data. SPSS statistical package version 20.0 (SPSS Inc. Chicago, Illinois) was used.

RESULTS

The clinical characteristics of the exclusively formula fed and breastfed infants are presented in Table 1.

Exclusively formula fed (FF) infants had a lower weight SDS and BMI SDS at 1 month and less weight gain between birth and 1 month than exclusively breastfed (BF) infants (p=0.05, p=0.02, p=0.001, resp.). The formula fed infants had a higher gain in weight SDS between 1 and 3 months (p=0.04, p=0.03, resp.) and less decline in weight SDS between 3 and 6 months than breastfed infants.

Levels of sIgA and short chain fatty acids

Results of short chain fatty acids (SCFA) are presented in Table 2.

Formula feeding vs. breastfeeding

Secretory IgA was lower in the stools of FF infants at 1 and 3 months, compared to the BF infants (both p<0.001)(Table 2.). FF infants had higher levels of acetic and propionic

Table 1. Clinical characteristics

	Total group (n=51)	Formula fed infants (n=21)		Breastfed infants (n=30)		p-value
	Median	Median	IQR	Median	IQR	
Gestational age	40.1	38.4	37.9-40.4	40.1	39.1-41.0	**0.002**
Gender (female) [n(%)]	18(35)	6(29)		12(40)		0.98
Mode of delivery (vaginal) [n(%)]	33(65)	14(70)		22(73)		0.41
Birth weight SDS	-0.04	-0.03	-0.60-0.40	-0.14	-0.93-0.32	0.48
Birth length SDS	0.13	0.13	-1.10-0.76	0.13	-1.03-0.60	0.80
At 1 month						
Weight SDS	0.61	0.04	-0.40-0.86	0.94	-0.21-1.40	**0.05**
Length SDS	-0.02	-0.07	-0.31-0.74	0.37	-0.37-0.71	0.52
BMI SDS	0.51	0.10	-0.70-0.78	0.65	0.04-1.23	**0.02**
Fat mass percentage	17.8	17.1	13.9-20.3	18.3	15.8-21.4	0.37
Weight gain SDS 0-1mo	0.71	0.11	-0.23-0.78	0.91	0.59-1.48	**0.001**
At 3 months						
Weight SDS	0.64	0.54	0.21-0.92	0.73	0.13-1.35	0.42
Length SDS	0.65	0.65	0.31-0.80	0.57	0.16-1.01	0.86
BMI SDS	0.33	0.07	-0.22-0.79	0.45	-0.53-1.13	0.62
Fat mass percentage	23.3	21.3	19.1-26.0	24.2	22.3-26.9	**0.04**
US-visceral fat (cm)	2.44	2.44	2.09-2.90	2.44	2.09-2.65	0.63
US-subcutaneous fat	0.42	0.40	0.36-0.52	0.43	0.40-0.52	0.25
Weight gain SDS 1-3mo	0.21	0.54	0.02-0.85	0.14	-0.34-0.46	**0.03**
Delta FM% 1-3 months	5.1	4.3	2.6-6.2	5.9	3.5-9.0	0.10
At 6 months						
Weight SDS	0.13	0.05	-0.20-0.85	0.23	-0.41-0.92	0.74
Length SDS	0.33	0.70	0.30-0.97	0.23	-0.12-0.42	0.03
BMI SDS	-0.08	-0.30	-0.91-0.81	0.19	-0.85-0.93	0.46
Fat mass percentage	23.5	22.7	18.5-28.3	25.4	22.7-28.3	0.17
US-visceral fat (cm)	2.14	2.15	2.04-2.86	2.14	1.95-2.81	0.69
US-subcutaneous fat	0.40	0.40	0.36-0.48	0.40	0.31-0.51	0.87
Weight gain SDS 3-6 mo	-0.44	-0.31	-0.65-0.06	-0.57	-0.93-0.15	**0.04**
Delta FM% 3-6 months	0.5	-0.1	-3.0-3.1	0.9	-1.4-1.8	0.91

Data are expressed as median(interquartile range) or percentage. Bolded p-values are p-values below 0.05. US-visceral fat = visceral fat thickness, measured by ultrasound. US-subcutaneous fat = abdominal subcutaneous fat, measured by ultrasound.

acid at 1 and 3 months (p=0.01, p=0.006, p<0.001, p<0.001, resp.), while butyric acid at 1 and 3 months was not different between the 2 feeding groups.

We also investigated the changes in sIgA and SCFAs between 1 and 3 months in FF vs. BF infants, but there were no significant differences between these groups (Table 2).

Associations of sIgA and SCFA with FM%, visceral and subcutaneous fat

Formula fed infants

Levels of sIgA at 1 and 3 months and the change in sIgA between 1 and 3 months did not correlate with FM% at 1, 3 or 6 months (Table 3). We found, however, that a higher change in sIgA correlated with the increase in FM% between 1 and 3 months of age and 3 and 6 months (r=0.53, p=0.02, r=0.72, p=0.003, resp.). SIgA at 1 and 6 months did not correlate with visceral and subcutaneous fat, but at 3 months, sIgA correlated positively with visceral fat at 3 months (r=0.46, p=0.04).

Levels of acetic acid at 1 and 3 months did not correlate with FM% at 1, 3 or 6 months, but the change in acetic acid between 1 and 3 months correlated positively with the increase in FM% between 3 and 6 months (r=0.66, p=0.01) and tended to be correlated with FM% at 6 months (r=0.50, p=0.07). Acetic acid at 1 month did not correlate with visceral and subcutaneous fat at 3 months, but acetic acid at 3 months correlated positively with visceral fat at 3 months (r=0.49, p=0.02).

Levels of butyric acid at 1 month did not correlate with FM% at 1, 3 or 6 months , but higher butyric acid at 3 months correlated with higher FM% at 3 and 6 months (r=0.62, p=0.02, r=0.81, p=0.005, resp.). butyric acid at 3 months and the change in butyric acid between 1 and 3 months correlated positively with subcutaneous fat at 3 months (r=0.77, p=0.001, r=0.70, p=0.03, resp.)

Levels of propionic acid at 1 and 3 months and the change between 1 and 3 months did not correlate with FM% at 1, 3 or 6 months. Higher propionic acid at 1 month correlated with a higher decrease in FM% between 3 and 6 months (r=-0.60, p=0.02) and higher propionic acid at 3 months tended to be correlated with a higher decrease in FM% between 1 and 3 months and 3 and 6 months (r=-0.42, p=0.07, r=-0.44, p=0.10, resp.).

Breastfed infants

We also investigated the correlations in the breastfed infants. In contrast to the formula fed infants, we could not find any significant correlation of sIgA and SCFAs with FM% and changes in FM% in the breastfed infants. There were also no significant correlations with visceral and subcutaneous fat at 3 and 6 months (data not shown).

Table 2. Amounts of short chain fatty acids per subgroup

	Total group		Formula fed infants		Breastfed infants		p-value* FF vs. BF	p-value** AWG vs. nAWG
	Median (IQR)		Accelerated weight gain	Non-accelerated weight gain	Accelerated weight gain	Non-accelerated weight gain		
1 month								
pH	5.5	5.0-6.0	5.54	6.1	5.4	5.1	**0.002**	0.29
sIgA	1575.1	551.6-2395.0	563.3	385.1	2108.9	2387.3	**<0.001**	0.19
Acetic acid	55.3	34.5-81.4	61.8	64.7	28.4	55.3	**0.01**	0.13
Butyric acid	2.9	1.8-4.9	2.0	2.7	0.7	5.2	0.82	**0.02**
Propionic acid	2.7	1.2-8.6	15.6	10.6	2.0	1.0	**<0.001**	0.51
Iso-butyric acid	1.1	0.8-1.7	0.8	0.9	NA	1.8	0.19	NA
Iso-valeric acid	1.0	0.8-1.2	1.1	0.8	1.0	0.9	0.72	0.16
Total SCFA	63.8	39.9-85.0	73.8	83.6	41.3	62.4	**0.001**	0.08
Total lactate	17.4	5.2-33.6	35.6	16.1	8.6	20.8	0.40	0.99
D-Lactate	1.5	1.3-12.5	13.8	5.2	2.5	1.4	**0.05**	0.71
L-Lactate	15.5	4.6-27.7	21.8	12.8	7.7	23.2	0.63	0.67
3 months								
pH	5.3	5.0-6.1	5.2	6.2	5.3	5.1	**0.02**	0.22
sIgA	1043.8	416.7-2126.5	658.4	367.8	2227.2#	1102.5	**<0.001**	**0.009**
Acetic acid	56.1	39.2-70.6	71.3	67.2	56.2	41.9	**0.006**	0.89
Butyric acid	2.8	1.5-2.0	3.5	2.9	0.8	3.4	0.26	0.26
Propionic acid	3.4	1.1-11.1	6.7	12.3	1.5	1.0	**<0.001**	0.41
Iso-butyric acid	0.9	0.7-2.0	1.8	1.2	0.5	0.7	**0.01**	0.36
Iso-valeric acid	1.0	0.5-1.4	1.8	1.3	0.5	0.5	**0.02**	0.28
Total SCFA	62.6	45.4-82.8	74.6	89.0	60.0	53.1	**<0.001**	0.79
Total lactate	27.4	9.1-38.7	42.4	24.8	29.1	24.5	0.46	0.40
D-Lactate	5.1	2.3-8.2	19.8	5.6	5.0	2.1	0.10	**0.04**
L-Lactate	24.6	7.4-32.4	22.5	21.6	28.1	30.0	0.57	0.73
Change between 1 – 3 months								
sIgA	-92.2	-1053.5-436.9	65.8	-36.6	164.0	-1472.4	0.24	0.07
Acetic acid	-1.7	-16.8-17.5	16.6	1.8	-2.5	-9.8	0.77	0.11
Butyric acid	-1.0	-2.0-1.0	1.8	0.4	-2.4	-1.5	0.08	1.00
Propionic acid	0.0	-1.7-3.6	-12.3	2.3	0.0	0.0	0.49	0.40
Iso-butyric acid	0.4	-0.19-..	-0.2	0.9	NA	NA	NA	NA
Iso-valeric acid	-0.4	-0.8-0.6	-0.2	0.6	-0.5	-0.6	0.14	0.28
Total SCFA	1.5	-15.4-17.8	5.0	3.0	-2.4	-9.1	0.66	0.24
Total lactate	6.6	-0.1-16.3	10.2	5.3	6.6	6.8	0.96	0.57
D-Lactate	0.9	-3.6-4.5	4.15	0.7	3.54	0.8	1.00	0.59
L-Lactate	5.7	-3.3-12.6	6.07	-1.2	11.0	5.9	0.19	0.49

Data are expressed as median interquartile range). All values are in mmol/kg. Bolded p-values are p-values below 0.05.

*: p-values comparing infants with formula feeding FF) vs. breastfeeding BF)

**: p-values comparing infants with accelerated weight gain AWG) vs. non-accelerated weight gain (nAWG)

#: p<0.05 compared to BF infants without accelerated weight gain

Differences in infants with and without accelerated weight gain

As accelerated weight gain in the first 3 months of life is associated with a higher risk for obesity and adult diseases, we also analyzed differences in sIgA and SCFAs in infants with and without accelerated weight gain. We found a significantly higher amount of sIgA at 3 months in infants with accelerated weight gain compared to those without accelerated weight gain (p=0.009). Infants with accelerated weight gain had a higher increase in sIgA levels between 1 and 3 months while those without accelerated weight gain had a decrease in sIgA (p=0.05)(Table 2). After dividing the FF and BF infants into two groups, either with accelerated weight gain or without, we found that in both groups infants with accelerated weight gain had higher levels of sIgA at 3 months, but this did not reach significancy in the formula group, due to low number of formula fed infants with accelerated weight gain (breastfed group: p=0.02[#]). We also found that breastfed infants with accelerated weight gain had a smaller decrease in sIgA between 1 and 3 months (p=0.05).

Table 3. Correlations of sIgA and SCFAs with FM%, visceral and subcutaneous fat and changes in fat in formula fed infants.

	At 1 month	At 3 months			At 6 months			Delta FM% 1 and 3 months	Delta FM% 3 and 6 months
	FM%	FM%	Visceral fat	Subcutaneous fat	FM%	Visceral fat	Subcutaneous fat		
1 month									
sIgA	0.13	0.07	0.40	0.27	-0.20	0.01	0.04	-0.27	-0.31
Acetic acid	-0.23	-0.18	0.14	0.03	-0.51	0.25	-0.07	0.02	-0.19
Butyric acid	-0.31	0.12	-0.31	<0.01	0.36	0.02	-0.09	0.53	0.55
Propionic acid	0.25	0.13	0.25	-0.16	-0.14	-0.17	-0.11	-0.22	**-0.60***
3 months									
sIgA	-0.38	-0.28	**0.46***	0.09	-0.24	0.32	0.27	0.10	0.21
Acetic acid	-0.06	0.07	**0.49***	0.29	0.24	0.36	0.29	0.24	0.43
Butyric acid	0.30	**0.62***	-0.09	**0.77****	**0.81****	0.01	0.36	0.40	0.29
Propionic acid	0.36	0.18	-0.15	-0.24	0.06	-0.33	0.12	-0.42	-0.44
Change between 1 - 3 months									
sIgA	**-0.60****	-0.37	0.21	0.07	0.07	0.42	0.43	**0.53***	**0.72****
Acetic Acid	-0.05	0.09	0.35	0.23	0.50	0.21	0.38	0.37	**0.66***
Butyric acid	0.47	0.32	-0.12	**0.70***	-0.04	0.05	0.24	-0.38	-0.57
Propionic Acid	0.22	0.17	0.35	-0.07	0.31	-0.38	0.18	-0.25	0.09

Significant correlation coefficients are indicated in boldface. *: p-value <0.05, **:p-value<0.01

Composition of high level microbiota, diversity and richness

Formula feeding vs. breastfeeding

At the phylum level, the relative abundances showed quite some variation but only the Firmicutes were significantly higher in FF infants at 3 months (p=0.01). We found no differences in changes in microbiota between 1 and 3 months between FF and BF infants (Table 4).

Table 4. Relative abundances (%) of the bacterial phyla detected in the fecal samples

	Formula fed infants		Breastfed infants		p-value*	p-value**
	Accelerated weight gain	Non-accelerated weight gain	Accelerated weight gain	Non-accelerated weight gain	FF vs. BF	AWG vs. nAWG
1 month	(n=5)	(n=14)	(n=15)	(n=14)		
Actinobacteria	64.06	70.17	50.32	34.77	0.22	0.74
Bacteroidetes	0.146	0.06835	0.0907	0.1594	0.35	0.48
Firmicutes	20.34	17.63	13.9	14.73	0.45	0.848
Fusobacteria	0	0	0	0	1.00	1.00
Proteobacteria	1.997	2.107	4.971	1.963	0.62	0.43
Verrucomicrobia	0	0	0	0	0.40	1.00
3 months	(n=6)	(n=15)	(n=14)	(n=13)		
Actinobacteria	16.98	31.35	26.52	45.22	0.69	0.37
Bacteroidetes	5.062	0.3273	0.02125	1.457	0.65	0.65
Firmicutes	29.53	15.22	8.159	8.102	**0.01**	0.21
Fusobacteria	0	0	0	0	0.19	0.75
Proteobacteria	14.91	9.877	19.39	16.12	0.24	0.12
Verrucomicrobia	0	0	0	0	0.08	0.71
Change between 1 -- 3 months	(n=5)	(n=14)	(n=14)	(n=13)		
Actinobacteria	-34.23	-1.576	-6.363	-6.169	0.59	0.53
Bacteroidetes	1.057	0	-0.003965	0.016	0.96	0.55
Firmicutes	9.067	1.391	-14.01	-4.235	0.23	0.51
Fusobacteria	0	0	0	0	0.17	0.75
Proteobacteria	9.612	7.838	14.41	11.36	0.35	0.24
Verrucomicrobia	0	0	0	0	0.21	1.00

Data expressed as medians %. Bolded p-values are p-values below 0.05. *: p-values comparing infants with formula feeding vs. breastfeeding **: p-values comparing infants with accelerated weight gain (AWG) vs. non-accelerated weight gain (nAWG).

Differences in infants with and without accelerated weight gain

Neither significant differences for the Firmicutes and Bacteroidetes phyla, nor for the other detected phyla, were found between the infants with and without accelerated weight gain neither at 1 month, nor at 3 months, nor over time (Table 4).

Actinobacteria was the most dominant phylum at both timepoints, followed by Firmicutes in all infants except for the breastfed infants with accelerated weight gain where Proteobacteria was the most dominant phylum followed by Actinobacteria (Table 4).

Although the samples of the FF infants at 1 month and 3 month showed a higher diversity than those of BF infants, independent of accelerated weight gain, this difference was only significant at 3 months ($p=0.001$). The number of the observed species at 1 and 3 months of age were significantly higher in the FF group without accelerated weight gain compared to the BF groups with or without accelerated weight gain ($p=0.033$, $p=0.002$, resp.).

Composition at lower level microbiota, diversity and richness

Next, the specific bacterial groups at lower phylogenetic levels were assessed.

Formula feeding vs. breastfeeding

No significant differences in the levels of *Bifidobacterium* were found between the FF and BF infants. *Staphylococcus* and *Haemophilus* were less abundant genera in FF infants, compared to BF infants at 1 month ($p=0.007$, $q=0.04$, $p=0.001$, $q=0.007$, resp.). No significantly more abundant genera were found at 3 months or over time in FF infants.

Characterizing bacteria in formula fed and breastfed infants with and without accelerated weight gain

The bacterial class of Coriobacteriia was significantly higher in the FF infants without accelerated weight gain compared to their breastfed counterparts. From this bacterial class only the genus *Atopobium* was significantly more abundant in FF infants without accelerated weight gain compared to their BF counterparts at 1 month ($p=0.008$, $q=0.041$). It should be noted that *Atopobium* was detected in six FF infants without accelerated weight gain, but not detectable in any breastfed infant without accelerated weight gain (Figure 1).

The *Enterococcus* genus was detected in most FF infants without accelerated weight gain and was significantly more abundant compared to BF infants without accelerated weight gain where it was detected in only three infants at 1 month ($p<0.001$, $q=0.001$) (Figure 1). *Enterococcus* appeared frequently and abundantly in the FF infants with accelerated weight gain as well. Moreover, in both FF groups, *Enterococcus* appeared to decrease over time but this was not significant.

The genus *Enterobacter*, as well as its corresponding higher level taxonomy groups up to phylum level Proteobacteria, increased in time in all infant groups. This increase of *Enterobacter* occurred significantly more in BF infants with accelerated weight gain compared to BF infants without accelerated weight gain (p= 0.048) and FF infants without accelerated weight gain (p= 0.035), but this resulted only in a nearly significant higher abundance level at 3 months of age compared to BF infants without accelerated weight

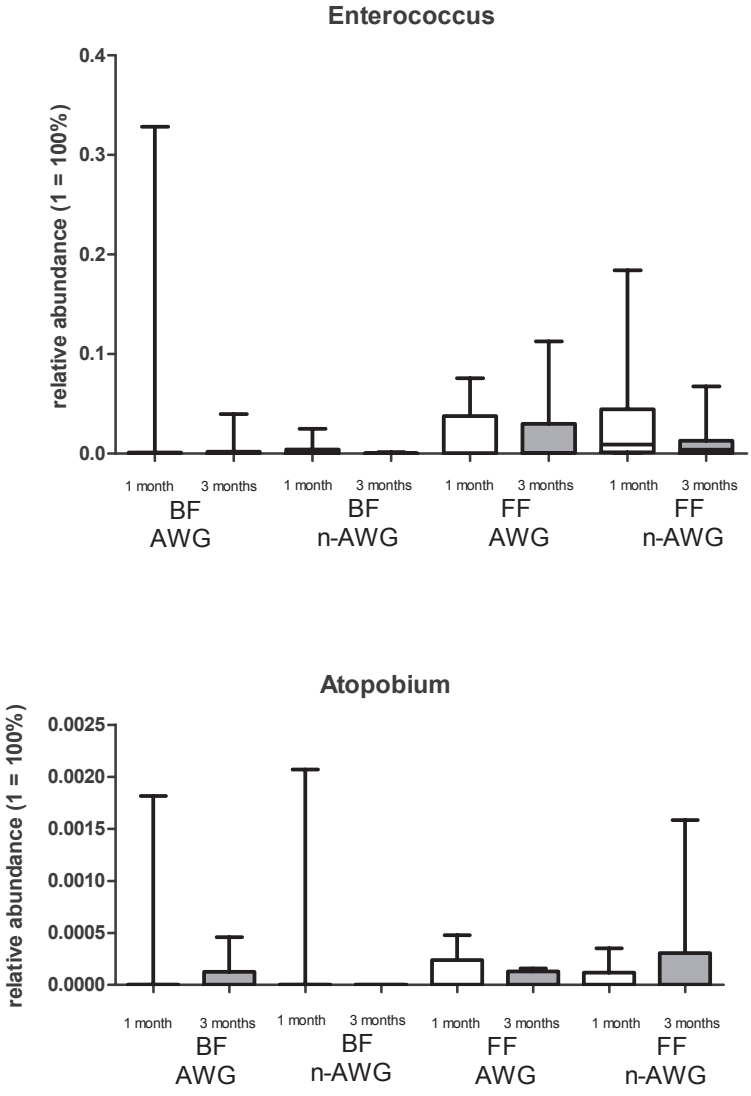

Figure 1. Bacteria more specific for formula-fed infants

gain (p= 0.038; q= 0.201; Figure 2). From the same family as *Enterobacter,* the *Escherichia-Shigella* genus group was significantly more abundant in BF infants with accelerated weight gain at 3 months of age compared to BF infants without accelerated weight gain (p<0.001; q= 0.020; Figure 2). In contrast, the *Rothia* genus group occurred significantly more in the BF infants without accelerated weight gain than in the BF infants with accelerated weight gain at 3 months. (p=0.03).

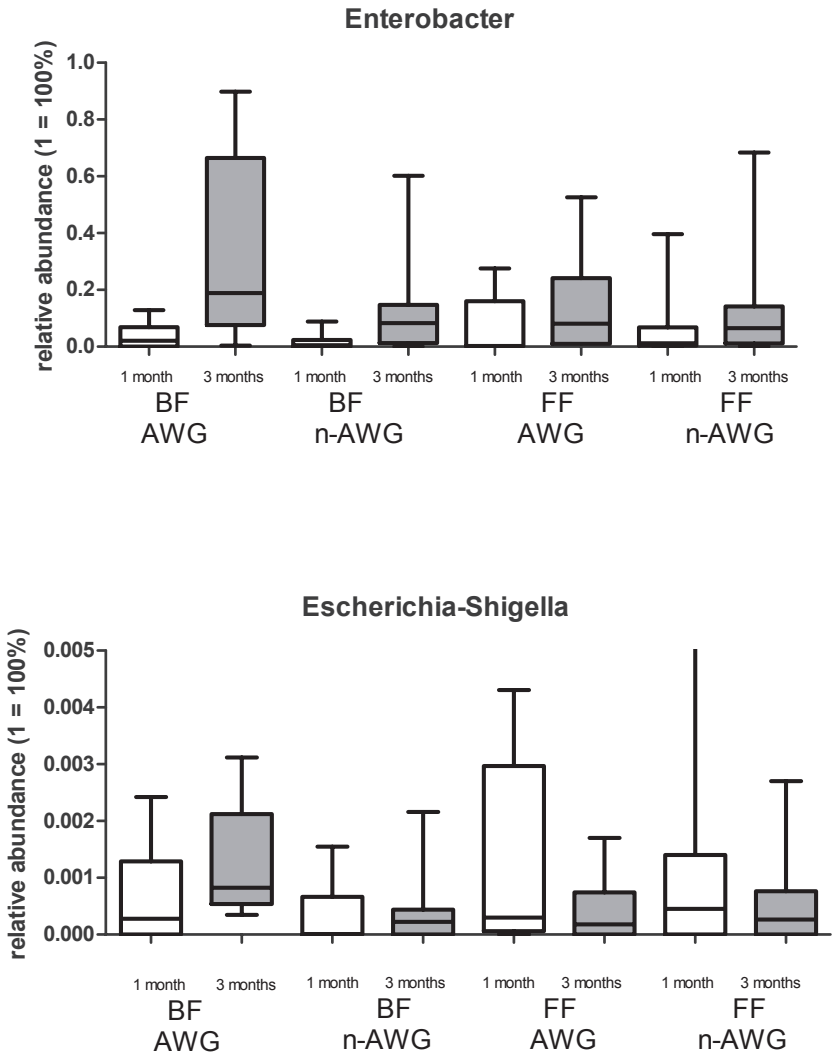

Figure 2. Bacteria more specific for accelerated weight gain in breastfed infants

DISCUSSION

Our study shows that exclusive formula fed (FF) infants have lower levels of IgA and higher levels of acetic and propionic acid at 1 and 3 months, compared to the exclusively breastfed (BF) infants. Formula fed infants had a higher change in sIgA between 1 and 3 months, which correlated with a higher increase in FM%. Formula fed infants, a higher sIgA and acetic acid at 3 months correlated with more visceral fat at that age, while a higher level of propionic acid correlated with a lower increase in FM%. In BF infants, there were no correlations of sIgA and SCFAs with body composition. There appeared to be no significant effect of type of feeding on the phylum level, although the FF infants had a significantly higher abundance of Firmicutes at 3 months of age compared to BF infants. Infants with accelerated weight gain had a higher increase in *Enterobacter*.

We identified differences in sIgA and short-chain fatty acids (SCFAs) between FF and BF infants. Breastfed infants had higher levels of sIgA. It is known that BF infants have higher levels of sIgA in their stools because breast milk contains sIgA.(41) In the first weeks of life, they receive the sIgA from the breastmilk and over time infants are going to produce sIgA by their own. The sIgA levels in BF infants decline during the first weeks and months of lactation.(42) The phenomenon of enhanced fecal sIgA in BF infants is, however, not solely caused by the presence of IgA in breast milk, it includes sIgA levels due to a stimulatory effect of breast milk on the gastrointestinal humoral immunologic development. In infants fed with formula without prebiotics, the gut will be less stimulated to produce its own sIgA, and in these infants higher amounts of sIgA are mostly related to low-grade inflammation or infection.(43) We demonstrated that BF infants with accelerated weight gain had even higher levels of sIgA than those without accelerated weight gain. This may imply that there could be an additional low grade inflammation in these infants. In infants with FF with accelerated weight gain, we also identified higher sIgA than in formula fed infants without accelerated weight gain, but this did not reach significance due to low number of FF infants with accelerated weight gain. Our data support the hypothesis that accelerated weight gain in the first months of life is related with higher sIgA levels in the stools.

We also studied the differences in microbiome in infants with and without accelerated weight gain because accelerated weight gain in early life is associated with unfavorable outcomes in later life, such as obesity, type 2 diabetes and cardiovascular diseases. Changes in microbiome in early life may be one of the links between these associations. To our knowledge, this is the first study investigating SCFA in combination with detailed data on body composition in human infants. A recently published review discussed the effects of SCFAs on energy homeostasis and metabolism, as well as how SCFAs can modulate adipose tissue and liver tissue function in adult life.(44) It was concluded that

SCFAs can modulate adipose tissue in adulthood. With our results, we demonstrate that associations between SCFAs and FM% can already be found in early life.

We only found small differences in microbiome between infants with and without accelerated weight gain. A possible explanation could be that the differences in microbiome are present but small and therefore not yet detectable in this group of infants. The microbiome of the infants at 1 and 3 months is still developing and possibly differences between the infants with and without accelerated weight gain become more prominent later in infancy or childhood. It remains unclear if the microbiome influences the gain in weight and fat mass in early life or that the gain in weight and fat mass influences the microbiome. It seems that there are interactions between these elements.

Interestingly, we found more Firmicutes at 3 months in the formula fed infants. This is in line with other studies, investigating microbiota in newborns and 4-weeks old infants.(28,45) Because higher levels of Firmicutes might be associated with obesity, this could be one of the links between formula feeding and obesity in later life.(17) Remarkably, we found no major differences in the levels of *Bifidobacterium,* a genus of Actinobacteria, between the FF and BF infants, in contrast to previous reports.(46) Over the last decades, many approaches have been used to improve infant formulas by inducing a more 'breastfed like' composition with higher *Bifidobacterium* levels.(34,47-49) Our study suggests that these approaches are effective with respect to the *Bifidobacterium* levels. On the other hand, the overall microbiota profile diversity remained different between FF and BF infants, which is in line with current literature, i.e. FF infants having a higher bacterial diversity compared to BF infants.(24) By adding prebiotics to formula feeding, infants have a lower stool pH and higher concentrations of *Bifidobacteria*.(50) Nowadays, most formula feedings contain prebiotics and in our study, only 2 FF infants did not receive prebiotic-containing formula feeding.

There were, however, bacteria that were more specific for FF infants or BF infants and bacteria more specific for accelerated weight gain. *Enterococcus* species are among the first colonizers in newborn infants and have even been detected on the first day of life.(60,61) Our results indicate that *Enterococcus*, a genus of Firmicutes, decreases in the first months of life, which is in line with current literature. The lower amount of *Enterococcus* in BF infants is likely due to the significantly lower pH of stools of BF infants. Lower amounts of *Enterococcus* have also been found in a study in 16 BF infants at 1 month of age.(62) *Staphylococcus* and *Haemophilus* were more abundant in BF infants. These findings are in line with the literature, and these bacteria are known as early colonizers and part of the normal breastfed microbiota. The *Atopobium* genus, a genus of Actinobacteria, harbors anaerobic species that mainly produce lactate (55) and the *Atopobium* cluster, which consists of *Atopobium*, *Coriobacterium*, *Eggerthella* and *Collinsella* species (56), has been detected in infants that were six weeks of age.(57) Our study shows that the *Atopobium* genus can already be detected in infants at 4 weeks of age. Interest-

ingly, it has been suggested that *Atopobium* species are active protein degraders in the gastrointestinal tract since significant associations between protein fermentation end products and the bacteria of the *Atopobium* cluster have been reported.(58,59) In our study, Bacteroides represented one of the most predominant bacterial genera in BF infants. The presence of high levels of *Bacteroides* may be beneficial, because members of *Bacteroides* have shown to exert immunomodulatory effects on the host.(63) We found a more pronounced increase in *Enterobacter* and *Escherichia-Shigella,* both genera of Proteobacteria, in infants with accelerated weight gain. Some bacterial strains belonging to these genera have been described as potentially harmful, already at moderately abundant levels. *Enterobacter* and *Escherichia-Shigella* are significant producers of lipopolysaccharide (LPS) endotoxins, which is the primary structural component of the outer membrane of Gram-negative bacteria. Several studies have shown that if endotoxins translocate from the gut into serum, they can cause low-grade inflammation that can contribute to the development of obesity, type 2 diabetes and cardiovascular diseases.(8,51,52) *Rothia*, a genus of Actinobacteria, was more present in infants without accelerated weight gain. *Rothia* is in adults an oral/upper gastrointestinal tract inhabitant. As *Rothia* was more present in stools of infants without accelerated weight gain, these infants might have had less intestinal absorption due the faster transit time.

In conclusion, formula fed infants had lower levels of sIgA, higher levels of SCFAs at 1 and 3 months and also higher levels of Firmicutes at 3 months of age compared to breastfed infants. Only in formula fed infants, we found correlations of sIgA and SCFAs with body composition. Infants with accelerated weight gain had higher sIgA levels at 3 months. No significant differences in the microbiota were found between the infants with and without accelerated weight gain at month 1, 3, or over time, although infants with accelerated weight gain in the critical window had a higher increase in Enterobacter which might contribute to a different metabolic programming and increased risks for adult diseases.

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Life						
Domain						
Kingdom	Bacteria	Bacteria	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	**Actinobacteria**	**Bacteroidetes**	**Firmicutes**	**Fusobacteria**	**Proteobacteria**	**Verrucomicrobia**
Class	Actinobacteria	Bacteroidetes	Bacilli	Fusobacteria	Gammaproteobacteria	Verrucomicrobia
Order	1. Bifidobacteriales 2. Coriobacteriales 3. Actinomycetales	Bacteroidales	1. Bacillales 2. Lactobacillales	Fusobacteriales	1. Enterobacteriales 2. Pasteurellales	Verrucomicrobialis
Family	1. Bifidobacteriaceae 2. Coriobacteriaceae 3. Actinomycetaceae	Bacteroidaceae	1. Staphylococcaceae 2. Enterococcaceae	Fusobacteriaceae	1. Enterobacteriaceae 2. Pasteurellaceae	Verrucomicrobiaceae
Genus	***1. Bifidobacterium*** ***2. Atopobium*** ***3. Rothia***	Bacteroides	1. Staphylococcus 2. Enterococcus	*Fusobacterium*	***1. Enterobacter* and *Escherichia-Shigella*** ***2. Haemophilus***	*Verrucomicrobium*
Species	*1. B. breves* *2. A. fossor* *3. R. aeria*	*B. fragilis*	*1. S. aureus* *2. E. faecalis*	*F.prausnitzii*	*1. E. cloacae* *2. H. influenza*	*V.spinosum*

Supplemental figure 1. Examples of different bacteria, subdivided by taxonomy
Phyla and genera that are indicated in the manuscript are in bold

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9

Chapter 9

General Discussion

GENERAL DISCUSSION

This thesis describes results of studies performed to investigate if infant growth is associated with risks for non-alcoholic fatty liver disease in term and preterm born adults. Also the effects of fetal, maternal and infant growth determinants on body composition during the first 6 months of life were investigated, as well as the associations with the serum appetite regulating hormones levels and gut microbiome in healthy infants participating in the Sophia Pluto Study.

In this chapter, results of the studies described in this thesis are discussed in view of current literature. In addition, the clinical implications of these data, as well as directions for future research are given.

9.1 Influence of infant growth on later health

Our research group, but also other researchers demonstrated that the first months of life are of influence on later health.(1-8) Accelerated gain in weight for length in early life has been associated with the prevalence of metabolic syndrome at age 21 years, independently from birth weight.(9,10) Given that non-alcoholic fatty liver disease (NAFLD) is considered the hepatic metabolic syndrome (11,12), it might well be that the previously found association between small size at birth and NAFLD can also be ascribed to accelerated early weight gain.(13,14)

In Chapter 2 we showed that gain in weight SDS in the first 3 months of life was associated with a higher fatty liver index (FLI) at 21 years, a validated measure to predict hepatic steatosis in the general population(15,16), whereas low birth weight SDS was not, independent of gestational age, indicating that these earlier described association between small for gestational age (SGA) and NAFLD is likely due to the gain in weight SDS in the first 3 months.(17) We also found that subjects with rapid catch-up in weight for length have a significantly higher FLI in early adulthood than subjects with slow catch-up in weight, also after adjustment for birth weight SDS. Furthermore, when a FLI score (low, intermediate, or high risk for NAFLD) was assigned to each participant and ordinal regression analysis was performed, it was shown that higher gain in weight for length in the first 3 months of life associates with more risk of having a higher FLI score in early adulthood (OR: 11.7, p=0.016), whereas low birth weight does not. The FLI refers to a clustering of risk factors representing a condition of underlying hepatic steatosis. Our study population consisted of healthy young adults, which made it impossible to perform liver biopsy to study hard endpoints such as NAFLD, steatohepatitis and fibrosis. We, therefore, used the FLI as a proxy. Although the study group consisted of healthy young adults, we found intermediate and high FLI-scores in a quite large number of participants. A high FLI-score has a positive predictive value of 72.2% for fatty liver.(18) It was striking that in young adults with catch-up significantly more intermediate and

high FLI-scores were found than in young adults without catch-up in weight for length in the first months of life.

In Chapter 3 we showed that rapid infant weight gain after term age in young adults born preterm was also associated with an increased risk of NAFLD in early adulthood. Subjects born preterm with rapid weight gain after term age had a significantly higher FLI in early adulthood than subjects without rapid catch-up in weight, independent of size at birth and gestational age. Previous studies demonstrated associations between a low birth weight and risk factors for NAFLD in adulthood, but associations between first year growth data and NAFLD were neither investigated in term born adults nor in preterm born adults.(17,19) Ayonrinde et al. showed in a large cohort of young adults that pre-school age growth was associated with the risk for NAFLD at age 17 years: the adiposity rate from 3 years and onwards was greater in adolescents diagnosed with NAFLD than in those without NAFLD.(20) These results demonstrate that pathways leading to NAFLD are established during early childhood. Our findings suggest that it might even occur during the first months of life.

Conclusions, clinical implications and directions for future research

To our knowledge, we are the first investigating the relationship of early life weight trajectories with risk factors for non-alcoholic fatty liver disease. Over the last decades, the number of patients with NAFLD is increasing (21), also in young adults and it is, therefore, important to identify persons who are at risk to develop NAFLD. We have shown that infants with rapid weight gain in the first months after term age are at increased risk. Based on the findings presented in Chapter 2 and 3, we conclude that accelerated gain in weight compared to length after term age, should be avoided to reduce the risk for NAFLD. Further studies examining the role of infant diet, as well as the contribution of emerging factors such as microbiome diversity and epigenetic variations are important to guide interventions to prevent future NAFLD.

9.2 Early life determinants of obesity, type 2 diabetes and cardiovascular disease

Previously and in the studies described in chapter 2 and 3, we investigated associations of birth size, preterm birth and early postnatal weight gain with risk factors for later disease.(2,9,22-24) However, the question remained whether combinations of determinants will lead to adult diseases in later life and how changes in early life can influence the risk for adult diseases.

Previous studies demonstrated that increased fat mass in early life can track to obesity in childhood and in adulthood.(25,26) We, therefore, investigated body composition at birth and during early life and its determinants.

In Chapter 4 we investigated body composition in 194 healthy newborns born term. These newborns had a large variation in fat mass percentage, ranging from 1.4 to 19.9%, also after correction for gender and gestational age, indicating that it is difficult to predict fat mass based on weight or ponderal index at birth. This large variation in FM% in newborns with a similar birth weight had not yet been reported. Estimated fetal weight (EFW) at 30 and 36 weeks and catch-up in fetal weight between 30 and 36 weeks of gestation was positively associated with more FM% of the newborn. A recently published systematic review showed various prenatal markers of newborn fat mass.(27) Two of the reviewed studies showed also a positive associations between EFW and newborn FM%, but catch-up in estimated fetal weight was not investigated.(28,29) In our study, pre-pregnancy BMI of the mother was positively associated with FM% of the newborn, which is in line with other studies, showing positive associations between pre-pregnancy BMI and newborn FM%.(30-32) It has been reported that the pre-pregnancy BMI influences the cardiovascular health of the offspring, which confirms the clinical importance of this marker.(33,34) Also maternal gestational weight gain has been reported to be positively related to newborn FM%, but in contrast to our expectations we could not identify this relationship.(35-37) A possible explanation of this contradiction is the difference in the timing of weight gain during pregnancy. One study showed that newborns of women who gained excessive weight in the first part of their pregnancy had higher FM% compared to newborns of women who gained excessive weight in the last part of their pregnancy.(38) In the Netherlands, it is common to gain more weight in the last trimester of pregnancy, so this might explain why we found no associations between maternal weight gain during gestation and newborn FM%.(39)

Although estimated fetal weight and pre-pregnancy BMI are significant contributors to the variance of FM% at birth, the highest explained variance of these models was 56%, indicating that other unidentified factors could play a role, such as (epi)genetics and feedings habits of mother.(40-42)

In Chapter 5 we describe longitudinal reference data on fat mass percentage (FM%) and fat-free mass (FFM), measured by Peapod, and subcutaneous and visceral fat thickness, measured by ultrasound, in a cohort of 203 healthy term infants during the first 6 months of life. We revealed a significant increment in FM% during mainly the first 3 months of life, without a change between 3 and 6 months. When girls and boys were analyzed separately, we observed the same pattern in boys but girls showed significant increases in FM% during both age periods. Also visceral fat and abdominal subcutaneous fat thickness remained similar between 3 and 6 months. Girls had a significantly higher FM% and lower FFM at 1 and 6 months than boys. FM% correlated with abdominal subcutaneous fat thickness, but not with visceral fat thickness. This is one of the first large studies investigating longitudinal fat mass percentage in healthy term infants. We showed that

the highest increase in FM% is in the first months of life, supporting the concept that the first 3 months of life are of major influence for establishing fat deposition. Also other researchers have showed evidence that the first months are important.(1,2,25) For that reason accurate body composition data in early life are essential to individualize dietary intake and to guide parents and caregivers, particularly of infants born SGA or being at risk for obesity development later in life.(43-45)

In Chapter 6 we investigated the influence of gain in weight, type of feeding and maternal characteristics on infant total body fat mass percentage (FM%), measured by Peapod, at 1, 3 and 6 months of age in combination with visceral fat and abdominal subcutaneous fat, measured by ultrasound, at 3 and 6 months of age. Our study shows a strong association of gain in weight for length SDS and FM% in the first 3 months of life with FM% at 6 months. Higher gain in weight SDS and in FM% between 1 and 3 months was associated with more visceral fat at 3 months and a higher gain in FM% between 3 and 6 months was associated with more visceral fat at 6 months. Exclusive breastfeeding was associated with more FM% at 6 months due to more subcutaneous fat, but not due to more visceral fat. This is reassuring because increased subcutaneous fat in childhood has been associated with a more beneficial phenotype for obesity and adult diseases.(46) A higher visceral fat is detrimental, because it will effluent to the portal vein which drains into the liver where hepatocytes are directly exposed to its metabolites and secretory products, whereas subcutaneous fat drains systemically.(47)

We showed that breastfed infants had a higher total body FM% at 6 months of age. Several studies showed different growth patterns in breast- and formula fed infants (48,49), but reports about different patterns in FM% are limited.(50-53) In general, breastfed infants tend to grow rapidly in the first 2 to 3 months, but they grow, subsequently, more slowly than their formula fed counterparts, which results in significantly heavier infants at the age of 12 months when formula fed.(49,54) Although formula fed infants had significantly lower fat mass than breastfed infants at the age of 3 and 6 months, which is in line with our results, by 12 months this was no longer apparent. There was a trend toward reversal with higher fat mass in 12-months-old formula fed infants.(53) Exclusive breastfeeding during infancy has been associated with a lower incidence of childhood obesity.(55-60) After the period of breastfeeding, it is, however, important for parents to be careful with food during weaning. Early introduction of solid foods, before 4 months, may increase the likelihood of obesity.(61-63) Unhealthy infant feeding practice, such as introducing of sugar-sweetened beverages, is the one the most important factors in relation to early childhood obesity.(64-66)

We could not demonstrate that maternal variables were of influence on infant body composition at 3 and 6 months. Literature data show conflicting results about this topic. Most of these studies were performed in newborns, including our own study (chapter 4). Some studies observed a higher FM% in newborns of obese mothers compared to

mothers with a normal BMI, but other studies did not find it either.(31,36,38,67-69) Apparently the effect of maternal variables on the body composition of the offspring disappears after the first months of life. This has also been found in a study from Ay et al, in a large group of 6-month-old infants.(70) Possibly this disappearance is only temporary, because studies showed associations between maternal pre-pregnancy BMI and body composition of children above the 2 years of age.(71-73) This might suggest that the fetal period and first months of life are critical windows for programming with effects on the longer term.

Conclusions, clinical implications and directions for future research

We found a wide variation in fat mass in newborns with a similar weight, indicating the necessity of measuring fat mass instead of only weight and length, particularly when infants are at risk to develop overweight or obesity later on.(24,58,74-76) Infants with catch-up in estimated fetal weight during pregnancy had a higher fat mass percentage at birth. Future studies, investigating the mechanisms underlying the variations in fat mass are needed and should be focused on both environmental and (epi)genetic variations in infants and their influence on body composition.

We provided reference data of fat mass percentage in healthy tem infants at birth and at 1, 3 and 6 months of age and reference data of subcutaneous and visceral fat at 3 and 6 months. These reference data are mandatory for an appropriate evaluation of the fat mass or visceral or subcutaneous fat in individual infants and for group analyses.

Breastfeeding was related to an increase in FM% at 6 months of age but this increase in FM% was largely due to an increase in subcutaneous fat instead of visceral fat. Future longitudinal studies are needed to examine the effects of various types of feeding on FM%, visceral and subcutaneous fat later in infancy and childhood.

We found that infants with accelerated weight gain during the first months of life had more FM% and visceral fat at 6 months. Follow-up studies are needed to examine if these infants have also a higher FM% and more visceral and subcutaneous fat later in childhood and eventually in adulthood.

In Chapter 7 we studied the serum levels of appetite regulating hormones, ghrelin, leptin, insulin, GLP-1, GIP, PP, PYY at 3 and 6 months and their associations with type of feeding and body composition (including FM% and visceral and subcutaneous fat). We found significantly higher serum levels of ghrelin, leptin, insulin, GIP and PP and lower serum levels of PYY in infants with exclusive formula feeding compared those with exclusive breastfeeding. This suggests that type of feeding can influence the appetite regulating hormones in very early life and could thereby have programming effects for later life. It has been shown that high levels of ghrelin induces adiposity in rodents (77) and that lower levels of PYY inhibit the effect of reducing food intake.(78) These actions could be explanations why formula fed infants are at higher risk for developing obesity

than breastfed infants.(79) Breastfeeding during the first year promotes satiety responsiveness in children aged 18-24 months.(80,81) We showed that only ghrelin and leptin were positively correlated with FM% at 3 months. It has been demonstrated in animal studies that neonatal ghrelin is important for normal maturation of hypothalamic neural circuits. The neural developmental activity of ghrelin is essentially restricted to the neonatal period.(82) Proper expression of ghrelin during neonatal life is, therefore, crucial for lifelong metabolic regulation and too high ghrelin levels during the first months of life result in lifelong metabolic disturbances.(83). The associations of leptin and body composition could be explained by the known physiology of adipose tissue. During early life, the adipocyte proliferate and differentiate (84) and leptin levels increase in parallel to the number of adipocytes in the first months of life.(85) The other appetite regulating hormones did not seem to be involved in the regulation of body composition in 3- and 6-months-old infants.

Conclusions, clinical implications and directions for future research

In conclusion, there are large differences in appetite regulating hormones between formula fed and breastfed infants at 3 months of age and these differences might contribute to differences in growth patterns between those infants. The formula fed infants had higher serum levels of ghrelin, leptin and insulin, which is remarkable for that early age. It is, therefore, important to stimulate mothers to give breastfeeding for at least 3 months in order to give the babies the benefits of a "better" profile of appetite regulating hormones. Future research is needed to elucidate the role of these appetite regulating hormones in early life with respect to adult health and disease.

In Chapter 8 we investigated the gut microbiome of healthy term infants in the first 3 months of life in relation with type of feeding and fat mass. In exclusively formula fed infants, we found lower levels of sIgA at 1 and 3 months, compared to the exclusively breastfed infants. It is known that breastfed infants have higher levels of sIgA in their stools because breastmilk contains sIgA.(86) In the first weeks of life, the levels of sIgA decreased in these infants. In formula fed infants, the gut contains less sIgA because it is dependent from the production of the gut. Higher amounts of sIgA will be produced in case of inflammation or infection. We showed that infants with accelerated weight gain had higher levels of sIgA, supporting our hypothesis about the relationship between accelerated weight gain and higher sIgA, a marker of inflammation, which associates with obesity. The microbial population established in the initial stages of life has a profound impact on epigenetic programming and future homeostasis and well-being of the individual.(87,88) It is known that nutrition, of mother and infant, may promote an inadequate gut microbiota composition and functionality, accounting for worse programming of later immunity and of regulation of genes involved in the develop-

ment of obesity and cardiovascular diseases.(89,90) Breast milk is the gold standard for infants, because it provides the most optimal nutritional, immunological and emotional nurturing for growth and development of infants.(91) However, in instances where breast-feeding is not possible, unsuitable or inadequate, formula feeding is used as breast milk substitute. Many attempts have been made to improve infant formula, not only with respect to the protein, carbohydrate and lipid content but also in immunologic aspects.(92-96) This has resulted in a gut microbiota composition with a higher level of *bifidobacteria*, as it is found in stools of breastfed infants.(97-101) Our data show that these approaches are quite effective, as we found no major differences in microbiota, and particularly similar levels of *bifidobacteria* in formula fed infants as in breastfed infants. We identified small differences in microbiota composition between infants with and without accelerated weight gain, infants with accelerated weight gain having more abundance of Proteobacteria.

Conclusions, clinical implications and directions for future research

As there were only small differences in sIgA, SCFA and microbiota between breast- and formula fed infants, we can conclude that approaches to create a more breastfed-like formula, with respect to the microbiota composition, are quite adequate. No significant differences in the microbiota were found between the infants with and without accelerated weight gain at month 1, 3, or over time, although infants with accelerated weight gain in the critical window had a higher increase in Enterobacter. Long-term follow-up studies are needed to evaluate if these differences persist during childhood and adulthood. Follow-up research is warranted to assess if changes in microbiota occur after weaning and later on in childhood.

General conclusions

Our studies show that the first 3 months of life are a critical period in which increased weight for length increases the risk for non-alcoholic fatty liver disease in adults born at term or preterm. Balanced weight gain during the first months of life is, therefore, important to reduce this risk. We also present body composition at birth and longitudinally during the first 6 months after birth, in healthy term born infants. Based on these data, we can conclude that fat mass percentage is highly variable between infants.

When data on infant body composition are required, it is, therefore, important to measure fat mass instead of only weight and length. Fat mass increases mainly during the first 3 months of life and stabilizes thereafter, supporting the concept of a critical window for programming of adiposity in the first three months of life. We demonstrated that a higher gain in estimated fetal weight in the third trimester is associated with a higher fat mass percentage at birth and that pre-pregnancy BMI of mother is of influence on fat mass percentage at birth, but that the influence of mother disappeared in the first

months of infancy. Breastfeeding is positively associated with fat mass percentage at 3 months but mainly through subcutaneous fat, not visceral fat, which is reassuring. Formula fed infants have a different profile of serum appetite regulating hormones than breastfed infants, suggesting that these hormones could contribute to the protective role of breastfeeding against obesity development. Besides associations with ghrelin, leptin and insulin, we could not identify associations with other appetite regulating hormones with anthropometrics and body composition in infants of 3 and 6 months of age, indicating that possible early programming effects of appetite regulating hormones do not have their influence on body composition in early life. In addition, we found differences in sIgA in the stools of formula fed and breastfed infants, with lower levels of sIgA at 1 and 3 months in the formula fed infants but we found no major differences in levels of *bifidobacteria* between those groups, indicating that approaches to create a more breastfed-like formula, with respect to the microbiota composition, are quite adequate. No significant differences in the microbiota were found between the infants with and without accelerated weight gain at month 1, 3, or over time, although infants with accelerated weight gain in the critical window had a higher increase in Enterobacter. This could imply that these bacteria could influence accelerated weight gain during early life and thereby increasing the risk for later adult diseases.

Directions for Sophia Pluto Study

In the coming years, we should also focus on the (epi)genetic influences on body composition and health profile, not only during infancy but also across childhood into adulthood.

It would be interesting to investigate the risks for metabolic syndrome and non-alcoholic fatty liver disease in our Sophia Pluto Study birth cohort and investigate if infants who showed accelerated gain in weight and particularly in fat mass in early life will have a higher fatty liver index than those who did not. Also the long-term effects of various types of formula feeding would be interesting to investigate.

We already investigated the influence of accelerated weight gain in the first 3 months of life and type of feeding on fat mass percentage at birth and on body composition (fat mass percentage, but also visceral and subcutaneous fat) in the first 6 months of life, but it would be interesting to examine if accelerated weight gain and type of feeding are associated with body composition later in infancy and in childhood. Also if there are effects of maternal determinants on later infant's body composition would be interesting to investigate. In this thesis we described the effect of fetal growth on body composition of newborns, but it would be valuable to also associate the fetal growth with later infant's body composition. In the Sophia Pluto Study, we included only healthy term infants. However, as preterm born infants have higher risks for accelerated post-term weight gain, it would also be interesting to explore the associations described in this

thesis in infants born preterm. We showed associations of serum appetite regulating hormones levels with type of feeding and body composition of infants, but associations with appetite regulating hormones in maternal human milk and their interactions with serum levels and gut microbiota would also be important. We explored associations of accelerated weight gain and type of feeding with gut microbiota. It would be valuable to confirm these associations with microbiota data during late infancy and childhood, to confirm the programming hypothesis. The results of this thesis show the relevance to continue the Sophia Pluto Study as it provides very detailed measurements and allows to find important determinants of adult health and disease.

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10

Chapter 10

Summary / Samenvatting

SUMMARY

Chapter 1

This chapter provides a general introduction to the different hypotheses with regard to influences of fetal size, birth weight and childhood growth on adult diseases. Potential early life determinants of these adult diseases are discussed. In addition, we describe our study populations, provide the aims of the studies performed, and present the outline of this thesis.

Chapter 2

Rapid catch-up in weight in early life has been associated with metabolic syndrome in adulthood. Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic metabolic syndrome, but its association with rapid catch-up remained unknown. The fatty liver index (FLI) is an estimation of risk for NAFLD in the general population. We studied in 323 young adults born at term associations of birth weight, first-year gain in weight for length and rapid catch-up in weight with the fatty liver index (FLI) at 21 years. We showed that gain in weight for length in the first three months of life was associated with FLI as a continuous variable, whereas low birth weight was not. There were no significant associations with γ-GT, ALT or AST. Of the subjects with rapid catch-up in weight for length, 27.8% had an intermediate or high FLI at the age of 21 years, compared with 5.3% of subjects with slow catch-up.

In conclusion, accelerated gain in weight for length in the first three months of life is associated with a higher risk for NAFLD in early adulthood, whereas small size at birth is not.

Chapter 3

We reported an association between accelerated catch-up in weight during infancy in subjects born at term and increased risk for NAFLD in early adulthood (see chapter 2), but this association had not been studied in subjects born preterm.

We describe, in 162 young adults born preterm, associations of birth weight, gain in weight for length and accelerated catch-up in weight in the first year after term age with the FLI and with individual biochemical markers of FLI as well as with other markers indicating liver damage. We showed that accelerated gain in weight in the first three months after term age was associated with FLI as a continuous variable, whereas gestational age and low birth weight were not. Of the subjects with accelerated catch-up in weight for length after term age, 7.3 % had a high FLI at the age of 21 years, whereas none of the subjects without accelerated catch-up in weight had a high FLI.

In conclusion, accelerated weight gain after term age is associated with an increased risk of developing NAFLD in young adults born preterm.

Chapter 4

Body composition in early life influences the development of obesity during childhood and beyond. Fetal growth and maternal factors might influence neonatal fat mass percentage (FM%), independent of birth weight. We studied in 194 healthy term neonates, within 24 hours after birth, body composition, measured by air-displacement plethysmography (Peapod), and its associations with estimated fetal weight (EFW), neonatal anthropometric data and maternal preconceptional BMI and weight gain during pregnancy. There was a large variation in neonatal FM%, even in case of a similar birth weight, corrected for gender and gestational age. Neonatal FM% was associated with EFW at 30 and 36 weeks of gestation and with catch-up in weight between 30 and 36 weeks of gestation, but not with EFW at 20 weeks. Neonatal FM% was also associated with preconceptional BMI of the mother. There was no correlation with maternal weight gain during pregnancy. This study shows that term neonates have a large variation in fat mass percentage.

In conclusion, neonatal FM% is associated with EFW at 30 and 36 weeks, catch-up in weight between 30 and 36 weeks of gestation and preconceptional BMI of the mother.

Chapter 5

We investigated in 203 healthy term infants longitudinal body composition, including fat mass percentage (FM%) and fat-free mass (FFM), by air-displacement plethysmography, at 1, 3 and 6 months of age, and abdominal visceral fat and abdominal subcutaneous fat, by ultrasound, at 3 and 6 months. We found a significant increase in FM% between 1 and 3 months, but not between 3 and 6 months. Girls had higher FM% than boys at 1 and 6 months and less FFM than boys at 1, 3 and 6 months. There was a large variation in FM% at all ages, even between infants with similar weight SDS. Visceral fat and abdominal subcutaneous fat did not change between 3 and 6 months. FM% was highly correlated with abdominal subcutaneous fat, but not with visceral fat.

In conclusion, changes in FM% mainly occur in the first 3 months of life, and FM%, visceral and abdominal subcutaneous fat do not change between 3 and 6 months, supporting the concept of a critical window for adiposity development in the first three months of life. In addition, this study provides longitudinal reference data of FM%, FFM, visceral fat and abdominal subcutaneous fat during the first 6 months of life.

Chapter 6

Accelerated gain in fat mass and particularly in visceral fat in the first months of life might be major risk factors for adult diseases, because fat mass tracks into adulthood. Infant growth, type of feeding and maternal variables might influence the gain in weight, fat mass and visceral fat in early life. We investigated in 300 healthy term infants, longitudinal changes in fat mass percentage (FM%), measured by air-displacement plethysmography

(Peapod) at 1, 3 and 6 months of age in combination with changes in abdominal visceral and subcutaneous fat, measured by ultrasound, at 3 and 6 months, and their associations with infant growth, type of feeding and maternal variables. A higher gain in weight and FM% in the first 3 months of life was associated with a higher FM% at 3 and 6 months of age, whereas size at birth was not associated with FM% at 3 and 6 months. A higher gain in weight and FM% between 1 and 3 months was associated with more visceral fat at 3 months and a higher gain in FM% between 3 and 6 months with more visceral fat at 6 months. Exclusive breastfeeding (EBF) duration was associated with more subcutaneous fat, but not with more visceral fat. Maternal characteristics did not associate with infant's fat mass and visceral fat at 3 and 6 months.

In conclusion, a higher gain in weight SDS and FM% in the first postnatal months leads not only to a higher FM%, but also to more visceral fat at 6 months of age and exclusively breastfed infants develop more subcutaneous fat.

Chapter 7

Appetite regulating hormones, such as ghrelin and leptin, play a role in the regulation of food intake and energy balance and might influence body composition in later life. Studies on associations between appetite regulating hormones and body composition in early life are limited. We, therefore, investigated serum fasting levels of ghrelin, leptin, insulin, glucose-dependent insulinotropic peptide (GIP), pancreatic polypeptide (PP) and peptide YY (PYY) in 197 healthy term infants at 3 and 6 months and associated these with type of feeding and body composition, including FM%, visceral and subcutaneous fat. Infants with exclusive formula feeding for 3 months had significantly higher serum levels of ghrelin, leptin, insulin, GIP and PP and lower serum levels of PYY at 3 months than breastfed infants. Leptin and ghrelin were positively correlated with FM% at 3 months and insulin with change in FM% between 1 and 3 months. Leptin at 3 months correlated with subcutaneous fat at 3 months but not with visceral fat. Other appetite regulating hormones did not correlate with body composition.

In conclusion, formula fed infants had a very different profile of serum appetite regulating hormones than breastfed infants, suggesting that lower levels of ghrelin, leptin and insulin in breastfed infants could contribute to the protective role of breastfeeding against obesity development.

Chapter 8

Studies suggest a relationship between the compositional pattern of the gut microbiota and obesity in adults, but such data are very limited in infants. We, therefore, investigated in 51 healthy infants several fecal parameters, such as secretory immunoglobulin A (sIgA) and short chain fatty acids (SCFA) contents, and the composition of the microbiota at 1 and 3 months of life, also in relation to type of feeding, infant's body composition and

accelerated weight gain. Formula fed (FF) infants had lower levels of sIgA at 1 and 3 months than their breastfed (BF) counterparts, but levels increased over time in contrast to BF infants. In FF infants, we found that a higher change in sIgA between 1 and 3 months correlated with a higher increase in FM% between 1 and 3, and 3 and 6 months and that higher sIgA and acetic acid at 3 months correlated with more visceral fat at that age, while a higher level of propionic acid at 1 month correlated with a lower increase in FM% between 3 and 6 months. Besides higher levels of Firmicutes at 3 months, FF infants had no different microbiota compared to BF infants. Infants with accelerated weight gain had higher amounts of sIgA at 3 months and lower levels of butyric acid at 1 month. Microbiota were not different between infants with and without accelerated weight gain at month 1, 3, or over time, although infants with accelerated weight gain had a significantly higher increase in *Enterobacter* compared to infants without accelerated weight gain, which might contribute to a different metabolic programming and increase the risk for adult diseases.

Chapter 9

In this chapter, we discuss our findings in a broader context. We emphasize clinical implications and give suggestions for further research.

SAMENVATTING

Hoofdstuk 1

Dit hoofdstuk geeft een algemene inleiding over de verschillende hypotheses ten aanzien van invloeden van foetale groei, geboortegewicht en groei in de kindertijd op ziekten op de volwassen leeftijd. Mogelijke vroege determinanten van deze ziekten worden ook besproken. Bovendien beschrijven we onze studiepopulaties, de doelstellingen van de uitgevoerde studies en de opzet van het proefschrift.

Hoofdstuk 2

Niet-alcoholische vervetting van de lever (non-alcoholic fatty liver disease (NAFLD)) wordt gezien als het hepatisch metabool syndroom. Sommige studies hebben laten zien dat er een associatie is tussen te klein zijn bij geboorte en NAFLD. Versnelde toename van gewicht is meestal het gevolg van te klein zijn bij geboorte en is geassocieerd met het metabool syndroom, maar associaties met NAFLD zijn onbekend. We beschrijven de studie waarin we de associaties hebben onderzocht tussen geboortegewicht, eerste jaar toename in gewicht t.o.v. lengte en versnelde gewichtstoename met de Fatty Liver Index (FLI), een maat om NAFLD te voorspellen. We verrichtten onze studie in 323 jongvolwassenen en lieten zien dat gewichtstoename in de eerste 3 maanden van het leven is geassocieerd met de FLI als continue variabele en laag geboortegewicht niet. Er was geen associatie met gamma-gt, ALAT of ASAT. Van de jongvolwassenen met versnelde gewichtstoename t.o.v. lengte had 27.8% een gemiddelde of hoge FLI op de leeftijd van 21 jaar, vergeleken met 5.3% van de jongvolwassenen met langzame gewichtstoename. We concluderen daarom dat versnelde gewichtstoename t.o.v. lengte in de eerste 3 maanden is geassocieerd met een hoger risico op NAFLD op jongvolwassen leeftijd, terwijl te klein zijn bij geboorte niet geassocieerd is.

Hoofdstuk 3

We hadden laten zien dat versnelde toename in gewicht t.o.v. lengte in op tijd geboren jongvolwassenen geassocieerd is met een verhoogd risico op NAFLD (zie hoofdstuk 2), maar deze associatie was nog niet onderzocht bij jongvolwassenen die prematuur geboren waren. We beschrijven de studie waarin we de associaties hebben onderzocht tussen geboortegewicht, eerste jaar groei in gewicht t.o.v. lengte en versnelde gewichtstoename met de FLI in 162 prematuur geboren jongvolwassenen. We laten zien dat versnelde gewichtstoename in de eerste 3 maanden na de uitgerekende datum geassocieerd is met een hogere FLI. Zwangerschapsduur en laag geboortegewicht waren dit niet. Van de jongvolwassenen met versnelde gewichtstoename t.o.v. lengte na de uitgerekende datum had 7.3% een hoge FLI-score, terwijl geen enkele jongvolwassene zonder versnelde gewichtstoename dit had. We concluderen daarom dat ook in prema-

tuur geboren jongvolwassenen versnelde gewichtstoename t.o.v. lengte in de eerste 3 maanden na de uitgerekende datum geassocieerd is met een hoger risico op NAFLD op jongvolwassen leeftijd.

Hoofdstuk 4

Lichaamssamenstelling in het vroege leven beïnvloedt de ontwikkeling van obesitas gedurende de jeugd en daarna. We beschrijven neonatale lichaamssamenstelling binnen 24 uur na de geboorte en determinanten daarvan zoals foetale groei en maternale factoren. In 194 gezonde pasgeborenen onderzochten we de lichaamssamenstelling met behulp van air-displacement plethysmografie (Peapod) en analyseerden associaties met geschat foetaal gewicht, neonatale antropometrische data en maternaal BMI voor de zwangerschap en gewichtstoename van moeder in de zwangerschap. Er was een grote variatie in neonataal vetpercentage, zelfs als er sprake was van een zelfde gewicht, ook na correctie voor geslacht en zwangerschapsduur. Neonatale FM% was geassocieerd met het geschatte foetale gewicht op 30 en 36 weken zwangerschapsduur en met snelle inhaalgroei in gewicht tussen 30 en 36 weken zwangerschapsduur, maar niet met het geschatte foetale gewicht op 20 weken. Neonataal vetpercentage was ook geassocieerd met de BMI van de moeder vóór de zwangerschap. Er was geen correlatie met gewichtstoename van moeder in de zwangerschap. Onze conclusies zijn dat er een grote variatie in neonataal vetpercentage kan zijn, zelfs als er sprake is van een zelfde gewicht en dat neonataal vetpercentage is geassocieerd het foetale gewicht op 30 en 36 weken zwangerschapsduur, catch-up in foetaal gewicht tussen 30 en 36 weken en met de BMI van moeder voor de zwangerschap.

Hoofdstuk 5

We onderzochten in 203 gezonde à terme zuigelingen longitudinaal de lichaamssamenstelling, inclusief het vetpercentage en de vetvrije massa, door middel van de Peapod, op 1, 3 en 6 maanden en abdominaal visceraal en subcutaan vet, door middel van ultrasound, op 3 en 6 maanden. We vonden een significante stijging in vetpercentage tussen 1 en 3 maanden, maar niet tussen 3 en 6 maanden. Meisjes hadden een hoger vetpercentage dan jongens op 1 en 6 maanden en minder vetvrije massa op 1, 3 en 6 maanden. Er was een grote variatie in vetpercentages op alle leeftijden, zelfs tussen zuigelingen met een zelfde gewicht SDS. Visceraal en subcutaan vet veranderde niet tussen 3 en 6 maanden. We zagen dat het vetpercentage sterk gecorreleerd was met subcutaan vet, maar niet met visceraal vet. We concludeerden dat veranderingen in vetpercentage met name optreden in de eerste 3 maanden van het leven en dat het vetpercentage, visceraal en subcutaan vet niet meer significant veranderen tussen 3 en 6 maanden. Onze data ondersteunen het concept van een kritische periode voor adipositas ontwikkeling in de eerste drie maanden van het leven. Bovendien voorziet

deze studie in longitudinale referentie data van vetpercentage, vetvrije massa, visceraal en subcutaan vet gedurende de eerste 6 maanden van het leven.

Hoofdstuk 6

Versnelde toename in vetmassa en met name visceraal vet in de eerste maanden van het leven kunnen risicofactoren zijn voor de ontwikkeling van ziekten op de volwassen leeftijd omdat vetmassa de neiging heeft tot "tracking" tot in de volwassenheid. Groei in de eerste maanden van het leven, alsmede het type voeding en maternale variabelen zouden de toename in gewicht, vetmassa en visceraal vet kunnen beïnvloeden. We onderzochten de ontwikkeling in vetpercentage, gemeten met de Peapod, op 1, 3 en 6 maanden en visceraal en subcutaan vet, gemeten met de ultrasound, op 3 en 6 maanden en de associaties met de toename in gewicht in de eerste maanden, het type voeding en maternale variabelen. We vonden dat een hoger gewicht en vetpercentage tussen 1 en 3 maanden geassocieerd was met een hoger vetpercentage op 3 en 6 maanden, terwijl geboortegewicht dat niet was. Een grotere toename in gewicht en vetmassa in de eerste 3 maanden was ook geassocieerd met meer visceraal vet op 3 maanden en een grotere toename in vetmassa tussen 3 en 6 maanden met meer visceraal vet op 6 maanden. De duur van het exclusief borstvoeding geven was geassocieerd met meer subcutaan vet, maar niet met meer visceraal vet. Maternale variabelen waren niet geassocieerd met het vetpercentage en het visceraal vet op 3 en 6 maanden. Onze conclusie is dat een grotere toename van gewicht SDS en vetpercentage in de eerste maanden van het leven niet alleen leidt tot een hoger vetpercentage, maar ook tot meer visceraal vet en dat zuigelingen die exclusief borstvoeding krijgen meer subcutaan vet ontwikkelen.

Hoofdstuk 7

Eetlustregulerende hormonen, zoals ghreline en leptine, spelen een rol in de regulatie van voedselinname en energiebalans en kunnen mogelijk de lichaamssamenstelling later in het leven beïnvloeden. Studies naar associaties tussen eetlustregulerende hormonen en lichaamssamenstelling in de eerste maanden van het leven zijn beperkt. Daarom onderzochten we in 197 gezonde zuigelingen op de leeftijd van 3 en 6 maanden de serum spiegels van ghreline, leptine, insuline, glucose-afhankelijke insulinotrope peptide (GIP), pancreatisch polypeptide (PP) en peptide YY (PYY) en associeerden deze met het type voeding en de lichaamssamenstelling, waaronder vetpercentage, visceraal en subcutaan vet. Zuigelingen met flesvoeding gedurende 3 maanden vanaf geboorte hadden op de leeftijd van 3 maanden significant hogere spiegels van ghreline, leptine, insuline, GIP en PP, en lagere spiegels van PYY dan de borstgevoede zuigelingen. Leptine en ghreline waren positief gecorreleerd met vetpercentage op 3 maanden en insuline met de verandering in vetpercentage tussen 1 en 3 maanden. Leptine was op 3 maanden ook gecorreleerd met subcutaan vet, maar niet met visceraal vet. De andere

eetlustregulerende hormonen waren niet gecorreleerd met lichaamssamenstelling. Onze conclusie is dat de flesgevoede zuigelingen een heel ander profiel van eetlustregulerende hormonen hebben dan borstgevoede zuigelingen, hetgeen suggereert dat de lagere spiegels van ghreline, leptine en insuline in borstgevoede zuigelingen een rol kunnen spelen in de gevonden bescherming tegen de ontwikkeling van overgewicht.

Hoofdstuk 8

Studies suggereren een relatie tussen de samenstelling van de darmflora en obesitas bij volwassenen, maar data in zuigelingen zijn beperkt. Daarom onderzochten we in 51 gezonde zuigelingen diverse fecale parameters, zoals secretoir IgA (sIgA) en korte keten vetzuren en de bacteriële samenstelling van de darmflora op 1 en 3 maanden en ook in relatie met type voeding, lichaamssamenstelling en versnelde gewichtstoename. Flesgevoede zuigelingen hadden lagere sIgA spiegels op 1 en 3 maanden dan borstgevoede zuigelingen, maar deze spiegels stegen in de tijd in tegenstelling tot bij de borstgevoede zuigelingen. In flesgevoede zuigelingen vonden we dat een grotere toename in sIgA tussen 1 en 3 maanden gecorreleerd was met een grotere toename in vetpercentage tussen 1 en 3 maanden en tussen 3 en 6 maanden en dat hogere sIgA en azijnzuur spiegels op 3 maanden gecorreleerd waren met meer visceraal vet op die leeftijd, terwijl een hogere waarde van propionzuur op 3 maanden gecorreleerd was met een kleinere toename in vetpercentage tussen 3 en 6 maanden. Behoudens grotere aanwezigheid van Firmicuten waren er geen verschillen in darmflora tussen fles- en borstgevoede zuigelingen. Zuigelingen met versnelde gewichtstoename in de eerste 3 maanden hadden hogere spiegels van sIgA op 3 maanden en lagere spiegels van butyraat op 1 maand. De darmflora op 1 en 3 maanden was niet verschillend tussen zuigelingen met en zonder versnelde gewichtstoename, al zagen we wel dat zuigelingen met versnelde gewichtstoename meer toename in *Enterobacter* hadden ten opzichte van de zuigelingen zonder versnelde gewichtstoename, en dat kan betekenen dat deze bacterie kan bijdragen aan de verschillen in metabole programmering en de toename in risico's op ziekten op de volwassen leeftijd.

Hoofdstuk 9

In dit hoofdstuk worden de resultaten van de verschillende studies besproken in een bredere context. Wij sluiten dit hoofdstuk af met algemene overwegingen en suggesties voor toekomstig onderzoek.

11

Chapter 11

List of abbreviations
List of publications
PhD portfolio
Dankwoord
Curriculum Vitae

LIST OF ABBREVIATIONS

ADP	air-displacement plethysmography
AGA	appropriate for gestational age
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AWG	accelerated weight gain
BF	breastfeeding
BMI	body mass index
CNS	central nervous system
CU	catch-up
CVD	cardiovascular disease
DM2	type 2 diabetes
DXA	dual-energy x-ray absorptiometry
EBF	exclusive breastfeeding
EFW	estimated fetal weight
FF	formula feeding
FFM	fat-free mass
FFM%	fat-free mass percentage
FLI	Fatty Liver Index
FM	fat mass
FM%	fat mass percentage
GIP	glucose-dependent insulinotropic peptide
γ-GT	gamma-glutamyltransferase
HDLc	high density lipoprotein cholesterol
IQR	interquartile range
LDLc	low density lipoprotein cholesterol
MR	multiple regression
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
n-AWG	non-accelerated weight gain
PI	ponderal index
PP	pancreatic polypeptide
PYY	peptide YY
SCFAs	short-chain fatty acids
SDS	standard deviation score
SES	socio-economic status

SGA	small for gestational age
sIgA	secretory immunoglobulin A
TG	triglycerides
US	ultrasound

LIST OF PUBLICATIONS

Breij LM, Kerkhof GF, Hokken-Koelega AC. Accelerated infant weight gain and risk for nonalcoholic fatty liver disease in early adulthood. J Clin Endocrinol Metab. 2014 Apr;99(4):1189-95.

Breij LM, Kerkhof GF, Hokken-Koelega AC. Risk for Nonalcoholic Fatty Liver Disease in Young Adults Born Preterm. Horm Res Paediatr. 2015;84(3):199-205.

Breij LM, Steegers-Theunissen RP, Briceno D, Hokken-Koelega AC. Maternal and Fetal Determinants of Neonatal Body Composition. Horm Res Paediatr. 2015;84(6):388-95.

Breij LM, Kerkhof GF, De Lucia Rolfe E, Ong KK, Abrahamse-Berkeveld M, Acton D, Hokken-Koelega ACS. Longitudinal fat mass and visceral fat during the first 6 months after birth in healthy infants: support for a critical window for adiposity in early life. Pediatr Obes *Accepted*

Breij LM, Abrahamse-Berkeveld M, Acton D, De Lucia Rolfe E, Ong KK, Hokken-Koelega AC. Impact of infant growth, type of feeding and maternal factors on total body fat mass and visceral fat at 3 and 6 months of age. *Submitted*

Breij LM, Mulder MT, Van Vark-van der Zee LC, Hokken-Koelega AC. Appetite regulating hormones in early life and relationships with type of feeding and body composition in healthy term infants. *Submitted*

Breij LM, Tims S, Hokken-Koelega AC. Microbiome in early life and relationships with type of feeding, accelerated weight gain and body composition in healthy term infants. *Submitted*

Zijlstra M, De Bie C, **Breij L**, van Pieterson M, van Staa A, de Ridder L, van der Woude J, Escher J. Self-efficacy in adolescents with inflammatory bowel disease: a pilot study of the "IBD-yourself", a disease-specific questionnaire. J Crohns Colitis. 2013 Oct;7(9):e375-85.

PHD PORTFOLIO

Name:	Laura Maria Breij
Erasmus MC Department:	Pediatrics, division Pediatric Endocrinology
PhD period:	October 2011 –February 2016
Promotor:	Prof.dr. A.C.S. Hokken-Koelega

Summary of PhD training

	Year	Workload (ECTS)
General courses		
Good Clinical Practice, Erasmus MC	2012	1.0
Biostatistical Methods, Basic Principles, NIHES, Erasmus MC	2012	5.7
Integrity in scientific research, Erasmus MC	2012	2.0
Biomedical English Writing and Communication, Molmed, Erasmus MC	2014	4.0
Specific courses		
Basic and Translational Endocrinology	2012	2.2
Pubmed and Endnote, Medical Library, Erasmus MC	2013	0.3
Radiation protection 5A, Zorgacademie, Erasmus MC	2012	1.0
Photoshop & Illustrator CS5, Molmed, Erasmus MC	2014	0.3
Indesign CS5, Molmed, Erasmus MC	2014	0.3
Microsoft Access 2010: Basic & Advanced	2014	0.7
Seminars and workshops		
Annual PhD day	2012-2013	0.6
Annual Research Day, Sophia Children's Hospital	2013-2014	0.6
Annual Interclinical evening	2012-2015	0.3
Weekly research meetings, department of Pediatric Endocrinology	2011-2015	4.0
Research and social media	2014	0.3
International and national conferences		
Developmental Origins of Health and Disease Satellite Meeting 2012: New Developments in Developmental Epidemiology, Rotterdam (poster presentation)	2012	0.3
8th World Congress on Developmental Origins of Health and Disease, Singapore, Singapore (2x Poster presentation)	2013	1.0
53rd Annual European Society for Pediatric Endocrinology Meeting, Dublin, Ireland (oral presentation)	2014	1.0

48th Annual meeting of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition, Amsterdam (poster presentation)	2015	1.0
54rd Annual European Society for Pediatric Endocrinology Meeting, Barcelona, Spain (poster presentation)	2015	1.0
1st Peapod User meeting, Rome (expert-opinion)	2015	0.3
Lecturing		
Lecture Refer evening Metabolism, Endocrinology and Feeding	2015	0.5
Annual IMC Weekendschool day "Growth and Development", Rotterdam	2013-2014	1.0
Other		
Planning weekly research meetings of the department of Pediatric Endocrinology	2011-2015	2.0

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Laura

CURRICULUM VITAE

Laura M. Breij was born in Gouda, the Netherlands on July 12, 1986. She received her Gymnasium degree at the St.-Antoniuscollege in Gouda in 2004. In the same year she started her medical training at the Medical Faculty of the Erasmus University of Rotterdam. She finished the theoretical part of medical school in 2008. Her graduate research focused on transition in care in children with Inflammatory Bowel Diseases (supervisor Prof. dr. J.C. Escher). In 2008, she started her medical internships and as part of her clinical training, she did an elective internship at the department of pediatrics at the Mater Dei Hospital in Malta. She concluded her internships at the department of pediatrics at the Reinier de Graaf Gasthuis in Delft.

Laura worked at the department of pediatrics in the Maasstad Ziekenhuis for almost a year, after which she started her PhD in 2011. She was supervised by Prof. dr. A.C.S. Hokken-Koelega, pediatric endocrinologist at the Erasmus Medical Center-Sophia Children's hospital in Rotterdam. The research performed during this PhD is presented in this thesis.

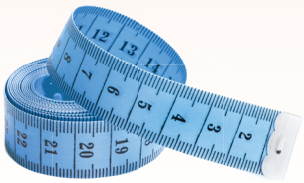
Laura is currently working as a resident at the department of pediatrics in Noordwest Ziekenhuis Groep (MCA Alkmaar) and lives in Heerhugowaard with the love of her life Stefan Kowalski.

Infant Body Composition and Other Early Life Determinants of Obesity and Adult Diseases

Childhood obesity is a significant public health problem. It is not only associated with short-term, but also with long-term morbidity, such as obesity, type 2 diabetes and cardiovascular disease in adulthood. Increasing adiposity in the first months of life is a major risk factor for these adult diseases, as it tracks into adulthood.

This thesis presents:

- Accelerated weight gain in the first 3 months of life in association with the risk for non-alcoholic fatty liver disease, in young adults born term or preterm.
- Longitudinal reference data of body composition during the first 6 months of life.
- Fetal growth, infant characteristics, type of feeding and maternal data and their associations with infant's body composition, at birth and at 1, 3 and 6 months of age.
- Levels of appetite regulating hormones in association with type of feeding and body composition at 3 and 6 months of age.
- Composition of fecal microbiome at 1 and 3 months of age and associations of microbiota with type of feeding, early weight gain and infant's body composition.



Laura M. Breij