

ADIPOSIITY PROGRAMMING IN EARLY LIFE

ADVANCING KNOWLEDGE ABOUT POTENTIAL
DETERMINANTS IN HEALTHY INFANTS



KIRSTEN DE FLUITER

Adiposity programming in early life

Advancing knowledge about potential determinants in healthy infants

Kirsten S. de Fluiter

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Adiposity programming in early life

Advancing knowledge about potential determinants in healthy infants

Programmering van lichaamsvet in het vroege leven

Voortschrijdend inzicht in potentiële determinanten in gezonde, jonge kinderen

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Chapter 1

General introduction

INTRODUCTION

In 2012 our research group started the Sophia Pluto Study, investigating growth, detailed body composition and feeding patterns in healthy, term-born infants during the first two years of life, with a focus on finding determinants of adiposity programming in early life. This thesis describes the results of six new studies embedded in the Sophia Pluto Study.

This introduction describes the current knowledge concerning the influence of infant growth on adult health and the potential factors that might influence infant body composition. In addition, the objectives of the studies presented in this thesis are described.

Infant weight gain influencing adult health

Accelerated weight gain during early life has been associated with an increased risk for adult diseases (1-4). In the PROGRAM-study, initiated in 2002, our research group showed that rapid gain in weight-for-length during the first months of life was associated with 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 (5). These findings suggested the presence of a critical window for adiposity programming in the first postnatal months and indicated that accelerated weight-for-length standard deviation score (SDS) should be avoided to reduce the risk for obesity, type 2 diabetes and cardiovascular diseases in later life (5-7). Others also showed that the first postnatal months are important for adiposity programming (8).

In 2019, ~13% of the Dutch children between age 4 and 17 years was overweight. At age 2 years already ~8% of the infants had moderate or severe overweight (9). Obesity during childhood is associated with short-term morbidity, such as asthma and psychological problems. It has also been associated with an increased risk for adolescent and adult obesity (10-13), as most adolescents with obesity will have excessive adiposity during adulthood. This in turn puts them at risk for later cardiovascular diseases and cancer (13).

Over the last years, progress has been made in unraveling the genetics and epigenetics of obesity (14), but only a low proportion of the heritability of obesity has now been explained (15). The established loci, the position of genes on a chromosome, involved in obesity development explain only a small part of the variance and can only poorly predict obesity (16). It can, therefore, not be clinically used as a predictive tool (15). Since obesity has a multifactorial etiology (13), single treatment strategies are not likely to be effective for all obesity patients.

In addition, obesity later in life requires lifelong treatment (13). Treatment options range from nutritional diet with increased physical activity to drugs and surgery (14), but the

success rate is generally very low in obtaining and maintaining a healthy weight. It is, therefore, crucial to elucidate which factors influence adiposity programming in early life as this will help to develop prevention strategies for childhood obesity in the future.

Infant body composition

There is increasing evidence that excessive weight gain in early life increases the risk of more fat mass in childhood and in later life. It is important to specify weight gain in terms of gain in fat mass and fat-free mass in early life. Most studies, however, have mainly focused on longitudinal anthropometric outcomes like weight-for-length SDS, BMI and skinfold measurements as proxy for adiposity during infancy (17). As the first 1000 days, from conception until age 2 years, are an important period for the development of the body and brain of the infant (18, 19), it is crucial to obtain longitudinal values of fat mass and fat-free mass during the first 2 years of life. Nowadays, there are various tools to measure detailed body composition in infants.

Methods for measuring infant body composition

For many years weight and the ponderal index after birth, calculated based on birth weight and birth length, were used as a proxy for body composition. These measures, however, do not reflect actual body composition (20). Techniques to determine body composition by the multi-component model for quantifying fat, water, mineral and protein or by magnetic resonance imaging (MRI) have been applied in infancy and childhood (21-23). These methods can accurately determine body composition, but are very expensive and cannot be used routinely in large studies.



Figure 1. PEA POD by Cosmed. derived from the brochure on www.cosmed.com.

Body composition by air-displacement plethysmography

Body composition in infants can nowadays be assessed by air-displacement plethysmography (PEA POD, Infant Body Composition System, COSMED, Italy). ADP assesses fat mass (FM), fat mass percentage (FM%), fat-free mass and fat-free mass percentage by direct measurements of body mass and body volume, based on the whole-body densitometry principle (24). Body mass is measured on the integrated scale of the PEA POD and body volume is

measured inside the closed test chamber by applying pertinent gas laws that relate pressure changes to volumes of air. Details of the principle and operating procedure of the PEA POD have been described (25, 26). Studies have shown that ADP is a valid, non-invasive and fast technique for measuring body composition in infants from birth until the upper limit of ap-

proximately 8 kg (27-29). Another ADP system, BOD POD, is available from approximately 2-3 years onward (30), but there are no ADP systems for children between age 6 months (\pm 8 kg) and 2 years, which complicates obtaining longitudinal body composition by ADP during the important first 2 years of life.



Figure 2. DXA <https://www.acertys.com/nl/producten/ge-lunar-prodigy>

Body composition by dual-energy X-ray Absorptiometry

Dual-energy X-ray absorptiometry (DXA) is an alternative measurement technique for body composition in infants (29, 31-33). DXA has good reproducibility in infants in case of a successful scan (31), but successful measurements are extremely difficult to obtain of infants, especially between the age of 1 and 2 years, due to movement artifacts (34-36). Also, it has been reported that DXA might overestimate FM (35). Reference values for body composition measured by DXA scan only exist from age 4 years onwards (37).



Figure 3. DXA with vacuum cushion. Photo depicted with permission from parents.

As abovementioned, obtaining successful measurements of infant body composition by DXA scan is limited due to movement artifacts. The use of a vacuum cushion might be a solution in preventing movement during DXA scan, which might improve the chance of obtaining successful measurements. It was unknown if this would indeed be the case. An evaluation of the use of a vacuum cushion was, therefore, required.

In addition, it was unknown whether measurements of body composition by ADP and DXA, either with or without a vacuum cushion, would be comparable at the transition point at age 6 months. This is essential for using longitudinal measurements of body composition during infancy by ADP and DXA.

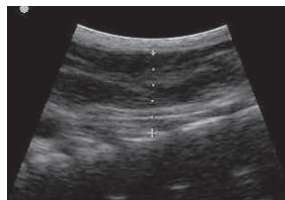


Figure 4. Visceral fat mass thickness by ultrasound.

Abdominal subcutaneous and visceral fat mass by ultrasound

Not only the total amount of fat mass, but also the location of fat mass is important. Particularly increased abdominal visceral FM has been associated with an unfavorable metabolic health profile during childhood and later on (38, 39).

Ultrasound is a non-invasive method to estimate abdominal fat mass. Ultrasound measurements of subcutaneous and

visceral fat mass thickness are reliable and reproducible estimates of abdominal subcutaneous fat mass and intra-abdominal (visceral) fat mass (40, 41).

Body composition – potential influencing factors

Several factors might influence the development of infant body composition (Figure 5), which are explained point-by-point below.

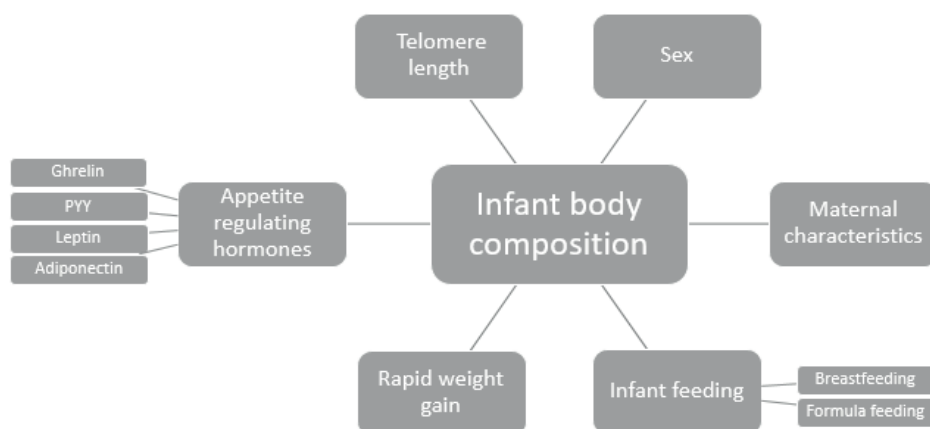


Figure 5. Potential influencing factors of infant body composition development.

Boys and girls

Sex differences in body composition development during the first 2 years of life might be present due to different sex hormone levels in boys and girls in early life. It was already known that girls have higher fat mass and lower fat-free mass compared to boys after birth and we previously described higher fat mass in girls at age 6 months (27, 42-44), but the fat mass development until age 2 years was unknown.

Maternal characteristics

Maternal pre-pregnancy body mass index (BMI) and gestational weight gain are determinants of fetal growth, infant birth weight and fat mass percentage at birth (45-47). Associations between these maternal factors and body composition in infants and children were not found during the postnatal period until age 6 months (48, 49), but independent relationships of maternal early pregnancy BMI with childhood BMI and adiposity have been found at age 6 years (50). However, little is known about the effect of maternal pre-pregnancy BMI and gestational weight gain on early infant body composition trajectories.

Infant feeding

Breastfeeding and formula feeding are two types of infant feeding. Breastfeeding is considered the gold standard infant feeding, as it can result in health benefits for mother and child (51). Breastfeeding lowers the risk for adiposity during childhood (52-56) and is a protective factor against several infections (51), asthma development (57), eczema and allergic rhinitis (58) by supporting the development of the immune system and microbiota (59). For mothers, breastfeeding lowers the risk of breast cancer (51).

Breastfeeding is, however, not always possible, for example due to maternal disease and/or use of medication or is not desired by parents. In such cases, formula feeding is another option. Differences between breastfeeding and formula feeding exist in macronutrient composition and bioactive factors (60). In addition, there are also different formula feeding options, in brand and compound (61). Formula feeding has been associated with altered body composition in infancy compared to breastfeeding, as shown in small cohorts and short-term follow-up periods (60).

Human milk is composed of macronutrients, micronutrients and bioactive factors (62). Different techniques exist for analyzing macronutrient composition, with infrared human milk analyzers (HMA) being a method to estimate this composition. These methods are mainly used for optimizing feeding for preterm infants. It is, however, unknown to what extent human milk macronutrients might be involved in early adiposity programming, since studies investigating human milk macronutrients in association with (changes in) body composition in early life are very limited.

Rapid weight gain in early life

Accelerated gain in weight-for-age SDS during the first postnatal months has been associated with an increased risk for overweight and obesity in childhood and adulthood (63-67), unfavorable metabolic health profiles in young adults (6, 7, 68) and cardiovascular diseases in later life (4, 69). In addition, associations between early weight gain and childhood obesity have been described (55, 70, 71).

We have previously shown that newborns with similar weight and weight-for-length SDS might have different fat mass (72). Until now, data on associations between gain in fat mass, instead of gain in weight, and body composition trajectories in early life do not exist.

Longitudinal reference values for FM% until the age of 2 years are also lacking due to the different measurement techniques at different ages. Obtaining these longitudinal measurements is of great importance as this period in early life is important for infant development (18, 19). In addition, it is important to compare body composition during the first 6 months of life, a critical window for adiposity programming, with the period from 6 months to 2 years. These data are of interest since two studies showed that fat mass accretion until age 6 months associated with higher fat mass index at age 4 years and that fat mass accretion

until age 8 months associated with overweight/obesity at age 6-11 years in a small group of children (73, 74).

Appetite regulating hormones

Appetite regulating hormones (ARH) are involved in the regulation of food intake through specific brain centers. The hypothalamus plays a key role in controlling glucose and energy homeostasis and food intake (75, 76). Active ghrelin, a stimulating hormone, increases food intake, while other hormones like leptin and PYY decrease food intake and increase metabolic rate and adiponectin increases the uptake of fatty acids and carbohydrates (76, 77). Furthermore, the (active) ghrelin/PYY ratio is a marker of orexigenic drive (78, 79).

Data on ARH trajectories during early life are very limited. ARH have been associated with later growth and adiposity, but most studies used cord blood (80-85) instead of blood samples obtained during early infancy and specifically during the first 6 postnatal months. Investigating ARH and their trajectories in early life is of interest as they might play a role in adiposity programming.

Three studies compared ARH levels between breastfed and formula fed infants and reported different ARH levels in early life between both groups (86-88), but associations of ARH with human milk macronutrients, infant appetite and body composition have not been reported.

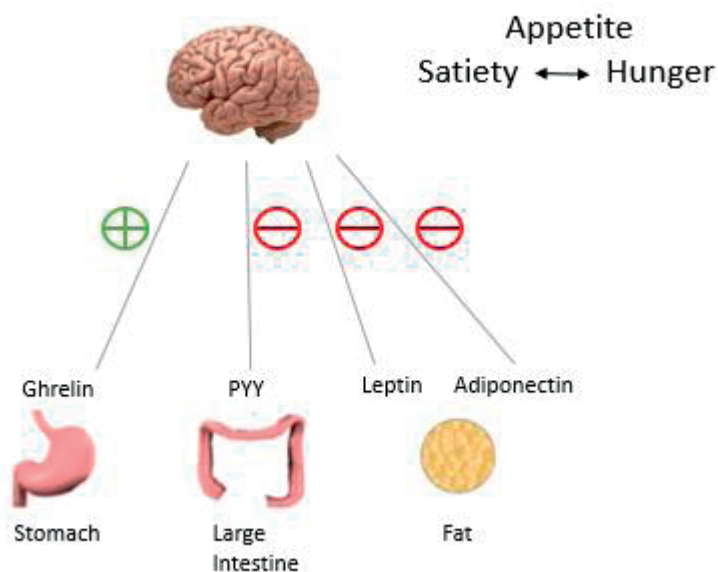


Figure 6. Appetite regulating hormones (partly adapted from www.sigmanutrition.com).

Leukocyte telomere length

Telomeres are non-coding repetitive DNA sequences located at the end of chromosomes, protecting DNA in maintaining stability (89). Leukocyte telomere length (LTL) is a marker of biological aging as shortening occurs over time, because DNA polymerase is not able to fully replicate the end of chromosomes. When telomeres are reduced to a critical length, cells enter a state of arrest (90). By using a quantitative PCR technique, telomere length can be measured in leukocytes (90, 91). Shorter LTL has been associated with adiposity and a higher risk of cardiovascular diseases (92, 93). Until now, only one study has investigated longitudinal LTL during the first two years of life (94), which is an important period for infant development (18). Their first LTL measurement, however, was at a mean age of 8.6 months, thus not during the critical window for adiposity programming until age 6 months. It is important to specifically investigate LTL during this period in early life in association with changes in body composition until the age of 2 years.

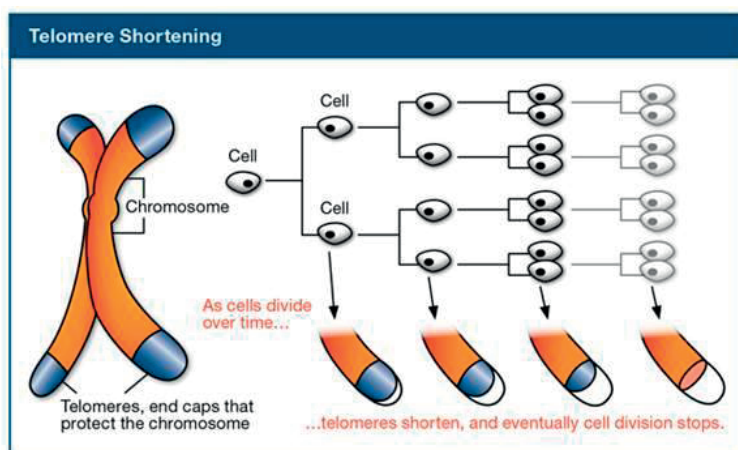


Figure 7. Telomere shortening (adapted from <http://www.wholehealthinsider.com/newsletter/2012/a-genetic-solution-to-slowng-aging-and-preventing-disease/> and thesis Lin Smeets: Silver-Russell Syndrome & Small for Gestational Age – long-term health perspectives.)

HYPOTHESES

We hypothesized that a rapid increase in fat mass percentage in the first 6 postnatal months leads to a higher fat mass percentage at the age of 2 years. We also hypothesized that infant feeding, milk macronutrient composition, leukocyte telomere length and appetite regulating hormones associate with adiposity development in the first two years of life.

The Sophia Pluto Study birth cohort

The Sophia Pluto Study was initiated in 2012 to prospectively identify determinants of adiposity programming in early life in healthy, term-born infants during the first 2 years of life. The in- and exclusion criteria are described in Appendix A.

Aims of the studies

This thesis presents the results of 6 studies in healthy, term-born infants participating in the Sophia Pluto Study.

1. *Longitudinal body composition assessment in early life*

To evaluate the reliability of using a vacuum cushion during dual-energy X-ray absorptiometry (DXA) to prevent movement artifacts and to compare fat mass (FM) measured by DXA with FM measured by air-displacement plethysmography (ADP).

To construct sex-specific longitudinal body composition values and charts from age 1 month until 2 years.

2. *Rapid increase in fat mass in early life and later body composition*

To investigate in which postnatal months a change in FM% is associated with FM% at age 2 years

To investigate whether a rapid increase in FM% in the first months of life is associated with higher trajectories of body fat mass during the first 2 years of life.

3. *Human milk macronutrients, body composition and appetite*

To investigate human milk macronutrients at age 1 and 3 months in association with body composition and appetite until age 2 years in healthy, term-born infants.

4. *Leukocyte telomere length and body composition*

To obtain longitudinal LTL measurements and determine the shortening of LTL during the first 2 years of life in healthy, term-born infants and to associate LTL shortening with potential stressors and body composition.

5. *Appetite regulating hormones and body composition until age 6 months*

To investigate longitudinal serum ghrelin (acylated), PYY, ghrelin/PYY ratio and leptin levels until age 6 months and their associations with body fat mass, infant feeding, human milk macronutrient composition and infant appetite until age 6 months.

6. *Appetite regulating hormones and body composition until age 2 years*

To investigate longitudinal appetite regulating hormone levels from age 3 months to 2 years in association with FM parameters at age 2 years and their predictive value for FM development until age 2 years.

To investigate associations of appetite regulating hormone trajectories until 6 months and from 6 months to 2 years with trajectories of FM parameters in the same periods.

APPENDIX A

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

Healthy infants are included in the Sophia Pluto Study. The inclusion into this study is still ongoing and the total number will be 1250 infants.

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 disease 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 age 28 days and visited the outpatient clinic at age 1, 3, 6, 9 months and 1 year, 18 months and 2 years. During the visits, anthropometrics, body composition and various other parameters were measured and blood samples were collected. Until and including age 6 months, FM% was measured by air-displacement plethysmography (PEA POD) and from 6 months onward by DXA-scan. Abdominal subcutaneous and visceral fat mass were measured by abdominal ultrasound.

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Chapter 2

Longitudinal body composition assessment in healthy
term-born infants until 2 years of age using ADP and
DXA with vacuum cushion

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ABSTRACT

Objectives Accelerated gain in fat mass (FM) in early life increases the risk for adult diseases. Longitudinal data on infant body composition are crucial for clinical and research use, but very difficult to obtain due to limited measurement tools and unsuccessful measurements between age 6–24 months. We compared FM% by dual-energy X-ray absorptiometry (DXA), with cushion to reduce movement artifacts, with FM% by air-displacement plethysmography (ADP) and evaluated the reliability of this cushion during DXA by comparing FM% with and without cushion. Subsequently, we constructed sex-specific longitudinal body composition charts from 1–24 months.

Methods In 692 healthy, term-born infants (Sophia Pluto Cohort), FM% was measured by ADP from 1–6 months and DXA with cushion from 6–24 months. At 6 months, FM% was measured in triplicate by ADP and DXA with and without cushion (n=278), later on in smaller numbers.

Results At 6 months, mean FM% by DXA with cushion was 24.1 and by ADP 25.0, mean difference of 0.9% (Bland–Altman $p=0.321$, no proportional bias). Mean FM% by DXA without cushion was 12.5% higher compared to ADP (Bland–Altman $p<0.001$). DXA without cushion showed higher mean FM% compared to DXA with cushion (+11.6%, $p<0.001$) at 6 months. Longitudinally, FM% increased between 1–6 months and decreased from 6–24 months (both $p<0.001$).

Conclusions In infants, DXA scan with cushion limits movement artifacts and shows reliable FM%, comparable to ADP. This allowed us to construct longitudinal body composition charts until 24 months. Our study shows that FM% increases from 1–6 months and gradually declines until 24 months.

INTRODUCTION

The first 1000 days window, from conception until 24 months, is an important period for body and brain development and hence an optimal time for early obesity prevention (1). Determining sex-specific changes in longitudinal body composition, i.e., fat mass (FM) and fat-free mass (FFM), during this period is crucial for clinical and research use, but challenging due to movement artifacts when infants become older and stronger and to the limited measurement tools between the age of 6 months and 2 years.

Earlier studies focused mainly on longitudinal anthropometric outcomes, such as weight-for-length, body mass index, and skinfolds as a proxy for infant adiposity (2). We and others demonstrated that rapid weight gain in infancy, and specifically in the first 3 months, was strongly associated with determinants of cardiovascular disease, type 2 diabetes, and overweight in early adulthood (3-6). It is essential to obtain longitudinal data on body composition in early life, as we previously showed that infants with similar weight and weight-for-length standard deviation scores (SDS) might have different fat mass percentage (FM%) (7).

Nowadays, methods exist to assess detailed body composition in infants (8). Techniques to determine body composition by the multi-component model for quantifying fat, water, mineral, and protein or by magnetic resonance imaging have been applied in infancy and childhood (9-11). However, these methods are laborious, expensive, and cannot be applied routinely in larger study settings. Air-displacement plethysmography (ADP) is a noninvasive, fast, and accurate technique for measuring longitudinal body composition in infants from birth until the upper limit of 8 kg (PEA POD) (12-15) and from ~2–3 years onward (BOD POD, pediatric option) (16), but there are no ADP systems for children between 6 months and 2 years (12, 17).

Dual-energy X-ray absorptiometry (DXA) is an alternative measurement technique (15, 18-20), but reference values exist only from age 4 years onward (21). Also, it has been reported that DXA overestimates FM (22). DXA has good reproducibility in infants in case of a successful scan (18), but successful measurements are extremely difficult to obtain in infants due to movement artifacts (8, 22, 23). Unsuccessful scans are reported in newborns up to 69% (19, 24), which could be higher when infants become older and stronger. The use of a vacuum cushion prevents movement in infants during DXA, but the effect on body composition measurement was unknown.

Two studies compared ADP and DXA in infancy, with conflicting results and performed in small populations (23, 25). Since both have different measurement techniques, it is important to determine whether measurements are comparable at the transition point at

age 6 months. Combining measurements of ADP and DXA would allow the construction of longitudinal data on body composition during the first 2 years of life, which is essential for the clinical and research use.

We hypothesized that infant body composition measured by DXA with vacuum cushion would be comparable with ADP measurements at the age of 6 months, because DXA measurements with vacuum cushion provide more reliable FM%, due to less movement artifacts, compared to DXA measurements without vacuum cushion. Consecutively, we wanted to construct sex-specific longitudinal body composition charts from age 1 until 24 months, based on our large group of healthy, term-born boys and girls.

MATERIALS AND METHODS

Study settings and subjects

The study population consisted of 692 healthy, term-born infants, participating in the Sophia Pluto Study, a birth cohort study in Rotterdam area (The Netherlands; Supplemental Fig. 1). Between January 2013 and October 2018, infants were recruited to obtain detailed data on body composition and growth during early life. The Medical Ethics Committee of Erasmus Medical Center approved the Sophia Pluto Study (MEC-2012-164) and parental written informed consent was obtained (13). All participants fulfilled the following inclusion criteria: term born (≥ 37 weeks of gestation), age < 28 days, uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score < 3 after 5 min), sepsis, or long-term complication of respiratory ventilation. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy, or a significant maternal medical condition that could interfere with the study results.

Data collection and measurements

Outpatient clinic visits were scheduled at the age of 1, 3, 6, 9, 12, 18, and 24 months (Table 1). Pregnancy and birth data were obtained from midwife and hospital records. Measurements were performed by trained staff.

Anthropometrics

Weight was measured to the nearest 5 g by using an electronic infant scale (SECA 717, Hamburg, Germany). Length was measured twice by two-person technique to the nearest 0.1 cm using an infantometer (SECA 416) and head circumference was measured twice as the widest frontal–occipital circumference, to the nearest 0.1 cm using a measuring tape (SECA 201).

Table 1. Clinical characteristics.

	Gender	1 month	3 months	6 months	9 months	12 months	18 months	24 months
N	Total	692	617	550	505	473	396	346
	M	375	342	300	281	260	225	200
	F	317	275	250	224	213	171	146
Weight (kg)	M	4.42 [4.03 – 4.77]	6.19 [5.81 – 6.72]	7.90 [7.39 – 8.55]	9.15 [8.51 – 9.81]	9.98 [9.20 – 10.76]	11.48 [10.53 – 12.30]	12.83 [11.70 – 13.80]
	F	4.08 [3.67 – 4.42]	5.72 [5.20 – 6.18]	7.33 [6.83 – 7.87]	8.44 [7.83 – 9.06]	9.35 [8.74 – 9.99]	10.80 [10.10 – 11.46]	12.06 [11.30 – 12.85]
Length (cm)	M	55.0 [53.6 – 56.5]	62.2 [60.5 – 63.5]	69.0 [67.0 – 70.5]	73.2 [71.9 – 74.9]	77.0 [75.0 – 79.0]	83.9 [81.5 – 86.0]	90.0 [87.5 – 91.9]
	F	54.0 [52.4 – 55.3]	60.5 [59.0 – 61.5]	67.0 [65.5 – 68.4]	71.3 [69.6 – 72.6]	75.0 [73.5 – 76.5]	82.0 [79.8 – 83.4]	88.0 [85.9 – 90.0]
Head circumference (cm)	M	37.5 [36.5 – 38.2]	40.5 [40.0 – 41.3]	43.5 [42.7 – 44.3]	45.3 [44.5 – 46.2]	46.3 [45.5 – 47.2]	48.0 [47.0 – 48.5]	48.6 [47.5 – 49.8]
	F	36.4 [35.7 – 37.2]	39.5 [38.6 – 40.2]	42.0 [41.5 – 43.0]	44.0 [43.2 – 44.5]	45.0 [44.2 – 45.5]	46.3 [45.5 – 47.0]	47.5 [46.5 – 48.0]

Data expressed as median [IQR] for male and female.

Body composition measurements

Until the age of 6 months, body composition was assessed by ADP (ADP by PEA POD, COSMED, Italy). ADP assesses FM, fat mass percentage (FM%), fat-free mass (FFM), and fat-free mass percentage (FFM%) by direct measurements of body mass and body volume, based on the whole-body densitometric principle (26). Body mass was measured on the integrated scale of the PEA POD and body volume was measured inside the closed test chamber by applying pertinent gas laws that relate pressure changes to volumes of air. The PEA POD was calibrated every day, according to the protocol recommended by the supplier. At the age of 6 months, body composition was assessed in triplicate in 278 infants. Once by PEA POD and twice by DXA (Lunar Prodigy, GE Healthcare, UK): once with a vacuum cushion (465 75100, Schmidt, Germany) to prevent movement and once without. During all DXA scans, infants were wearing only a disposable diaper and were swaddled in a cotton blanket.

From 6 months onward, DXA with vacuum cushion to prevent movement was used in all infants at every visit. All DXA scans were performed with the same machine with daily quality controls. We used enCORE software version 14.1 and for analysis the enhanced analysis algorithm (27). FM% was calculated as total FM (gram) divided by total weight (gram) $\times 100$ [FM/weight $\times 100$]. As dependent on the position of the infant, a variable part of the vacuum cushion is considered as FFM by DXA. FFM was calculated by subtracting total FM (gram) from total weight (gram). Fat mass index (FMI) was determined by dividing FM (kg) by height squared (m^2) and fat-free mass index (FFMI) by dividing FFM (kg) by height squared (m^2).

Statistical analysis

Clinical characteristics are expressed as median and interquartile range [IQR]. Differences in clinical characteristics were determined by independent Student's t-test or Mann–Whitney U test for non-parametric parameters. Correlations were determined by Pearson's correlation coefficient, or Spearman for non-parametric parameters. Related Samples Wilcoxon signed-rank test was used to compare median FM% by ADP and DXA. Bland–Altman analysis was used for the level of agreement between PEA POD and DXA measurements. SPSS statistical package version 24 (SPSS Inc. Chicago, Illinois) was used. P values <0.05 were considered statistically significant. For the creation of sex-specific curves for FM%, FM, FMI, FFM, and FFMI, generalized additive models for location, scale, and shape (28) were used. To fit the parameters of kurtosis, a four parameter (μ , σ , v , and τ) Box–Cox power exponential distribution was applied to construct the final curves (29). The distribution expresses the mean (μ), variance (σ), skewness (v), and kurtosis (τ) that change as a function of age.

RESULTS

Clinical characteristics of the subjects are presented in Table 1. Fifty-four percent of the infants was male and 69.2% had Caucasian ethnicity. Median [IQR] birthweight was 3.37 [3.06–3.71] kg at a gestational age of 39.9 [38.9–40.7] weeks in the total group, and 3.46 [3.13–3.77] kg at 39.7 [38.9–40.6] weeks in boys and 3.29 [2.97–3.65] kg at 39.9 [39.0–40.7] weeks in girls.

ADP versus DXA with and without cushion at age 6 months in 278 infants

Mean FM% was 24.1 by ADP and 25.0 by DXA with cushion, with a mean difference of 0.9% between both measurements ($p=0.004$; Fig. 1). Bland–Altman analysis did not show a significant correlation ($p=0.321$) for the difference in FM% with the mean in FM% (Fig. 2), indicating that there is no proportional bias. Mean FM% was 24.1 by ADP and 36.6 by DXA without cushion, showing a much higher mean difference of 12.5% between both measurements ($p<0.001$). Bland–Altman analysis showed a significant correlation ($p<0.001$) for the difference in FM% with the mean in FM% (Fig. 2), indicating proportional bias.

DXA with versus without cushion at 6, 9, 12, 18, and 24 months

At age 6 months, measurements of FM% by DXA with and without cushion in 278 infants were significantly different ($p<0.001$), with a much higher mean FM% of +11.6% measured by DXA without cushion compared to DXA with cushion. Bland–Altman analysis showed a significant correlation ($p=0.01$) for the difference in FM% with the mean in FM%, indicating proportional bias. After the age 6 months, it proved extremely difficult to perform reliable DXA measurements in duplicate (with and without cushion) as it was almost impossible to

acquire reliable results of DXA without cushion due to movement artifacts. Movement artifacts were present in ~70% of DXA without cushion compared to ~15% of DXA with cushion at age 9, 12, 18, and 24 months. Mean FM% measured by DXA without a cushion was, in infants with successful scans, 13.3% higher compared to DXA with cushion at age 9 months (n=8), 10.8% at age 12 months (n=5), 14.1% at 18 months (n=3), and 13.9% at age 24 months (n=4). It was not possible to perform Bland–Altman analyses due to the small group of infants with duplicate measurements after the age of 6 months.

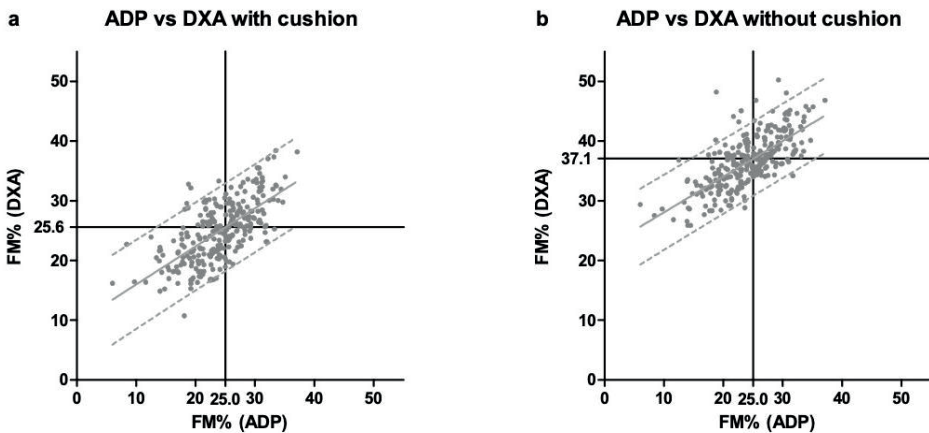


Figure 1. FM% by ADP versus DXA at 6 months of age.

Mean and 95% prediction interval for FM% a) ADP versus DXA with cushion $R=0.659$, $p<0.001$ and b) ADP versus DXA without cushion $R=0.711$, $p<0.001$.

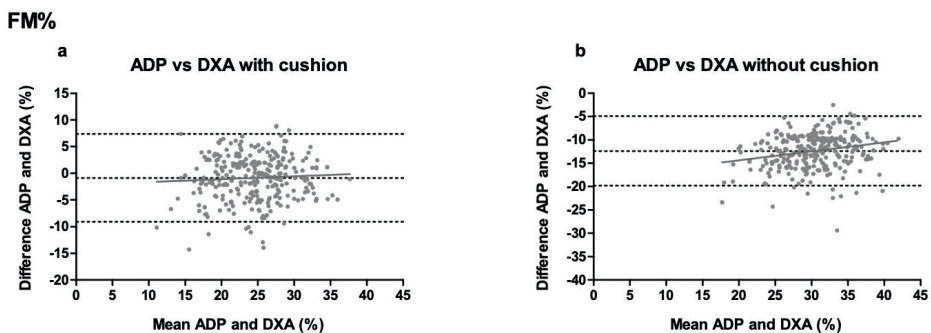
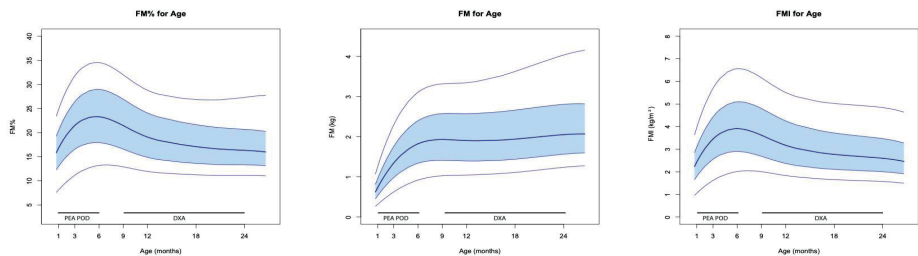


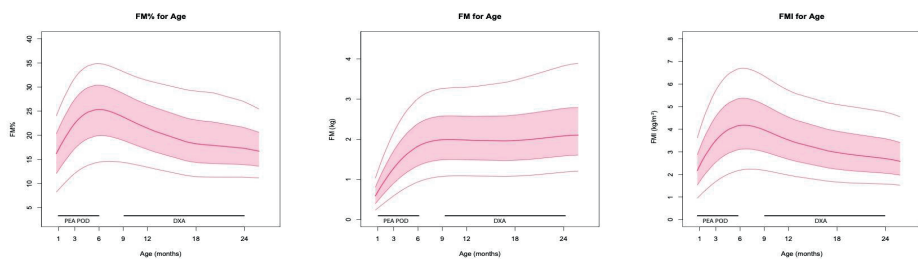
Figure 2. Bland–Altman analyses between ADP and DXA at 6 months of age, $n=278$.

The middle dashed line represents the mean difference between ADP and DXA. The upper and lower dashed lines represent ± 2 SD and the solid line represents the regression line. FM% by a) ADP versus DXA with cushion with $R=0.061$, $p=0.321$, and b) ADP versus DXA without cushion with $R=0.227$, $p<0.001$.

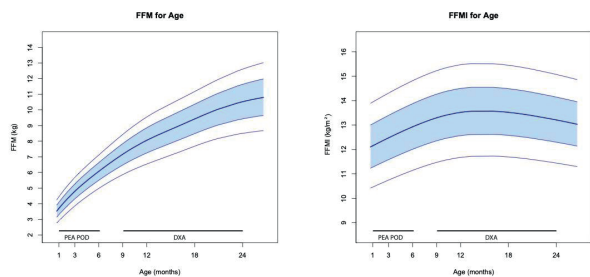
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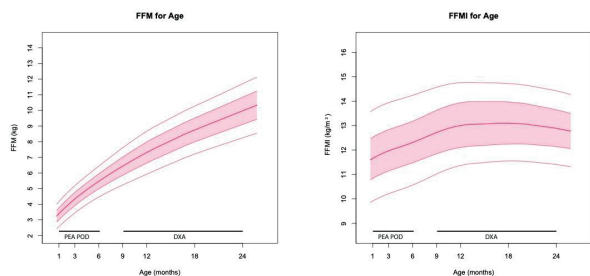


Figure 3. Longitudinal values of FM% (fat mass percentage), FM (fat mass), FMI (fat mass index), FFM (fat-free mass) and FFMi (fat-free mass index) for male and female.

Table 2. Longitudinal values of FM%, FM, FMI, FFM and FFMI.

	Male					Female				
	-2 SD	-1 SD	0 SD	+1 SD	+2 SD	-2 SD	-1 SD	0 SD	+1 SD	+2 SD
FM%										
1 month	8.1	13.0	16.8	20.4	24.8	8.7	12.8	17.1	21.3	25.2
3 months	11.2	16.5	21.5	26.4	31.7	12.1	17.2	22.5	27.4	31.8
6 months	13.2	17.9	23.3	29.0	34.5	14.4	19.9	25.4	30.4	34.9
9 months	12.9	16.6	21.5	26.9	32.0	14.4	18.9	23.8	28.7	33.2
12 months	12.0	14.9	19.1	24.1	28.8	13.4	17.1	21.5	26.3	31.4
18 months	11.3	13.6	17.1	21.7	26.9	11.4	14.4	18.2	23.2	29.2
24 months	11.1	13.3	16.3	20.7	27.3	11.3	13.9	17.3	21.6	26.9
FM (kg)										
1 month	0.32	0.53	0.73	0.95	1.25	0.28	0.47	0.69	0.94	1.20
3 months	0.63	0.98	1.35	1.76	2.30	0.59	0.91	1.28	1.71	2.18
6 months	0.92	1.34	1.82	2.40	3.10	0.94	1.34	1.83	2.39	3.01
9 months	1.03	1.41	1.93	2.57	3.31	1.08	1.49	1.99	2.58	3.26
12 months	1.04	1.39	1.90	2.57	3.34	1.09	1.49	1.98	2.57	3.30
18 months	1.11	1.44	1.94	2.66	3.63	1.08	1.48	1.97	2.60	3.47
24 months	1.24	1.56	2.05	2.81	4.03	1.18	1.58	2.08	2.77	3.83
FMI (kg/m²)										
1 month	1.06	1.79	2.43	3.10	3.96	1.05	1.68	2.38	3.13	3.94
3 months	1.61	2.55	3.45	4.43	5.69	1.68	2.54	3.50	4.56	5.71
6 months	2.01	2.90	3.91	5.10	6.56	2.19	3.11	4.17	5.36	6.69
9 months	2.01	2.69	3.62	4.80	6.16	2.18	3.00	3.97	5.08	6.35
12 months	1.84	2.37	3.17	4.25	5.51	1.97	2.67	3.52	4.54	5.72
18 months	1.65	2.11	2.77	3.72	5.03	1.66	2.23	2.97	3.91	5.09
24 months	1.57	2.00	2.60	3.47	4.84	1.57	2.05	2.70	3.58	4.76
FFM (kg)										
1 month	2.91	3.30	3.70	4.10	4.45	2.57	3.01	3.39	3.77	4.17
3 months	3.86	4.31	4.80	5.27	5.69	3.45	3.92	4.34	4.76	5.19
6 months	4.98	5.50	6.07	6.63	7.12	4.47	4.99	5.47	5.95	6.44
9 months	5.86	6.48	7.17	7.84	8.43	5.23	5.86	6.44	7.02	7.62
12 months	6.54	7.25	8.06	8.86	9.55	5.92	6.63	7.31	7.98	8.67
18 months	7.68	8.48	9.42	10.36	11.18	7.21	7.99	8.75	9.51	10.27
24 months	8.50	9.43	10.52	11.64	12.61	8.25	9.10	9.97	10.84	11.70
FFMI (kg/m²)										
1 month	10.46	11.28	12.15	13.05	13.94	9.91	10.83	11.65	12.52	13.63
3 months	10.75	11.59	12.48	13.40	14.31	10.21	11.14	11.97	12.83	13.94
6 months	11.15	12.01	12.93	13.88	14.82	10.57	11.48	12.31	13.18	14.24
9 months	11.47	12.35	13.29	14.26	15.22	11.02	11.84	12.72	13.62	14.57
12 months	11.67	12.57	13.52	14.50	15.47	11.37	12.10	13.00	13.93	14.76
18 months	11.69	12.58	13.52	14.49	15.45	11.55	12.24	13.09	13.96	14.72
24 months	11.44	12.30	13.21	14.14	15.07	11.41	12.14	12.89	13.65	14.42

Data expressed as median, ± 1 and ± 2 SD scores for male and female for FM% (fat mass percentage), FM (fat mass), FMI (fat mass index), FFM (fat-free mass) and FFMI (fat-free mass index), obtained by ADP (1, 3 and 6 months) and DXA with vacuum cushion (9, 12, 18 and 24 months). Comparison of triplicate measurements (ADP and DXA with versus without vacuum cushion) at age 6 months is described in the results section.

Longitudinal body composition measurements

Since FM% measured by DXA with cushion was comparable to FM% by ADP, all infants were measured by DXA with cushion from age 6 to 24 months. Table 2 presents the longitudinal values for FM and FFM as median ± 1 and ± 2 SD scores for boys and girls from age 1 to 24 months. Figure 3 shows the sex-specific longitudinal values for FM%, FM, FMI, FFM, and FFMI. Girls had a higher median FM% at age 6, 9, 12, and 18 months (p -values <0.001 , <0.001 , <0.001 and 0.009 , respectively) and lower median FFM ($p < 0.001$, all time points) than boys. In the total group, FM% was highest at age 6 months, with highest increment between 1 and 3 months of age. After 6 months, FM% decreased gradually until 24 months (Table 2, Fig. 3). We found a significant increase in FM% between 1 and 3 months ($p < 0.001$), and 3 and 6 months ($p < 0.001$), which were similar in boys and girls. Between 6 and 24 months, there was a significant decrease in FM% ($p < 0.001$) in boys and girls alike.

DISCUSSION

To our knowledge, this is the first study to describe the use of a vacuum cushion in infants to prevent movement during DXA scan, showing reliable results for FM% comparable to FM% measured by ADP. This allowed us to construct longitudinal charts on body composition in infants from 1 until 24 months of age, which addresses an important topic and gap in research due to limited measurement options. We found a significant increase in FM% in the first 6 months after birth, followed by a gradual decline until 24 months, in both sexes.

Longitudinal data on body composition in infants are essential for clinical and research use, but very difficult to construct as ADP is available from birth until the upper limit of 8 kg (PEA POD) and from ~2–3 years of age onward (BOD POD, pediatric option) (17, 30). DXA reference data are only available from 4 years onward [21]. DXA scans can be performed in infants and have good reproducibility for total FM (intraclass correlation coefficient (ICC) 0.94 (0.89–0.97)) between DXA measurements in infants with successful scans (18), but reliable results are extremely difficult to obtain in infancy with a very high proportion of unusable scans due to movement artifacts (19, 24).

Since ADP and DXA measure body composition with a different technique, it is important to investigate whether body composition results of these two techniques are comparable at the transition point of 6 months. Previously, this comparison between ADP and DXA was determined in small groups and results were contradictory (23, 25). Fields et al. (23) showed that body composition measurements by DXA and ADP were highly correlated in 84 infants at age 6 months, but DXA measurements showed significantly higher FM%, which we also found when FM% was measured by DXA without cushion. However, the opposite was found

in a South African study in 92 infants at 2 weeks of age, with DXA measurements having lower FM% compared to ADP (25). Both studies measured FM% by DXA without vacuum cushion.

From age 6 months to 2 years, most children do not want to be held in place and it is impossible to swaddle them only in a cotton blanket and then obtain a reliable DXA scan without movement artifacts. The use of a vacuum cushion prevents infants from movement. Our study shows that at the age of 6 months, FM% measured by DXA with a vacuum cushion provides comparable FM% results as FM% measured by ADP, in contrast to DXA without cushion. Unfortunately, we were only able to determine the difference in FM measured by DXA with versus without vacuum cushion at ages after 6 months in a small group of infants, because of a high percentage (70%) of unsuccessful DXA scans without cushion due to movements artifacts, which is a limitation of the study. However, this indicates the difficulties in obtaining reliable DXA scans without cushion, as was previously described in other studies, where only 9 out of 578 newborns had reliable duplicate measurements without cushion (24) and also high percentages of unsuccessful scans without cushion were reported (19, 24).

With increasing strength when infants become older, it is even more difficult at later ages. We found that measurements with cushion are more successful (85%) than those without (30%) and were able to determine the correction factors between DXA measurements with and without cushion at age 9, 12, 18, and 24 months with the same DXA machine, software, and cushion, which need to be interpreted with caution. It is important to investigate if this factor remains stable in a larger group and is similar if other DXA machines, software, and cushions are used.

Strengths of this study are the large number of boys and girls with detailed body composition measurements and duplicate measurements at the transition point at 6 months in 278 infants, given the lack of a true gold standard suitable for longitudinal studies in young infants. Furthermore, a consistent approach was used by measuring body composition with the same DXA machine, software, and cushion in all infants.

We did not start the study directly after birth, but at age 1 month, because the neonates had to visit the hospital for the body composition measurements. This was too much of a burden for the infants and mothers in the first days after birth.

Our longitudinal data show a significant increase in FM% during the first 6 months and a gradual decrease in FM% from the age of 6 to 24 months. Similar to our findings, some studies reported a longitudinal increase in FM% during the first 6 months of life (31-34). The

decline in FM% after 6 months of age might, at least partially, be explained by the increase in physical activity from ~6 months onward when infants start to roll over, crawl, and walk. We also found that girls had higher FM and lower FFM than boys, as found previously at birth and until 6 months (35-37).

In conclusion, the use of a vacuum cushion to prevent movements during DXA scan in infants, provides reliable measurements of body composition and results are comparable to ADP measurements. This allows longitudinal measurements of body composition in infants until 2 years of age, which addresses a gap in research. We found that FM% increases during the first 6 months and gradually declines until 24 months.

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Chapter 3

Association between fat mass in early life and later fat
mass trajectories

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ABSTRACT

Importance A rapid increase in weight in early life is associated with an increased risk for adiposity and cardiovascular diseases at age 21 years and beyond. However, data on associations of early change in measured fat mass percentage (FM%) with adiposity development are lacking.

Objective To investigate whether a rapid increase in FM% in the first months of life is associated with higher trajectories of body fat mass during the first 2 years of life.

Design, setting, and participants A birth cohort consisting of 401 healthy, term-born infants of the Sophia Pluto Cohort Study was analyzed. Participants were born between January 7, 2013, and October 13, 2017. Data were analyzed from February 1, 2020, to May 20, 2020.

Interventions Longitudinal measurements of FM% by air-displacement plethysmography and dual-energy x-ray absorptiometry, and abdominal subcutaneous and visceral fat mass (FM) by ultrasonography in infants at ages 1, 3, 6, 9, 12, 18, and 24 months. A rapid increase in FM% was defined as a change in FM% of greater than 0.67 standard deviation scores (SDS).

Main outcomes and measures Associations between change in FM% SDS in the first and second 6-month period of life with body composition at age 2 years and whether a rapid increase in FM% SDS during the first 6 months leads to higher body FM and abdominal FM trajectories during the first 2 years of life.

Results Of the 401 participants, 228 infants (57%) were male. Change in FM% SDS from age 1 to 6 months was positively associated with FM% (β , 0.044; 95%CI, 0.017-0.068), FMI (β , 0.061; 95%CI, 0.032-0.091), and abdominal subcutaneous FM (β , 0.064; 95%CI, 0.036-0.092) at age 2 years, but not with visceral FM. In contrast, no associations were found within the 6- to 12-month period. Infants with a rapid increase in FM% of greater than 0.67 SDS in the first 6 months of life had higher trajectories of FM%, FM index, and subcutaneous FM during the first 2 years of life (all $p=0.001$), but visceral FM index was not significantly different compared with infants without a rapid increase ($p=0.12$).

Conclusions and relevance In this study, only the change in FM% in the first 6 months of life was associated with more adiposity at age 2 years. Infants with a rapid increase in FM% had higher trajectories of FM% and FM index during the first 2 years of life. These findings appear to support a critical window for adiposity programming in early life.

INTRODUCTION

The first 1000 days of life, from conception until age 2 years, are important for the development of body and brain (1). A rapid increase in this period, in literature defined as a change in weight-for-age standard deviation score (SDS) greater than 0.67 between 2 times (2, 3) has been associated with an increased risk of overweight and adiposity (2, 4-7) unfavorable cardiovascular and metabolic health profiles in early adulthood (8-11) and cardiovascular diseases in later life (12, 13).

We noted in the Programming Factors for Growth and Metabolism (PROGRAM) study that a rapid increase in weight-for-age greater than 0.67 SDS during the first year of life appeared to be positively associated with waist circumference, acute insulin response, total cholesterol to high-density lipoprotein cholesterol ratio, triglyceride levels, and a higher fat mass percentage (FM%), more central obesity, and reduced insulin sensitivity at age 21 years, and was inversely associated with insulin sensitivity and serum high-density lipoprotein levels (8). First-year weight gain was postulated to be important for adiposity programming (8, 14). Most studies, however, have used weight SDS, height SDS, body mass index (BMI) SDS, or skinfolds as a proxy of body composition (3, 15-17). Based on the results of the PROGRAM study, we conducted an observational cohort study to investigate longitudinally measured body composition in term-born infants.

To our knowledge, this is the first study to evaluate the associations of change in measured FM% SDS, instead of weight-for-age SDS, in early life with longitudinal body composition by detailed fat mass (FM) measurements in healthy infants during the first 2 years of life. As increased abdominal visceral FM has been specifically associated with an unfavorable metabolic health profile during childhood and later on (18, 19), we also measured abdominal subcutaneous and visceral FM thickness by noninvasive ultrasonography (20-22).

In this study, we investigated the time within postnatal months when a change in FM% is associated with FM% at age 2 years. Subsequently, we assessed whether a rapid increase in FM% during that period would associate with higher trajectories of body FM and abdominal FM during the first 2 years of life. We hypothesized that infants with a rapid increase in FM% SDS would have more body FM and visceral FM at age 2 years.

METHODS

Participants

The study population consisted of healthy, term-born infants participating in the Sophia Pluto Study, a birth cohort study in the Rotterdam, the Netherlands, area. Ninety-eight percent of the births were singleton. Between January 7, 2013, and October 13, 2017, infants were recruited from obstetric departments of regional hospitals and primary health care centers to obtain detailed data on body composition and growth during early life. The Sophia Pluto Study obtained approval from the medical ethics committee of Erasmus University Medical Center, and parents gave written informed consent. Participants did not receive financial compensation. Data were analyzed from February 1, 2020, to May 20, 2020. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for cohort studies.

All participants fulfilled the following inclusion criteria: term born (> 37 weeks of gestation), age less than 28 days, and uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score <3 after 5 minutes), sepsis, or long-term complications of respiratory ventilation. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy, or a significant maternal medical condition that could interfere with the study results.

Data Collection and Measures

Outpatient clinic visits were scheduled at ages 1, 3, 6, 9, 12, 18, and 24 months (Table 1). Data on pregnancy and birth were obtained from records and measurements were performed by trained staff.

Weight was measured with an electronic infant scale to the nearest 5 g (Seca 717, Seca). Length was measured twice by 2-person technique with an infantometer to the nearest 0.1 cm (Seca 416) and head circumference was measured twice as the widest frontal-occipital circumference with a measuring tape to the nearest 0.1 cm (Seca 201). Weight-for-length, weight-for-age, and height-for-age SDS were calculated by Growth Analyser (23).

Until age 6 months, body composition was assessed by air-displacement plethysmography (ADP) (Pea Pod, COSMED) as described in detail elsewhere (20). The ADP system was calibrated daily, according to standard protocol (24).

From 6 months onward, a dual-energy x-ray absorptiometry (DEXA) scan was performed in all infants at every visit. All DEXA scans were performed with the same device (Lunar Prodigy, GE Healthcare) and software (enCORE software, version 14.1, enCORE).

At 6 months, median FM% was 24.1 as measured by ADP and 25.0 by DEXA, with a median difference of 0.9% between both measurements. Bland-Altman analysis showed no proportional bias ($p=0.32$) (25). Fat mass index (FMI) was determined by dividing fat mass (kilograms) by height squared (meters squared) and fat-free mass index (FFMI) by dividing fat-free mass (kilograms) by height squared.

Abdominal subcutaneous and visceral fat thickness were measured by ultrasonography at every visit starting from age 3 months, because earlier measurements are unreliable (20, 21). Unsuccessful ultrasonographic measurements of visceral FM, without visualization of the lumbar vertebra, were excluded from analyses.

Infant feeding was classified as exclusively breastfed if an infant received breastfeeding for at least 3 months and nonexclusively breastfed if an infant received either formula feeding or a mixture of breastfeeding and formula feeding. Information on the timing of solid food introduction was obtained from questionnaires.

Statistical Analysis

The total population consisted of 401 infants with 5 or more measurements of body composition during the first 2 years of life. Clinical characteristics were expressed as pooled means and SDs. Differences in clinical characteristics were determined by independent *t* test or Mann-Whitney test for nonparametric parameters.

Missing data, mainly for infants who had not yet reached age 2 years or showed resistance at measurements, were imputed using a multiple imputation approach in SPSS, version 25 (SPSS Inc) to generate 20 imputed data sets. We performed multiple linear regression analyses to investigate the associations of change in FM% standard deviation scores (SDS) during the first year of life with body composition at age 2 years, with adjustments for sex, gestational age, age, and gain in length in the same period. Although small differences in some effect estimates were observed between analyses with imputed missing data and complete cases only, the main conclusions of the results were similar. For determining rapid increase in FM% SDS of greater than 0.67, we could only include infants with measured FM% at 1 and 6 months. As previously reported, an increase greater than 0.67 SDS represents the width of a percentile band on standard growth charts and is used to define a rapid increase in weight (8). We used the same definition for a rapid increase in FM% SDS. As there are no published references for FM% from birth until age 2 years using ADP and DEXA, we calculated SDS for FM% in our large cohort (25). The study group was divided in 2 subgroups: one with a rapid increase in FM% SDS from age 1 to 6 months and one without (Figure 1). Longitudinal growth and body composition development in infants with and without a rapid increase in FM% SDS were analyzed using linear mixed model analysis, with adjustment for

sex. Time was modeled by entering hospital visits at ages 1, 3, 6, 9, 12, 18, and 24 months into the linear mixed models. For comparison with our PROGRAM study and literature data, we also analyzed data on changes in weight, length, and weight-for-length SDS.

For the analyses of abdominal FM, we used ultrasonographic measurements of abdominal FM at 4 or more times during the first 2 years of life. A χ^2 test was performed to determine whether the percentage of breastfeeding differed between infants with and without a rapid increase in FM% SDS. SPSS statistical package version was used for analysis. P-values <0.05 were considered statistically significant.

RESULTS

Clinical characteristics are presented in Table 1. Of the total group (n=401), 228 infants (56.9%) were male and 173 infants (43.1%) were female. Median (interquartile range [IQR]) birth weight was 3.38 (3.05-3.74) kg at a gestational age of 39.9 (39.0-40.7) weeks in the total group, 3.44 (3.12-3.77) kg at a gestational age of 39.8 (38.9-40.6) weeks in boys, and 3.33 (2.98-3.71) kg at a gestational age of 40.0 (39.0-40.9) weeks in girls.

In the total group of 401 infants, we divided the first year of life into 6-month periods. Table 2 presents associations between change in FM% SDS and outcomes at age 2 years. Change in FM% SDS during the first 6 months of life was positively associated with FM% (β , 0.044; 95% CI, 0.017-0.068), FMI (β , 0.061; 95%CI, 0.032-0.091), and abdominal subcutaneous FM (β , 0.064; 95% CI, 0.036-0.092) at age 2 years, but not with visceral FM. No associations were found for the following 6- to 12-month period (i.e., after the first 6 months of life).

When subdivided in 3-month periods, change in FM% SDS in the 3- to 6-month period was positively associated with FM% (β , 0.065; 95%CI, 0.029-0.101), FMI (β , 0.084; 95%CI, 0.041-0.127), and abdominal subcutaneous FM (β , 0.090; 95% CI, 0.046-0.133) at age 2 years, but not with visceral FM. Change in FM% SDS from 1 to 3 months was associated only with subcutaneous FM (β , 0.043; 95%CI, 0.008-0.078). We found no associations for the other 3-month periods.

For comparison with literature data, we also analyzed the associations between change in weight-for-length SDS, which is often used as a proxy for body composition, with body composition at age 2 years. Similar to the change in FM%, changes in weight-for-length SDS from 1 to 6 months were associated with FM% (β ,0.057; 95%CI, 0.026-0.087),FMI (β ,0.092; 95% CI, 0.056-0.128), and abdominal subcutaneous FM (β , 0.091; 95%CI, 0.058-0.124), but not with visceral FM.

Of the total group, 87 infants (26.1%) had a rapid increase in measured FM% (>0.67 SDS) during the first 6 months of life (Figure 1). Birthweight SDS, corrected for gestational age and sex, was significantly lower in infants with a rapid increase in FM% SDS compared with those without a rapid increase (-0.47 vs -0.10 SDS, $p=0.004$), albeit 95.4% had a birth weight well within the reference range. Gestational age did not differ significantly (39.6 vs 39.8 weeks, $p=0.09$).

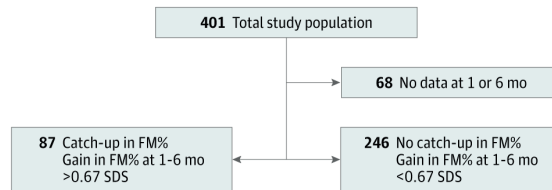


Figure 1. Study flowchart.

FM%: fat mass percentage, SDS: standard deviation score.

Table 1. Clinical characteristics.

Age	1 month	3 months	6 months	9 months	12 months	18 months	24 months
N [Male]	401 [228]	401 [228]	401 [228]	401 [228]	401 [228]	401 [228]	401 [228]
Weight (kg)	M 4.37 (0.53)	6.20 (0.67)	7.92 (0.85)	9.14 (0.99)	10.01 (1.12)	11.46 (1.26)	12.76 (1.45)
	F 4.10 (0.60)	5.69 (0.70)	7.35 (0.79)	8.49 (0.87)	9.37 (0.95)	10.86 (1.04)	12.17 (1.17)
Length (cm)	M 55.0 (2.20)	62.0 (2.07)	68.7 (2.18)	73.3 (2.36)	77.0 (2.62)	83.6 (2.96)	89.3 (3.30)
	F 53.9 (2.32)	60.3 (2.20)	66.8 (2.08)	71.1 (2.24)	75.0 (2.39)	81.8 (2.62)	87.9 (2.93)
FM (%)	M 16.1 (4.34)	22.1 (4.80)	23.0 (5.15)	20.9 (5.35)	19.7 (5.00)	17.6 (4.81)	17.4 (4.74)
	F 16.6 (4.57)	23.0 (5.08)	25.2 (5.35)	23.7 (4.90)	21.5 (4.88)	18.4 (5.33)	17.5 (4.97)
FFM (kg)	M 3.66 (0.41)	4.82 (0.48)	6.07 (0.56)	7.20 (0.69)	8.02 (0.83)	9.41 (0.89)	10.50 (1.07)
	F 3.41 (0.42)	4.37 (0.44)	5.49 (0.51)	6.47 (0.64)	7.33 (0.69)	8.83 (0.80)	10.01 (0.89)
Abdominal subcutaneous FM (cm)	M NA	0.41 (0.11)	0.41 (0.11)	0.37 (0.10)	0.34 (0.10)	0.32 (0.10)	0.32 (0.10)
	F NA	0.40 (0.12)	0.41 (0.12)	0.38 (0.10)	0.34 (0.09)	0.31 (0.09)	0.32 (0.09)
Visceral FM (cm)	M NA	2.46 (0.63)	2.33 (0.64)	2.46 (0.67)	2.46 (0.67)	2.32 (0.65)	2.15 (0.58)
	F NA	2.34 (0.59)	2.28 (0.58)	2.46 (0.70)	2.44 (0.64)	2.29 (0.61)	2.15 (0.57)

Data expressed as pooled means (pooled SD) for male (M) and female (F).

Abbreviations: FM, fat mass. FFM, fat-free mass. NA, not applicable.

The FM% and FMI trajectories during the first 2 years of life were higher in infants with a rapid increase in FM% SDS compared with those without a rapid increase ($p \leq 0.001$), resulting in a higher FM% and FMI at age 2 years (both $p \leq 0.001$) (Figure 2). The FFM trajectories during the first 2 years of life were not significantly different between both groups ($p=0.16$), but when corrected for length, FFM trajectories were higher in infants with a rapid increase in FM% SDS ($p=0.007$). Length-for-age SDS trajectories during the first 2 years of life were significantly lower in infants with a rapid increase in FM% SDS ($p=0.006$).

Table 2. Regression coefficients for change in FM% SDS during the first year of life and body composition at age 2 years.

Outcome at age 2 years	Change in FM% SDS 1-6 months	Change in FM% SDS 6-12 months	Change in FM% SDS 1-3 months	Change in FM% SDS 3-6 months	Change in FM% SDS 6-9 months	Change in FM% SDS 9-12 months
	β [95%CI]	p-value	β [95%CI]	p-value	β [95%CI]	p-value
FM (%)	0.044 [0.017 - 0.068]	0.001	0.019 [-0.014 - 0.052]	0.26 [0.029 - 0.101]	0.011 [-0.019 - 0.041]	-0.004 [-0.038 - 0.031]
FM index (kg/m ²)	0.061 [0.032 - 0.091]	<0.001	0.033 [-0.006 - 0.071]	0.084 [0.041 - 0.127]	0.016 [-0.020 - 0.051]	-0.005 [-0.045 - 0.035]
Abdominal subcutaneous FM (cm)	0.063 [0.036 - 0.091]	<0.001	-0.024 [-0.056 - 0.008]	0.084 [0.041 - 0.126]	-0.029 [-0.061 - 0.003]	0.003 [-0.032 - 0.038]
Abdominal visceral FM (cm)	0.016 [-0.011 - 0.043]	0.25	0.003 [-0.030 - 0.037]	0.021 [-0.018 - 0.060]	-0.006 [-0.038 - 0.026]	0.028 [-0.007 - 0.063]

Abbreviation: CI, confidence interval. FM%, fat mass percentage. SDS, standard deviation score. All associations were based on log-transformed outcomes, adjusted for sex, gestational age, age and gain in length in the same period. A positive β score means that when 1 SD in FM% SDS is gained from 1 to 6 months, for example, FM% increases with 4.4% at 2 years.

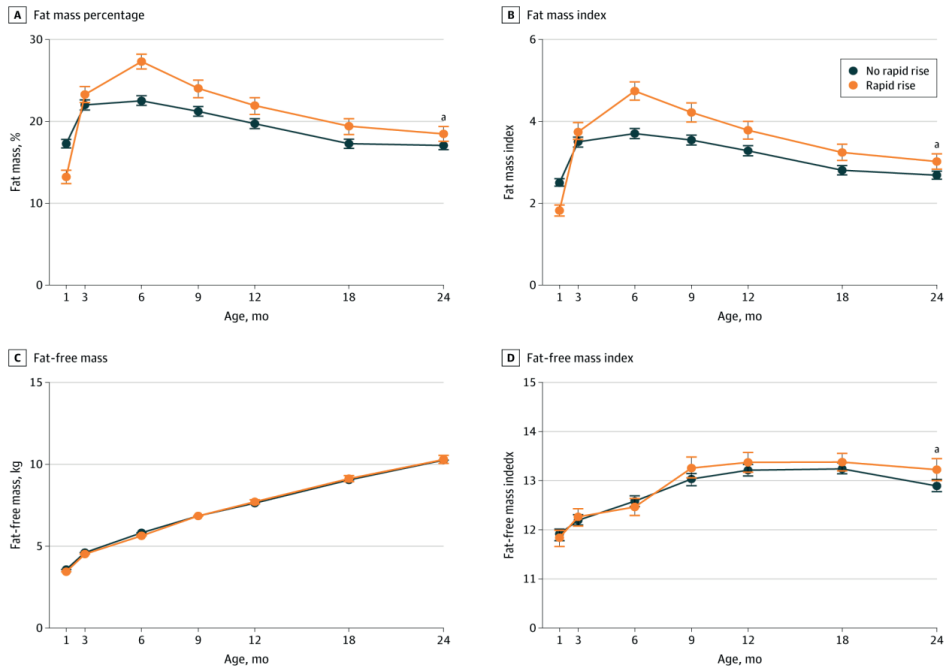


Figure 2. Trajectories during the first 2 years of life in infants with a rapid increase in fat mass percentage standard deviation score versus no rapid increase.

Values are estimated marginal means (lower-upper bound), corrected for sex.

Abdominal subcutaneous FM trajectories were higher in infants with a rapid increase in FM% SDS compared with those without a rapid increase ($p < 0.001$), but visceral FM trajectories were not significantly different from age 3 months until 2 years ($p = 0.12$) (Figure 3). At age 2 years, abdominal subcutaneous FM was higher in infants with a rapid increase in FM% SDS compared with infants without a rapid increase ($p < 0.001$), but visceral FM was similar ($p = 0.85$).

In addition, we analyzed the anthropometric data, which are often used as a proxy for body composition. At age 2 years, weight-for-length SDS and weight-for-age SDS were higher in infants with a rapid increase in FM% SDS (weight-for-length: 0.16 vs -0.69 , $p < 0.001$ and weight-for-age: 0.05 vs -0.39 , $p = 0.017$). Length-for-age SDS was not different (0.33 vs 0.36, $p = 0.21$).

Pre-pregnancy BMI of the mothers and maternal weight gain during pregnancy were not significantly different between infants with and without a rapid increase in FM% SDS (BMI: 23.8 vs 23.4, $p = 0.12$ and weight gain: 13.0 vs 14.0 kg, $p = 0.62$). When stratified by infant sex, however, pre-pregnancy BMI of the mothers was significantly higher in girls with vs without catch-up in FM% SDS (25.8 vs 23.8, $p = 0.04$).

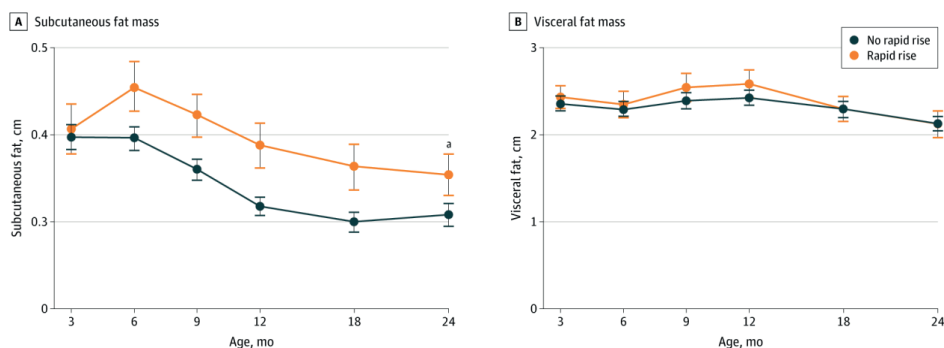


Figure 3. Abdominal subcutaneous and visceral fat mass trajectories during the first 2 years of life in infants with a rapid increase in fat mass percentage standard deviation score versus no rapid increase. Values are estimated marginal means (lower-upper bound), corrected for sex.

The percentage of infants with exclusive breastfeeding vs non-exclusive breastfeeding was not different between groups ($p=0.81$), and the duration of breastfeeding was similar in breastfed infants with and without a rapid increase in FM% SDS (5.91; 95% CI, 3.94-10.18 vs 7.49; 95% CI, 4.96-11.19 months; $p=0.12$). Timing of introduction of solid foods was not significantly different (4.04; 95% CI, 4.01-5.03 vs 4.04; 95% CI, 3.98-4.96 months; $p=0.26$).

DISCUSSION

To our knowledge, this is the first study to note that not only the change in weight-for-age or weight-for-length SDS, but specifically, the change in FM% SDS in the first 6 months of life vs the subsequent months is associated with FM% and FMI at age 2 years in healthy infants. In addition, a rapid increase in the FM% SDS resulted in higher trajectories of longitudinal FM% and FMI during the first 2 years of life. Our findings, therefore, appear to support a critical window for adiposity programming.

Our findings are in line with the PROGRAM study data, in which participants with a rapid increase in weight in early life had a significantly higher FM% and unfavorable metabolic and cardiovascular profiles at age 21 years (8, 26, 27). Also, other studies suggested that first-year rapid increase in weight of greater than 0.67 SDS resulted in a higher BMI SDS at age 2 years (28), while a rapid increase in weight during the first 2 years of life resulted in a higher BMI SDS and FM% at ages 6 and 7 years (15, 29). In these studies, however, FM% was calculated by equations from skinfolds, because detailed body composition was not measured.

Our findings are in line with those of 2 studies suggesting that FM accretion until 6 months measured by ADP correlated with higher FMI at 4 years (30) and FM accretion until 8 months measured by bioelectrical conductivity with overweight/obesity at 6 to 11 years (31). We can now present apparent trajectories of measured body composition based on longitudinal data in infants with and without a rapid increase in FM% SDS.

We found higher trajectories of abdominal subcutaneous FM in infants with a rapid increase in FM% SDS compared with those without. Other studies noted that children with a rapid increase in weight during the first 2 years of life had more FM and more central fat distribution based on skinfolds and waist circumference at age 5 years (3, 7), but abdominal subcutaneous and visceral FM were not measured in early life.

In contrast to our hypothesis, we found no difference in visceral FM until age 2 years between infants with and without a rapid increase in FM% SDS. This finding is in line with literature data describing that total FM in young children is associated with abdominal subcutaneous FM rather than visceral FM (32, 33).

Mice studies reported that most adipocytes arise from proliferating progenitors, which are committed in the prenatal or early postnatal period (34). After birth, adipose tissue grows mainly by an increase in the number of small adipocytes, which is set during childhood and adolescence (35). When a certain number of adipocytes is reached, the number cannot be decreased by a reduction in body weight (35).

We showed that a rapid increase in FM% SDS results in more adiposity at age 2 years. This increase might stimulate the proliferation of the progenitor cells in early life and thus increase the number of adipocytes in infants with a rapid increase in FM% SDS. Because we measured visceral FM thickness, we could not distinguish between the number or size of adipocytes. An alternative explanation could be that visceral adipocytes have a different time and rate of development than subcutaneous adipocytes. This difference could explain why the variability in the first 2 years of life is found in subcutaneous FM rather than in visceral FM. Further studies are required to investigate whether visceral fat cells increase in size at a later age and whether children with a rapid increase in FM% SDS in early life develop more visceral fat at an older age than those without the rapid increase.

Furthermore, we noted that FFM trajectories were similar in infants with and without a rapid increase in FM% SDS; however, when corrected for length, the FFMI trajectories were higher in infants with a rapid increase in FM% SDS, suggesting that these infants gained more muscle mass. Higher trajectories of FFMI in infants with a rapid increase in FM% SDS are likely due to lower length trajectories in these infants.

Infants with a rapid increase in FM% SDS had a lower birth weight SDS compared with infants without a rapid increase, but most of them (95.4%) had a birth weight well within the reference range of -2 to $+2$ SDS. Differences in birth weight might contribute to neonatal body composition (36), but other studies suggested that associations between a rapid increase in weight and obesity were independent of birth weight (15, 29, 37). Our findings thus suggest that a rapid increase in FM% SDS is relatively common in healthy, term-born infants with a normal birth weight SDS. No significant differences were found for mother's pre-pregnancy BMI and weight gain during pregnancy in the total group.

Notably, the percentage of infants with exclusive breastfeeding until age 3 months was not substantially different between infants with and without a rapid increase in FM% SDS. This nonsignificant finding might be explained by the fact that the median duration exceeded 3 months in both groups. It has been reported that exclusive breastfeeding of less than 3 months may be associated with rapid weight gain, leading to higher BMI and FM development in later life (11); thus, it remains important to support exclusive breastfeeding for at least 3 months.

We found no difference in the age at introduction of solid foods between both groups, which is in line with previous studies reporting no associations between age at solid food introduction and later body composition (38, 39).

Limitations

This study has limitations. Detailed eating patterns after introduction of solid food have not been investigated, which is a limitation of the study, as this might also influence body composition trajectories in early life. In addition, we did not start the study directly after birth, but at age 1 month. Recruited neonates had to visit the hospital for measurements, which was too much of a burden for both infants and mothers in the first days after birth.

Conclusions

It has been reported that a rapid increase in weight in early life associates with more adiposity and a less favorable health profile at age 21 years and beyond. The findings of this study suggest that a rapid increase in FM% SDS in the first 6 months of life leads to more adiposity at 2 years. These findings apparently support a critical window for adiposity programming in the first 6 months of life.

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Chapter 4

Longitudinal human milk macronutrients, body composition and infant appetite during early life

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ABSTRACT

Background & aims Breastfeeding is the gold standard infant feeding. Data on macronutrients in relation to longitudinal body composition and appetite are very scarce. The aim of this study was to investigate longitudinal human milk macronutrients at 1 and 3 months in association with body composition and appetite during early life in healthy, term-born infants. We hypothesized that infants receiving higher caloric human milk would have more body fat mass and satiate earlier.

Methods In 133 exclusively breastfed infants (Sophia Pluto Cohort), human milk samples at 1 and 3 months were analyzed for macronutrients (fat, protein, carbohydrate) by MIRIS Human Milk Analyzer, with appetite assessment by Baby Eating Behavior Questionnaires. Fat mass (FM) and fat-free mass (FFM) were measured by PEA POD and DXA, and abdominal FM by ultrasound.

Results Milk samples showed large differences in macronutrients, particularly in fat content. Protein and energy content decreased significantly from 1 to 3 months. Fat and carbohydrate content tended to decrease ($p=0.066$ and 0.081). Fat (g/100 ml) and energy (kcal/100 ml) content at 3 months were associated with FM% at 6 months (β 0.387 and 0.040, resp.) and gain in FM% from 1 to 6 months (β 0.088 and 0.009, resp.), but not with FM% at 2 years. Carbohydrate content at 3 months tended to associate with visceral FM at 2 years (β 0.290, $p=0.06$). Infants receiving higher caloric milk were earlier satiated and finished feeding faster.

Conclusions Our longitudinal data show decreasing milk protein and energy content from age 1 to 3 months, while fat and carbohydrate tended to decrease. Macronutrient composition, particularly fat content, differed considerably between mothers. Milk fat and energy content at 3 months associated with gain in FM% from age 1 to 6 months, indicating that higher fat and energy content associate with higher gain in FM% during the critical window for adiposity programming. As infants receiving higher caloric breastfeeding were earlier satiated, this self-regulatory mechanism might prevent intake of excessive macronutrients.

INTRODUCTION

Breastfeeding is considered to be the gold standard infant feeding, because it can result in health benefits for mother and child (1). Breastfeeding lowers the risk of adiposity during childhood (2-6). Breastfeeding is also a protective factor against several infections (1), asthma development (7), eczema and allergic rhinitis (8) by supporting the development of the immune system and microbiota (9). For mothers, breastfeeding lowers the risk of breast cancer (1).

Human milk is a dynamic fluid, with changes in composition from early to late lactation (10). It is likely that human milk composition affects infant growth (11). Human milk is composed of macronutrients, micronutrients and bioactive factors (10). Different techniques exist for analyzing human milk macronutrient composition (12). Nowadays, infrared human milk analyzers (HMA), a fast method to estimate macronutrient composition, are being used in clinical settings (13), mainly to determine adequate fortification of donor human milk for feeding preterm infants (14) [14]. In research, some studies have used HMA to assess macronutrient human milk composition in term-born infants (11, 15-17).

The first 1000 days period, from conception until age 2 years, is important for body and brain development, and obesity prevention (18). Studies investigating human milk macronutrients in association with longitudinal body composition (fat mass percentage (FM) and fat-free mass (FFM)), and appetite during this period are very scarce in healthy term-born infants (11). One study stated that human milk protein inversely associated with the rate of body fat gain until age 4 months in 41 infants (19). Another study presented the effect of only carbohydrates on body composition measured by skinfolds and bioelectrical impedance measurements in 20 infants until age 12 months and concluded that human milk carbohydrate concentrations associated with decreased FM% and FMI (20).

Within this 1000 days period, the first 6 postnatal months are considered a critical window for adiposity programming (21-24). Accelerated weight gain during that period associated with more adiposity and a less favorable health profile at 21 years (21). We previously found that not only weight gain, but specifically accelerated gain in FM% in the first 6 months of life associated with higher FM%, already at age 2 years (22). We now present data on human milk macronutrients and their associations with longitudinally measured body composition, abdominal FM and appetite during this critical window for adiposity programming and at age 2 years in healthy term-born infants.

The primary objective of this study was to investigate longitudinal human milk macronutrient composition and the associations with body composition and abdominal fat mass in

infants during the first 6 months of life and at age 2 years. We hypothesized that human milk with higher caloric value would result in infants having more body fat mass and visceral fat mass during early life compared to those receiving low caloric human milk. The secondary objective was to investigate the associations between human milk macronutrient composition and infant appetite.

METHODS

Study settings and subjects

The study population consisted of healthy, term-born infants, participating in the Sophia Pluto Study, a birth cohort study in the Rotterdam area (The Netherlands). Between January 2013 and March 2020, infants were recruited to obtain detailed data on body composition and growth during early life. The Sophia Pluto Study obtained approval by the Medical Ethics Committee of Erasmus University Medical Center and parents gave written informed consent.

All participants fulfilled the following inclusion criteria: term born (37 weeks of gestation), age < 28 days, uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score < 3 after 5 min), sepsis or long-term complication of respiratory ventilation. For this study, infants who were exclusively breastfed during the first 3 months of life, with breastmilk samples of their mothers at age 1 and 3 months, were included. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy or a significant maternal medical condition that could interfere with the study results.

Data collection and measurements

Outpatient clinic visits were scheduled at age 1, 3, 6, 9, 12, 18 and 24 months (Table 1). Data on pregnancy and birth were obtained from medical records and measurements were performed by trained staff. Ethnicity groups were classified as White/Caucasian [1], Black/African [2], Asian [3], Latin-American [4] or other [5].

Anthropometrics

Weight was measured with an electronic infant scale to the nearest 5 g (SECA 717, Hamburg, Germany). Length was measured twice by the two-person technique with an infantometer to the nearest 0.1 cm (SECA 416). Head circumference was measured twice as the widest frontal-occipital circumference with a measuring tape to the nearest 0.1 cm (SECA 201). Weight-for-length, weight-for-age and height-for-age standard deviation scores (SDS) were calculated using Growth Analyser ([https:// growthanalyser.org/](https://growthanalyser.org/); Talma, 2010).

Body composition measurements

Up to and including age 6 months, body composition was assessed by air-displacement plethysmography (ADP by PEA POD, COSMED, Italy) as described in detail elsewhere (25). The PEA POD was calibrated daily, according to standard protocol (26).

From 6 months onwards, a Dual Energy X-ray Absorptiometry (DXA) scan was performed at every visit in all infants, with the use of a vacuum cushion (465 75100, B.u.W Schmidt GmbH, Germany) to reduce movement artifacts (27). All DXA scans were performed with the same device (DXA, Lunar Prodigy, GE Healthcare, UK) and software (enCORE software version 14.1). We previously reported that FM% was measured in triplicate at the transition point of 6 months in 278 infants; by PEA POD and DXA with versus without vacuum cushion. Median FM% was 24.1 by ADP and 25.0 by DXA with vacuum cushion, with a median difference of 0.9% between both measurements and no potential bias (Bland-Altman analysis: $p=0.32$) (27). DXA without vacuum cushion did show potential bias, most likely due to movement artifacts and was thus inaccurate (27).

Fat mass index (FMI) was determined by dividing fat mass (kg) by height squared (m^2).

Abdominal fat mass measurements

Abdominal subcutaneous and visceral fat thickness (cm) were measured by ultrasound at every visit starting from age 3 months, because earlier measurements are unreliable (25, 28). Unsuccessful ultrasound measurements of visceral fat mass, without visualization of the lumbar vertebra, were excluded from analyses.

Breastmilk samples

Mothers were instructed to collect hind milk samples, thus after their infants were breastfed (BF), at infant's age of 1 and 3 months. Samples were frozen at 18 °C at home until study visits at the hospital and thereafter at 80 °C until analysis. Breastmilk concentrations of fat, crude and true protein, carbohydrate and energy were analyzed using a Human Milk Analyzer (HMA, MIRIS, Uppsala, Sweden). Before analysis, samples were warmed to 40 °C and homogenization was obtained by an ultrasonic processor (MIRIS, Uppsala, Sweden). The HMA was cleaned and calibrated according to manufacturer's protocol and samples with protein values <0.5 g/100 ml were classified as 'bad samples'. We used the same MIRIS HMA device for all analyses, which were performed by the same investigator (KF).

Human milk composition was measured in triplo. The intra-assay mean [95% confidence interval] coefficients of variance were 1.1% [1.0-1.2] for fat, 2.9% [2.5-3.2%] for crude protein, 2.9% [2.5-3.3] for true protein, 1.7% [1.1-2.3] for carbohydrate and 1.1% [0.9-1.2] for energy. The samples were divided in groups based on time of collection to investigate circadian

variations: during night/ early morning (00:00-09:00) and morning (09:00-12:00). For the comparison of fasting versus non-fasting human milk samples, we defined samples as fasting samples if mothers collected the milk sample in the morning before their breakfast. For comparison of macronutrient content in breastmilk versus formula feeding, we calculated median macronutrients per 100 ml of 6 common formula feeding brands available in the Netherlands.

Baby Eating Behavior Questionnaires (BEBQ)

At age 1 and 3 months, mothers were asked to fill out the Baby Eating Behavior Questionnaire (BEBQ) to assess infant appetite (29). Each item was answered using a five-point Likert frequency scale (1=never, 2=rarely, 3=sometimes, 4=often and 5=always). To investigate whether exclusively breastfed infants receiving high caloric human milk felt satiated faster and finished feeding earlier, we used items of “satiety responsiveness” (SR, e.g. “my baby gets full up easily”), slowness in drinking tempo called “slowness in eating” (SE, e.g. “my baby finishes feeding quickly”, “my baby takes more than 30 min to finish feeding” and “my baby sucks more and more slowly during the course of a feed”) and “food responsiveness” (FR, e.g. “my baby frequently wants more milk than I provide”, “even when my baby has just eaten well, he/she is happy to feed again if offered” and “my baby is always demanding a feed”).

Statistical analysis

Clinical characteristics are expressed as median (IQR). Differences in clinical characteristics were determined by independent Student t-test or Mann-Whitney U-test for non-parametric parameters. Differences between multiple groups were determined by one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks for non-parametric parameters. Differences between related samples at two time points were determined by Wilcoxon matched-pair signed-rank in infants with 2 milk samples (n=121) and categorical variables were determined by chi-squared test. Correlations were determined by Pearson’s correlation coefficient or Spearman’s correlation coefficient for nonparametric variables.

Missing data on growth and body composition, mainly because of infants had not yet reached age 2 years or showed resistance at measurements, were imputed using a multiple imputation approach in SPSS to generate 20 imputed datasets. Although small differences in some effect estimates were observed between analyses with imputed missing data and complete cases only, the main conclusions of the results were similar (22). Linear regression analyses were performed to investigate the associations between human milk macronutrients and infant body composition, with adjustments for sex, parity, gestational age and (postnatal) age. For determining FM% SDS, we could only use infants with measured FM% and SDS scores for FM% were calculated based on reference data of our large, total cohort (27).

SPSS statistical package version 25 (SPSS Inc. Chicago, Illinois) was used. P-values <0.05 were considered statistically significant at the two-sided level.

RESULTS

The study population consisted of 133 infants receiving exclusive breastfeeding during the first 3 months, of whom 49.6% was male and 63.2% Caucasian. Median (IQR) gestational age at birth was 40.1 (39.3-40.7) weeks. Clinical characteristics of the subjects by sex are presented in Table 1.

Human milk macronutrient composition in exclusively breastfed infants at age 1 and 3 months

Median (IQR) concentrations of human hind milk macronutrients at 1 and 3 months are presented in Table 2. Median crude and true protein decreased from 1 to 3 months from 1.3 to 1.0 g/100 ml ($p<0.001$) and 1.0 to 0.8 g/100 ml ($p<0.001$), respectively. Energy decreased from 1 to 3 months from 81.6 to 74.6 kcal/100 ml ($p=0.016$). Median fat and carbohydrate tended to decrease over time ($p=0.066$ and 0.081 , respectively). Of all macronutrients, milk fat showed the largest differences in concentration between mothers.

Human milk macronutrient composition compared to formula macronutrient composition

For comparing macronutrients in breastmilk versus formula, we calculated median (g/100 ml) macronutrient levels in the 6 most common formula feeding brands available in our country; 3.5 g fat, 7.4 g carbohydrate and 1.4 g protein and 66 kcal energy per 100 ml formula feeding. In contrast, macronutrients in human hind milk samples showed a large difference compared to macronutrients in formula feeding (Fig. 1). Of the 133 hind milk samples received by exclusively BF infants, 68.4% had higher fat, 78.2% higher energy, 92.5% higher carbohydrates and 31.6% higher protein per 100 ml compared to the average macronutrient levels in formula feeding at age 1 month. At age 3 months, 55.6% of hind milk samples received by exclusively BF infants had higher fat, 66% higher energy, 92.5% higher carbohydrates and 3.8% higher protein per 100 ml compared to the average macronutrient levels in formula feeding.

Associations of human milk macronutrient composition and body composition during early life in exclusively breastfed infants

Table 3 shows the associations of milk macronutrient composition with infant body composition, adjusted for sex, gestational age, parity and age at visit. Human milk macronutrients at 1 month were not associated with FM% at 1, 3 and 6 months. Human milk fat (g/100

Table 1. Clinical characteristics of exclusively breastfed infants.

N [Male]	Birth	1 month		3 months		6 months		9 months		12 months		18 months		24 months			
		133	[66]	133	[66]	133	[66]	133	[66]	133	[66]	133	[66]	133	[66]		
Weight (kg)	M	3.51	[0.46]	4.52	[0.58]	6.29	[0.68]	7.85	[0.82]	8.96	[0.94]	9.79	[1.09]	11.22	[1.22]	12.50	[1.43]
	F	3.41	[0.50]	4.20	[0.53]	5.75	[0.65]	7.21	[0.73]	8.35	[0.80]	9.20	[0.87]	10.71	[0.98]	12.01	[1.19]
Length (cm)	M	51.4*	[2.32]	55.3	[2.03]	62.3	[1.98]	68.4	[2.48]	72.6	[2.38]	76.4	[2.72]	82.8	[3.07]	88.5	[3.78]
	F	50.3*	[2.23]	54.3	[2.13]	60.6	[2.30]	66.8	[2.35]	70.9	[2.62]	74.8	[2.60]	81.5	[2.76]	87.7	[3.20]
Head circumference (cm)	M	NA		37.6	[1.30]	40.7	[1.34]	43.5	[1.43]	45.3	[1.41]	46.4	[1.44]	48.0	[1.41]	48.7	[1.72]
	F	NA		36.5	[1.04]	39.5	[1.12]	42.1	[1.11]	44.0	[1.30]	45.0	[1.22]	46.6	[1.25]	47.6	[1.29]
FM (%)	M	NA		16.3	[4.20]	23.0	[4.50]	24.5	[4.28]	22.1	[4.84]	20.6	[4.02]	17.9	[4.54]	17.2	[3.91]
	F	NA		17.3	[3.89]	22.9	[4.76]	25.3	[5.06]	23.5	[5.14]	21.0	[4.76]	19.5	[5.06]	18.8	[4.34]
FM index (kg/m ²)	M	NA		2.44	[0.76]	3.76	[0.97]	4.13	[0.93]	3.78	[1.02]	3.46	[0.85]	2.96	[0.91]	2.76	[0.78]
	F	NA		2.48	[0.67]	3.60	[0.90]	4.13	[0.99]	3.93	[1.00]	3.47	[0.95]	3.17	[0.95]	2.95	[0.81]
Subcutaneous FM (cm)	M	NA		NA		0.43	[0.12]	0.45	[0.12]	0.40	[0.10]	0.35	[0.09]	0.31	[0.09]	0.33	[0.09]
	F	NA		NA		0.39	[0.12]	0.44	[0.13]	0.38	[0.09]	0.33	[0.09]	0.33	[0.10]	0.34	[0.10]
Visceral FM (cm)	M	NA		NA		2.34	[0.65]	2.18	[0.56]	2.22	[0.56]	2.40	[0.70]	2.40	[0.70]	2.23	[0.53]
	F	NA		NA		2.36	[0.62]	2.11	[0.61]	2.36	[0.69]	2.44	[0.52]	2.30	[0.54]	2.16	[0.55]

Data expressed as pooled means [pooled standard deviation of the means] for male (M) and female (F). * Available for 37 boys and 44 girls. NA; not applicable.

Table 2. Macronutrient composition of human milk received by exclusively breastfed boys and girls at the age of 1 and 3 months.

	Total study group			Male		Female		Male		Female	
	1 month	3 months	p-value*	1 month	3 months	p-value	1 month	3 months	p-value	3 months	
Energy (kcal/100ml)	81.6 [71.4 – 94.4]	74.6 [62.6 – 90.3]	0.016	84.5 [72.6 – 99.6]	79.1 [67.7 – 90.7]	0.018	73.7 [64.4 – 90.3]	76.7 [61.8 – 92.0]	0.92		
Fat (g/100ml)	4.4 [3.3 – 5.8]	3.9 [2.6 – 5.6]	0.066	4.7 [3.6 – 6.3]	4.1 [3.3 – 5.5]	0.048	3.7 [2.6 – 5.6]	4.0 [2.6 – 5.6]	0.92		
Carbohydrate (g/100ml)	8.7 [8.5 – 8.9]	8.7 [8.5 – 8.8]	0.081	8.8 [8.6 – 8.9]	8.7 [8.5 – 8.9]	0.24	8.7 [8.4 – 8.8]	8.6 [8.5 – 8.8]	0.39		
Crude protein (g/100ml)	1.3 [1.1 – 1.5]	1.0 [0.9 – 1.2]	<0.001	1.3 [1.1 – 1.5]	1.3 [1.1 – 1.5]	0.59	1.0 [0.9 – 1.2]	1.0 [0.9 – 1.2]	0.59		
True protein (g/100ml)	1.0 [0.9 – 1.2]	0.8 [0.8 – 0.9]	<0.001	1.1 [0.9 – 1.2]	1.0 [0.9 – 1.2]	0.61	0.8 [0.7 – 0.9]	0.8 [0.7 – 1.0]	0.59		

Data expressed as median [IQR]. * comparison of samples from mothers who collected 2 breastmilk samples at age 1 and 3 months.

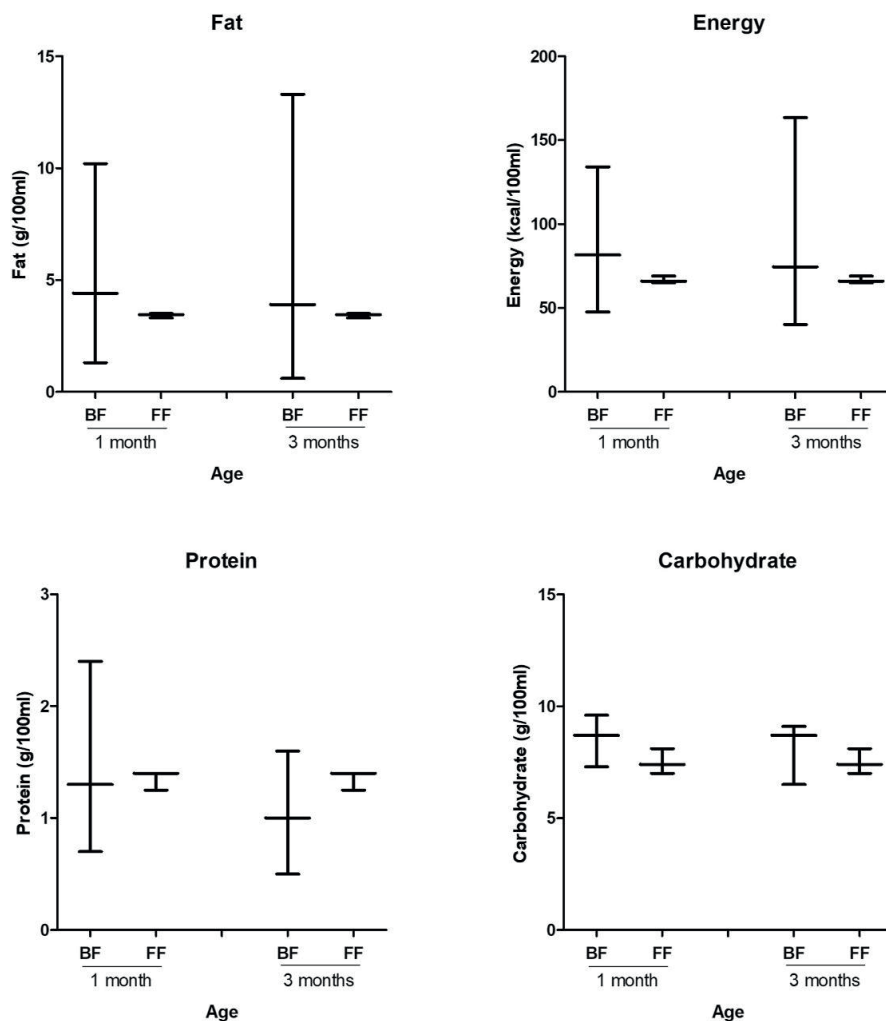


Figure 1. Macronutrient composition in human hind milk samples versus formula feeding. Data are expressed as median (total range: min-max). Abbreviations: BF: breastfeeding, FF: formula feeding.

ml) and energy (kcal/100 ml) at 3 months were associated with FM% at 6 months (β 0.387 (0.006-0.767) and β 0.040 (0.000-0.081), respectively, both $p \leq 0.049$), but not with FM% at 3 months.

Human milk fat (g/100 ml) and energy (kcal/100 ml) at 3 months were also associated (β 0.088 (0.005-0.171), $p=0.039$ and β 0.009 (0.000-0.018), $p=0.045$, respectively) with the change in FM% SDS from 1 to 6 months, the critical window for adiposity programming.

Human milk fat (g/100 ml) and energy (kcal/100 ml) at 1 month were associated with subcutaneous FM (cm) at 3 months (β 0.013 (0.002-0.025) and β 0.001 (0.000-0.003), respectively, both $p \leq 0.027$). Crude and true protein (g/100 ml) at 3 months were inversely associated with visceral FM (cm) at 6 months (β -0.271 (-0.539 to 0.004) and β -0.335 (-0.670 to 0.001), respectively, both $p \leq 0.049$). As ultrasound measurements were performed from age 3 months onwards, only the change in abdominal fat mass from age 3 to 6 months was investigated. Human milk macronutrients at 1 and 3 months were not associated with the change in subcutaneous and visceral FM (cm) from 3 to 6 months.

Human milk macronutrients at both 1 and 3 months were not associated with FM% and subcutaneous FM (cm) at age 2 years. Only milk carbohydrate (g/100 ml) content at 3 months tended to associate with visceral FM (cm) at age 2 years (β 0.290 (-0.014 to 0.595), $p=0.06$).

Human milk macronutrient composition and infant appetite

Infant's appetite at 1 and 3 months was assessed by using the items of the Baby Eating Behavior Questionnaire (BEBQ), regarding satiety and slowness in drinking, to investigate if exclusively breastfed infants receiving higher caloric human milk would satiate faster (Table 4). At age 1 month, milk fat and energy content correlated positively with "my baby gets full up easily", and inversely with "even when my baby had just eaten well, he/she is happy to feed again if offered", indicating that if milk fat concentration is higher, infants get full up more easily and are less eager to feed again if offered. Furthermore, milk fat correlated inversely with "my baby is always demanding a feed" and "my baby sucks more and more slowly during the course of a feed", also indicating that a higher fat content is associated with more satiety and less frequently demanding a feed. At age 3 months, milk fat and energy content correlated with "my baby finishes feeding quickly" and "my baby gets full up easily", indicating that also at age 3 months higher fat and energy content are associated with faster satiety and infants finishing feeding earlier.

Variables with potential influence on human milk macronutrient composition in infants with exclusive breastfeeding at 1 and 3 months

In addition, we investigated variables with potential influence on human milk macronutrient composition.

Sex

At age 1 month, only milk fat and energy content were higher in samples received by boys compared to girls (both $p < 0.05$). At 3 months, all macronutrients were similar between boys and girls (all $p = 0.38$) (Table 2).

Table 3. Univariate linear regression analyses for human milk macronutrient composition and (Δ) FM% during the first 6 months and at age 2 years in infants with exclusive breastfeeding.

	FM% 1 month		FM% 3 months		FM% 6 months		FM% 2 years		Δ FM% SDS 1-6 months		Δ FM% SDS 1 month – 2 years	
	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value
<i>At 1 month</i>												
Energy (kcal/100ml)	0.014	0.52	0.020	0.40	0.012	0.66	-0.001	0.74	-0.004	0.47	-0.013	0.17
Fat (g/100ml)	0.154	0.44	0.206	0.36	0.105	0.68	-0.004	0.79	-0.046	0.42	-0.121	0.18
Carbohydrate (g/100ml)	-0.385	0.68	-0.054	0.96	1.033	0.39	-0.015	0.85	0.177	0.52	-0.036	0.94
Crude protein (g/100ml)	-2.139	0.18	-1.565	0.39	-1.702	0.39	-0.102	0.39	0.024	0.96	-0.233	0.75
True protein (g/100ml)	-2.716	0.18	-1.996	0.38	-2.158	0.38	-0.129	0.38	0.026	0.96	-0.287	0.76
<i>At 3 months</i>												
Energy (kcal/100ml)			0.045	0.09	0.040	0.049	0.001	0.31	0.009	0.045	-0.002	0.77
Fat (g/100ml)			0.298	0.08	0.387	0.047	0.014	0.30	0.088	0.039	-0.020	0.80
Carbohydrate (g/100ml)			-0.171	0.86	0.444	0.68	-0.014	0.84	-0.094	0.71	-0.087	0.84
Crude protein (g/100ml)			0.502	0.83	-0.443	0.86	0.040	0.80	0.426	0.45	-0.410	0.69
True protein (g/100ml)			0.625	0.83	-0.597	0.85	0.048	0.81	0.523	0.46	-0.499	0.69

Adjusted for sex, gestational age, parity and age at visit (Δ age at visits was used for Δ FM% SDS). β represents unstandardized regression coefficients. Associations for FM% 2 years are presented based on log-transformed outcomes.

Table 4. Baby Eating Behavior Questionnaire items in relation to human milk macronutrient composition in exclusively breastfed infants.

	Energy		Fat		Carbohydrate		Crude protein		True protein	
	R	p-value	R	p-value	R	p-value	R	p-value	R	p-value
At age 1 month										
My baby frequently wants more milk than I provide	0.025	0.79	0.033	0.72	-0.018	0.85	0.126	0.171	0.125	0.17
Even when my baby has just eaten well, he/she is happy to feed again if offered	-0.238	0.009	-0.236	0.009	0.092	0.31	-0.024	0.80	-0.026	0.78
My baby is always demanding a feed	-0.172	0.06	-0.195	0.034	-0.021	0.82	0.022	0.81	0.021	0.82
My baby gets full up easily	0.241	0.008	0.202	0.026	-0.148	0.10	0.145	0.11	0.143	0.12
My baby finishes feeding quickly	0.136	0.14	0.081	0.38	0.075	0.41	0.165	0.07	0.165	0.07
My baby takes more than 30min to finish feeding	-0.077	0.41	-0.059	0.52	-0.165	0.07	-0.126	0.17	-0.128	0.17
My baby sucks more and more slowly during the course of a feed	-0.172	0.06	-0.184	0.044	0.098	0.29	-0.153	0.10	-0.152	0.10
At age 3 months										
My baby frequently wants more milk than I provide	0.111	0.23	0.100	0.28	0.143	0.12	0.070	0.45	0.069	0.46
Even when my baby has just eaten well, he/she is happy to feed again if offered	0.039	0.67	0.040	0.67	0.105	0.26	-0.039	0.68	-0.040	0.67
My baby is always demanding a feed	0.079	0.39	0.077	0.40	0.110	0.23	-0.014	0.88	-0.013	0.88
My baby gets full up easily	0.271	0.003	0.272	0.002	0.001	0.99	0.139	0.13	0.138	0.13
My baby finishes feeding quickly	0.215	0.019	0.219	0.016	-0.058	0.53	0.162	0.08	0.162	0.08
My baby takes more than 30min to finish feeding	-0.114	0.21	-0.121	0.18	0.100	0.27	-0.031	0.74	-0.028	0.76
My baby sucks more and more slowly during the course of a feed	-0.104	0.25	-0.116	0.21	0.015	0.87	-0.012	0.90	-0.012	0.89

Data are presented as correlations with R; correlation coefficient.

Fasting versus non-fasting samples

At age 1 month, there were no differences in macronutrient composition between fasting and non-fasting milk samples. At 3 months, only carbohydrate concentrations were higher in non-fasting samples compared to fasting samples (8.9 vs 8.7 g/100 ml, $p=0.002$).

Circadian variation

At age 1 and 3 months, no differences were found between macronutrients in milk samples collected during night/early morning or during late morning (all $p=0.23$).

Delivery mode, birthweight and ethnicity

Milk macronutrient composition at both time points were not different between infants born via normal vaginal delivery or caesarean section (all $p=0.19$). No correlations were found between birthweight SDS and macronutrient composition (all $p=0.24$) and macronutrients at both time points were not different across ethnicity groups (all $p=0.26$).

Parity

At age 3 months, milk fat content was higher in human milk samples collected from mothers of second children ($n=44$) compared to first ($n=72$) or third ($n=12$) children (4.5 vs 4.0 and 2.7, respectively, $p=0.048$). No differences were found for other macronutrients nor for human milk samples collected at the age of 1 month.

Maternal characteristics

No correlations were found between pre-pregnancy BMI or maternal weight gain during pregnancy and macronutrient composition.

DISCUSSION

In 133 healthy, term-born, exclusively breastfed infants, we showed that human milk protein and energy content decreased significantly from age 1 to 3 months, while fat and carbohydrate content tended to decrease. Human milk macronutrient composition showed remarkable differences between mothers, particularly in fat levels. Human milk fat and energy content at 3 months were associated with FM% at 6 months and the gain in FM% SDS from 1 to 6 months, the critical window for adiposity programming. Infants receiving higher caloric human milk were satiated earlier and finished feeding faster.

Human milk protein and energy decreased from age 1 to 3 months, which is in line with literature (15-17, 30, 31), while fat and carbohydrate content tended to decrease. Differences in macronutrient levels in breastmilk samples were considerable and more than half of the

breastfed infants would receive more fat, energy and carbohydrates until age 3 months compared to formula fed infants when they would drink the same amount of milk. Protein content decreased from 1 to 3 months in human milk, while formula feeding maintains the same concentration of proteins resulting in a higher protein per 100 ml intake in formula fed infants at age 3 months.

Our study is the first to investigate human milk macronutrient composition in relation to longitudinally measured body composition and abdominal FM in early life in a large group of term-born infants. Fat and energy content at 3 months were associated with FM% at 6 months and the change in FM% SDS from 1 to 6 months. Another study showed that human hind milk %fat at age 4-8 weeks, without assessment of intake, was inversely related to gain in weight and adiposity, which was assessed by skinfolds at age 3 and 12 months (32). The seemingly contradictory results could be explained by the physiological changes in body composition during the first year of life as we previously showed that FM% increases until 6 months and decreases thereafter (27). We now present data in a large group of exclusively breastfed infants with longitudinally measured FM% and show that higher human milk fat and energy content at age 3 months associates with a higher FM% at age 6 months and a higher gain in FM% during the critical window for adiposity programming.

Our findings are in line with those of our PROGRAM-study, where subjects with versus without a higher gain in weight in early life had significantly higher FM% and unfavorable metabolic and cardiovascular risk profiles at age 21 years (21, 33, 34). Also our previous study in infants showed that a higher gain in FM% from 1 to 6 months associated with a higher FM% at age 2 years (22). In current study, human milk fat and energy did associate with higher FM% at age 6 months and a higher gain in FM% from age 1 to 6 months, but not with a higher FM% at 2 years. A possible explanation for the latter might be that current study comprised of one third of the previously investigated infants as we only included exclusively breastfed infants with collected human milk samples.

Increased abdominal visceral FM has been specifically associated with an unfavorable metabolic health profile during childhood and thereafter (35, 36). The duration of exclusive breastfeeding has been associated with subcutaneous rather than visceral fat mass at age 3 and 6 months (37). We now show that specifically human milk fat and energy concentrations at 1 month associate with subcutaneous FM at 3 months, but not with visceral FM. Only milk carbohydrate content at 3 months tended to associate with visceral FM at 2 years. Since all associations are not strong, more research in a large group of infants is needed to investigate associations between human milk macronutrients and abdominal fat mass development during infancy and childhood.

To investigate whether exclusively breastfed infants receiving higher caloric human milk are feeling satiated faster and finish feeding earlier, we used BEBQ items of 'satiety', 'food responsiveness' and 'slowness in drinking' (29). The correlations of human milk fat and energy at 1 and 3 months with 'my baby gets full up easily' indicate that infants receiving higher fat and energy satiated faster. This was confirmed by the correlation at 3 months between human milk fat and energy with 'my baby finishes feeding quickly'. In breastfed infants, without use of expressed milk, it is difficult to determine the exact amount of human milk intake, and thus the total intake of macronutrients, which is a limitation. It could be a self-regulatory mechanism that higher caloric human milk leads to infants feeling satiated faster and longer while finishing feeding more quickly, thus preventing excessive intake of milk which in turn protects from adiposity programming. This finding could explain why some infants drink for a longer time and others finish earlier. It is our experience that this is reassuring for mothers, care takers, professionals and researchers. For future research, it is important to investigate these correlations in fore milk samples as well and to investigate body composition and metabolic health beyond age 2 years.

In addition, we investigated potential influencing factors of human milk macronutrients. Human milk samples of mothers with boys contained more fat and energy compared to girls at age 1 month, but all macronutrients were similar at 3 months. No differences were found for macronutrient composition between samples collected during night/early morning or during late morning. Others also reported similar concentrations of macronutrients throughout the day (38), but the difference between samples collected at night/early morning and during later in the morning had not been investigated before.

Macronutrient composition of human milk was not different between delivery mode. This is in contrast to two contradicting studies showing higher protein levels either after vaginal delivery (39) or Caesarian section (15). Both studies, however, investigated human milk samples after birth or until 1 month only.

Regarding parity, human milk samples received by second children contained more fat compared to samples received by first and third children, which is partly in line with a small study describing higher lipid levels with increasing parity (31). That study did, however, not use a Human Milk Analyzer, but weighed fat after using a modification of the gravimetric method which could affect comparison between studies.

Pre-pregnancy BMI and maternal weight gain during pregnancy were not correlated with macronutrient composition, which was in contrast to studies showing that human milk samples of overweight mothers had higher fat and protein and lower carbohydrate levels (40), and that maternal BMI was related to human milk fat and energy content (41).

In conclusion, longitudinal data on human milk macronutrient composition show that protein and energy content decrease from age 1 to 3 months, while fat and carbohydrate content tended to decrease. Human milk macronutrient composition shows remarkable differences between mothers, particularly in fat levels. Human milk fat and energy content at 3 months associate with FM% at 6 months and with the gain in FM% from age 1 to 6 months, the critical window for adiposity programming. Exclusively breastfed infants receiving higher caloric human milk satiate earlier and finish feeding faster. This self-regulatory mechanism might protect them from receiving excessive amounts of fat and energy.

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Chapter 5

Longitudinal telomere length and body composition in
healthy term-born infants during the first two years of
life

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ABSTRACT

Objective Leukocyte telomere length (LTL) is one of the markers of biological aging as shortening occurs over time. Shorter LTL has been associated with adiposity and a higher risk of cardiovascular diseases. The objective was to assess LTL and LTL shortening during the first 2 years of life in healthy, term-born infants and to associate LTL shortening with potential stressors and body composition.

Study design In 145 healthy, term-born infants (85 boys), we measured LTL in blood, expressed as telomere to single-gene copy ratio (T/S ratio), at 3 months and 2 years by quantitative PCR technique. Fat mass (FM) was assessed longitudinally by PEAPOD, DXA, and abdominal FM by ultrasound.

Results LTL decreased by 8.5% from 3 months to 2 years (T/S ratio 4.10 vs 3.75, $p < 0.001$). LTL shortening from 3 months to 2 years associated with FM% ($R = 0.254$), FM index ($R = 0.243$) and visceral FM ($R = 0.287$) at 2 years. LTL shortening tended to associate with gain in FM% from 3 to 6 months ($R = 0.155$, $p = 0.11$), in the critical window for adiposity programming. There was a trend to a shorter LTL in boys at 2 years ($p = 0.056$). LTL shortening from 3 months to 2 years was not different between sexes.

Conclusion We present longitudinal LTL values and show that LTL shortens considerably (8.5%) during the first 2 years of life. LTL shortening during first 2 years of life was associated with FM%, FMI and visceral FM at age 2 years, suggesting that adverse adiposity programming in early life could contribute to more LTL shortening.

INTRODUCTION

Telomeres are noncoding repetitive DNA sequences at the end of chromosomes, protecting genomic DNA by maintaining stability (1). Due to the inability of DNA polymerase to fully replicate the ends of chromosomes, telomeres shorten with each cell division, thus with increasing age. When telomeres are reduced to a critical length, cells enter a state of arrest (cell senescence) (2). Telomere length can thus be used as a proxy of biological aging and mortality (3), although it is not the only biomarker of aging.

The shortening of leukocyte telomere length (LTL) can be accelerated by multiple factors, such as inflammation, (oxidative) stress, obesity, toxins and radiation (4). Shorter telomeres are associated with an increased risk for cardiovascular diseases (CVD), but it is uncertain if telomere length can be seen as prognostic marker for CVD (3).

A rapid rise in weight during early life has also been associated with an increased risk for adiposity and CVD in adulthood (5-9). We have shown that a rapid rise in FM% SDS during the first 6 months of life, the critical window for adiposity programming, results in higher FM % trajectories during infancy (10). No associations were found between body size at birth and LTL in adulthood (11), but it is yet unknown whether LTL and its changes over time are associated with longitudinally measured body composition during infancy and the gain in FM% during the critical window for adiposity programming.

Until now, one other study has investigated leukocyte telomere length (LTL) longitudinally in healthy, term-born infants during the first two years of life (12), which is an important period for infant development (13). This study, however, did not investigate longitudinal LTL in association with body composition. Some studies in infants and children measured telomere length in cord blood directly after birth (14-16) or in childhood (17). Obtaining longitudinal values for LTL in healthy, term-born infants in early life in association with longitudinal body composition measures is important for clinical and research use. Various conditions and syndromes are linked to altered telomere length and adverse body composition, for example in infants born prematurely (18), small-for-gestational age (15) and infants with various syndromes (19).

The primary objective of this study was to investigate longitudinal LTL from age 3 months to 2 years. Our secondary objective was to investigate associations of LTL with potential influencing factors like gestational age, birth size and parity and with longitudinal body composition and abdominal fat mass during the first 2 years of life. We hypothesized that infants with more fat mass and particularly more visceral fat mass have more shortening in LTL during the first 2 years of life.

METHODS

Participants

The study population consisted of healthy, term-born infants, participating in the Sophia Pluto Study, a birth cohort study in Rotterdam area (The Netherlands). Between January 2013 and October 2019, infants were recruited from obstetric departments of regional hospitals and primary health care centers and detailed data on body composition and growth during early life were obtained. The Sophia Pluto Study obtained approval by the Medical Ethics Committee of Erasmus Medical Center and parental written informed consent was obtained for every participant.

All participants fulfilled the following inclusion criteria: term born (≥ 37 weeks of gestation), age < 28 days, uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score < 3 after 5 minutes) and no sepsis or long-term complication of respiratory ventilation. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy or a significant maternal medical condition that could interfere with the study results.

Data Collection and Measures

Outpatient clinic visits were scheduled at age 1, 3, 6, 9, 12, 18 and 24 months (Table 1). Data on pregnancy and birth outcomes were obtained and measurements were performed by trained staff. If an infant was ill at time of a scheduled study visit, parents were instructed to contact the study team in order to reschedule the appointment.

Anthropometrics

Weight was measured with an electronic infant scale to the nearest 5 grams (SECA 717, Hamburg, Germany). Length was measured twice by two-person technique with an infantometer to the nearest 0.1 cm (SECA 416) and head circumference was measured twice as the widest frontal-occipital circumference with a measuring tape to the nearest 0.1 cm (SECA 201).

Weight-for-length, weight-for-age and height-for-age SDS were calculated by Growth Analyser (<https://growthanalyser.org/>; Talma, 2010).

Body composition measurements

Until age 6 months, body composition was assessed by air-displacement plethysmography (ADP by PEA POD, COSMED, Italy) as described in detail elsewhere (20). According to standard protocol, the PEA POD was calibrated daily (21). From 6 months onwards, a Dual Energy X-ray Absorptiometry (DXA) scan was performed at every visit with the same device (DXA, Lunar Prodigy, GE Healthcare, UK) and software (enCORE software version 14.1). At the

transition point of 6 months, median FM% was 24.1 by ADP and 25.0 by DXA ($n=278$), with a median difference of 0.9% between both measurements. Bland-Altman analysis showed no proportional bias ($p=0.321$) (22).

Fat mass index (FMI) was determined by dividing fat mass (kg) by height squared (m^2) and Fat-free mass index (FFMI) by dividing fat-free mass (kg) by height squared (m^2).

Ultrasound measurement of abdominal fat mass

Subcutaneous and visceral fat thickness were measured by ultrasound at every visit starting from 3 months of age and described in detail elsewhere (20, 23). Unsuccessful ultrasound measurements of visceral fat mass, without visualization of the lumbar vertebra, were excluded from analyses.

Infant feeding

Infant feeding was classified as exclusive breastfeeding (BF) if an infant received breastfeeding for at least 3 months or no exclusive breastfeeding if they received formula feeding or mixed feeding before age 3 months. Information on the timing of solid food introduction was obtained from questionnaires.

Telomere length assessment

Genomic DNA was isolated from peripheral leukocytes using standard procedures and the same methods were used for all samples. All LTL measurements were made in the same laboratory at the University of Leicester. Mean LTL was measured by the quantitative PCR-based technique (2, 24). Telomere sequence copy number (T) was compared with a single copy gene number in the genome 36B4 (S) and telomere length expressed as a T/S ratio. All T and S values were calculated relative to a calibrator DNA (genomic DNA from the K562 cell line) that was included on every plate. This allowed correction for inter-run variation. To further minimize any technical variation in the LTL measurements for the 3 month and 2 year samples, both samples for each individual were run within the same assay plate. All samples were checked for concordance between duplicate values for T and S as quality control. Samples showing a difference of greater than 0.2 cycles in the take-off value or amplifying outside of the linear range of the assay were excluded and re-run alongside the second time point for that individual. Reproducibility of the assay was tested by re-running samples on separate days. The mean inter-run CV for the T/S ratio was 2.85%. T/S ratio in our cohort at 3 months and 2 years were compared with T/S ratio in our PROGRAM study at 21 years. Subjects of our PROGRAM study met the same inclusion criteria of the healthy, term-born infants of present study and samples were analyzed in the same laboratory using the same technique (18).

Statistical Analysis

Clinical characteristics were expressed as median and interquartile range [IQR], and pooled means (pooled SD) in Table 1. For this study, we included 145 infants with blood collection at age 3 months and/or 2 years with ≥ 4 body composition measurements during the first 2 years of life and without past or present serious illnesses. In total, 112 infants had blood collection at both time points in which the shortening in LTL from 3 months to 2 years was determined. Differences in clinical characteristics were assessed by independent student's *t*-test or Mann-Whitney U-test for non-parametric parameters. Correlations were determined by Spearman's correlation coefficient. Missing data on body composition, mainly because of infants showing resistance at measurements, were imputed using a multiple imputation approach in SPSS to generate 20 imputed datasets. Although small differences were observed between analyses with imputed.

RESULTS

Clinical characteristics of the subjects are presented in Table 1. Of the total group, 58.6% was male and 41.4% female. Median (IQR) birthweight was 3.38 (3.11–3.90) kg at 39.9 (39.0–40.9) weeks in boys and 3.19 (2.78–3.50) kg at 39.9 (38.8–40.5) weeks in girls.

Telomere length during the first 2 years of life

Median (IQR) LTL decreased from 3 months to 2 years (T/S ratio 4.10 (3.78–4.72) vs 3.75 (3.51–4.09), $p < 0.001$) (Table 2), which is a decrease of 8.5% from 3 months to 2 years (4.9% per year) (Fig 1). LTL at 3 months was associated with LTL at 2 years ($R = 0.641$, $p < 0.001$).

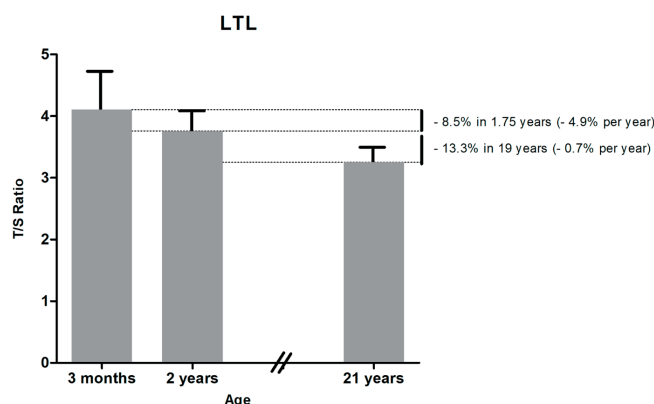


Figure 1. Longitudinal LTL development over time based on outcome of two separate birth cohort studies. Data are expressed as median LTL at 3 months and 2 years (current study) and mean LTL at 21 years (from our previous PROGRAM study (18)), both with upper interquartile range.

Table 1. Clinical characteristics of boys and girls.

Age N [Male]	1 month 145 [85]	3 months 145 [85]	6 months 145 [85]	9 months 145 [85]	12 months 145 [85]	18 months 145 [85]	24 months 145 [85]
Weight (kg)	M 4.39 [0.55]	6.31 [0.68]	8.12 [0.90]	9.44 [1.03]	10.34 [1.19]	11.78 [1.43]	13.18 [1.71]
	F 3.92 [0.62]	5.62 [0.75]	7.39 [0.83]	8.62 [0.96]	9.48 [1.00]	10.91 [1.15]	12.31 [1.26]
Length (cm)	M 55.0 [1.99]	62.2 [1.91]	68.9 [2.18]	73.7 [2.54]	77.3 [2.84]	84.0 [3.09]	89.9 [3.47]
	F 53.3 [2.51]	59.9 [2.47]	66.6 [2.33]	70.9 [2.53]	74.9 [2.68]	81.6 [2.58]	87.9 [3.08]
FM (%)	M 15.9 [4.31]	22.8 [4.75]	24.4 [4.95]	22.1 [5.55]	20.8 [4.81]	18.8 [4.57]	17.8 [4.10]
	F 16.0 [5.08]	23.1 [5.42]	24.6 [5.53]	24.9 [4.80]	21.8 [5.03]	19.0 [4.36]	18.6 [4.22]
Abdominal subcutaneous FM (cm)	M NA	0.41 [0.11]	0.43 [0.11]	0.39 [0.10]	0.35 [0.11]	0.34 [0.11]	0.35 [0.11]
	F NA	0.39 [0.12]	0.41 [0.11]	0.38 [0.10]	0.34 [0.10]	0.32 [0.11]	0.34 [0.10]
Visceral FM (cm)	M NA	2.42 [0.62]	2.28 [0.64]	2.42 [0.60]	2.43 [0.68]	2.31 [0.66]	2.11 [0.56]
	F NA	2.22 [0.58]	2.24 [0.56]	2.52 [0.62]	2.49 [0.59]	2.27 [0.63]	2.25 [0.54]

Data expressed as pooled means [pooled standard deviation of the mean] for boys (M) and girls (F). Abbreviations: N; number, FM; fat mass.

Telomere length and body composition during the first 2 years of life

The shortening in LTL from 3 months to 2 years associated with fat mass percentage (FM%, $R=0.193$, $p=0.048$), FM index (FMI, $R=0.243$, $p=0.016$) and visceral FM ($R=0.234$, $p=0.022$) at age 2 years, but not with abdominal subcutaneous FM. There was no association between LTL at age 3 months and 2 years and FM%, FMI, abdominal subcutaneous and visceral FM at the same ages.

As we previously found in the same study group that the gain in FM% from 3 to 6 months was associated with a higher FM% at 2 years, we investigated if the shortening in LTL from 3 months to 2 years was associated with the gain in FM% from 3 to 6 months. The shortening in LTL from 3 months to 2 years tended to associate with the gain in FM% from 3 to 6 months ($R=0.155$, $p=0.11$).

LTL at age 3 months, 2 years and the shortening in LTL from 3 months to 2 years did not associate with the change in FM% and visceral FM from 3 months to 2 years.

The shortening in LTL from 3 months to 2 years associated with fat-free mass index (FFMI) at 3 months ($R=0.223$, $p=0.019$), but not at age 2 years and not with FFM, and there was no association between LTL at age 3 months and 2 years and FFM and FFMI at the same ages.

Table 2. LTL (T/S ratio) at age 3 months and 2 years for the total group, boys and girls.

	LTL 3 months	LTL 2 years	P-value
Total group	4.10 [3.78 – 4.72]	3.75 [3.51 – 4.09]	<0.001
Boys	4.05 [3.67 – 4.84]	3.65 [3.34 – 4.05]	<0.001
Girls	4.20 [3.86 – 4.67]	3.86 [3.53 – 4.29]	<0.001
P-value	p=0.48	p=0.056	

Data expressed as median [IQR]. Abbreviations: LTL; leukocyte telomere length, T/S ratio; Telomere to single-gene copy ratio.

Variables with potential influence on telomere length

Boys and girls

Median LTL was not different between boys and girls at age 3 months ($p=0.48$), but boys tended to have shorter LTL than girls at age 2 years (3.65 vs 3.86, $p=0.056$) (Table 2). The shortening in LTL from 3 months to 2 years was not different between boys and girls ($p=0.38$).

Parental and infant variables

Neither gestational age, parity, mode of delivery (vaginally or caesarian section), maternal pre-pregnancy BMI and weight gain during pregnancy, nor birthweight and ethnicity were associated with LTL at age 3 months, 2 years and shortening in LTL from 3 months to 2 years. Maternal and paternal age at infant's birth did also not associate with LTL at age 3 months, 2 years and shortening in LTL from 3 months to 2 years.

Infant feeding

Of 145 infants, 77 were exclusively breastfed and 68 were not exclusively breastfed. Exclusively breastfed infants had longer LTL at 3 months compared to infants without exclusive breastfeeding (T/S Ratio 4.4 vs 4.1, $p=0.046$), but LTL at 2 years and the shortening in LTL from 3 months to 2 years were similar in infants with exclusive BF and nonexclusive BF ($p\geq 0.19$). Duration of BF and timing of introduction of solid foods were also not correlated with LTL at 3 months, 2 years and shortening in LTL from 3 months to 2 years.

Length and growth

Length SDS at 3 months, 2 years and change in length SDS from 3 months to 2 years did neither associate with LTL at these ages, nor with the shortening in LTL from 3 months to 2 years.

DISCUSSION

Our findings in healthy, term-born infants show that 8.5% of shortening of LTL occurs from age 3 months to 2 years. The shortening in LTL from 3 months to 2 years was associated with FM%, FMI and visceral FM at age 2 years. LTL shortening during the first 2 years of life tended to associate with the gain in FM% from 3 to 6 months, within the critical window for adiposity programming.

The period from conception until age 2 years, the first 1000 days of life, is an important period for infant development (13). Prenatal exposure to damaging environmental factors and maternal stress have been associated with shorter LTL in newborns (25, 26). Leukocyte telomere length is one the biomarkers of aging (27, 28), next to other biomarkers like oxidative stress, inflammation and aberrations in protein and lipid metabolism, which could also affect aging rate (28). To our knowledge, however, a very limited number of studies describe longitudinal data on LTL during infancy as most studies have used neonatal cord blood to investigate LTL at birth (15, 16) or investigated LTL cross-sectionally (17, 29). We investigated longitudinal values for LTL at age 3 months and 2 years based on a large group of healthy, term-born infants and show that LTL decreases considerably during the first 2 year of life. There is one other paper about the change in LTL from infancy to age 2 and 3 years (12). Our findings are in line in describing an impressive decline in LTL during the first 2 years of life. However, in contrast to the study by Bosquet Enlow et al., our first LTL measurement took place at age 3 months, thus during the critical window for adiposity programming from birth to age 6 months (7, 8), while they measured LTL for the first time at a mean age of 8.6 months.

Our findings show that LTL decreases with 8.5% from age 3 months to 2 years, which is a decline of 4.9% per year. In our PROGRAM study, LTL was investigated at age 21 years in 284 healthy, term-born subjects in the same laboratory using the same technique (18). These subjects met the same inclusion criteria as the healthy, term-born infants of present study. We showed that LTL declined with 13.3% from age 2 years to 21 years, which is 0.7% per year after infancy, indicating that telomeres might shorten more during the first 2 years of life compared to the period from age 2 years to 21 years. Longitudinal studies from birth to 21 years have to confirm the abovementioned decline in the same subjects instead of comparing two cohorts of healthy, term-born subjects, but our findings are in line with the study by Bosquet Enlow et al. describing a stable LTL from age 2 until 3 years (12).

The shortening in LTL from age 3 months to 2 years associated significantly with FM% and FMI at age 2 years, indicating that infants with more adiposity at age 2 years had more shortening of telomeres in the period from 3 months to 2 years. Shortening in LTL from

age 3 months to 2 years also associated with abdominal visceral FM at age 2 years. This is in line with a study in healthy children and adults showing that those with higher total and abdominal adiposity have lower telomere length (30). More visceral FM has been associated with unfavorable metabolic health profiles (31, 32) and we now show that shortening in LTL during infancy is associated with visceral FM at age 2 years.

As we have shown that particularly more gain in FM% SDS from 3 to 6 months, the critical window for adiposity programming, was associated with a higher FM% at age 2 years (10), we investigated whether the shortening in LTL in the first 2 years was associated with the gain in FM% from 3 to 6 months. We found that the shortening in LTL from 3 months to 2 years tended to associate with the gain in FM% from 3 to 6 months, which could indicate that a higher gain in FM% in the critical window for adiposity programming might accelerate the shortening of LTL. It has been reported that early life adiposity programming could potentially be an early life stressor by inducing oxidative stress, which would accelerate telomere shortening (33, 34). Also early onset of obesity has been associated with shorter LTL in children at a mean age of 11 years (35). Our findings suggest that adverse adiposity programming in early life could contribute to more shortening of LTL.

Shortening in LTL from 3 months to 2 years also associated with FFMI at 3 months, but not at 2 years. Longer telomeres at birth have been associated with more lean mass during late infancy (15), which is in line with our results. We have, however, no data on LTL at birth and were therefore not able to study LTL and FFMI from birth onwards.

LTL was similar in boys and girls at 3 months, which has been reported in newborns (36, 37). At 2 years, however, boys tended to have shorter LTL compared to girls, which is in line with findings during infancy, childhood and adulthood, when females have longer telomeres (12, 17, 38). For final conclusions about sex differences in LTL, more research is required in a larger study group.

No correlations were found between LTL and birthweight and gestational age in our large group of term-born, mainly appropriate-for-gestational age infants, which is in contrast to a study describing lower birthweight result in lower cord blood LTL (39). This study, however, also included premature infants where we only included term-born infants and in addition most of the infants in our study had a birthweight between -2 and +2 SDS. There was no correlation between cord blood LTL and gestational age, similar to our findings. Our findings are also in line with studies investigating LTL in healthy subjects at 11 years (40) and at adult age (11). Shorter LTL has mainly been found in children born with very low birthweight (41) or born prematurely (18).

Maternal pre-pregnancy BMI did not correlate with infant LTL. This is in contrast to a study describing an association between maternal pre-pregnancy BMI and shorter newborn LTL. We, however, investigated LTL at age 3 months and 2 years instead of birth. Future research is needed to investigate pre-pregnancy BMI and LTL in different pre-pregnancy BMI classes. Maternal and paternal age did not associate with LTL until age 2 years, which is in line with literature (12).

The strength of this study is the collection of longitudinal blood samples for investigating LTL in combination with the longitudinal body composition measurements in healthy infants until the age of 2 years. We did not adjust for social economic status (SES) as we and others have shown that SES did not associate with LTL at any age (12, 18, 42). The effect of SES on the decline of LTL during the first 2 years of life seems therefore limited, but a definite answer would require more research.

Conclusions

In conclusion, we present longitudinal values of LTL and show that telomere length decreases by 8.5% from age 3 months to 2 years. Shortening in LTL during the first 2 years of life was associated with FM%, FMI and visceral FM at 2 years. LTL shortening during the first 2 years of life tended to associate with a higher gain in FM% from 3 to 6 months, suggesting that adverse adiposity programming during the critical window for adiposity programming could contribute to more LTL shortening in early life.

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Chapter 6

Appetite regulating hormone trajectories
and relationships with fat mass development
in term-born infants during the first 6 months of life

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ABSTRACT

Background The first 6 months of life are a critical window for adiposity programming. Appetite regulating hormones (ARH) are involved in food intake regulation and might therefore play a role in adiposity programming. Studies examining ARH in early life are limited.

Purpose To investigate ghrelin, peptide YY (PYY) and leptin until 6 months and associations with fat mass percentage (FM%), infant feeding and human milk macronutrients.

Procedures In 297 term-born infants (Sophia Pluto Cohort), ghrelin (acylated), PYY and leptin were determined at 3 and 6 months, with FM% measurement by PEA POD. Exclusive breastfeeding (BF) was classified as BF ≥ 3 months. Human milk macronutrients were analyzed (MIRIS Human Milk Analyzer).

Main findings Ghrelin increased from 3 to 6 months ($p < 0.001$), while PYY decreased ($p < 0.001$), resulting in increasing ghrelin/PYY ratio. Leptin decreased. Leptin at 3 months was higher in girls, other ARH were similar between sexes. Leptin at 3 and 6 months correlated with FM% at both ages ($R \geq 0.321, p \leq 0.001$) and gain in FM% from 1-6 months ($R \geq 0.204, p = 0.001$). In BF infants, also ghrelin and ghrelin/PYY ratio correlated with this gain in FM%. Exclusively BF infants had lower ghrelin and higher PYY compared to formula fed infants at 3 months ($p \leq 0.039$). ARH did not correlate with macronutrients.

Conclusions Increasing ghrelin and decreasing PYY, thus increasing ghrelin/PYY ratio, suggest an increasing orexigenic drive until 6 months. ARH were different between BF and FF infants at 3 months, but did not correlate with human milk macronutrients. Ghrelin and leptin, but not PYY, correlated with more FM development during the first 6 months, suggesting that they might be involved in adiposity programming.

INTRODUCTION

Appetite regulating hormones (ARH) are involved in the regulation of food intake through specific brain centers, such as the hypothalamus that plays a key role in controlling glucose, energy homeostasis and food intake (1, 2). Ghrelin and peptide YY (PYY) are secreted from the gastrointestinal tract (2). Leptin is secreted mainly from adipose tissue (1, 3) and also from the stomach, but systemic effects of gastric leptin are negligible (4). Ghrelin stimulates food intake whereas PYY and leptin decrease appetite and increase metabolic rate (2, 3). In addition, the ghrelin/PYY ratio is of interest as a marker of orexigenic drive, rather than ghrelin and PYY levels separately (5, 6).

Early life rapid weight gain, and specifically during the first months of life, has been associated with an increased adiposity and cardiovascular disease risk in adulthood (7-11). In addition, we have shown that particularly the change in fat mass percentage (FM%) during the first 6 months, in contrast to the 6- to 12-month period, is associated with higher FM% and abdominal subcutaneous FM at the age of 2 years (12). These first 6 months after birth are considered a critical window for adiposity programming (9, 10). ARH trajectories might be of importance in unraveling this early adiposity programming. ARH have been associated with later growth and adiposity, but most studies used cord blood (13-18) or newborn blood spots (19, 20) to investigate ARH at birth or in specific groups (like infants born premature or small-for-gestational age) (21, 22). However, data on ghrelin, PYY and leptin trajectories during early life in healthy term-born infants are very limited.

Few studies have compared ARH levels between breastfed (BF) and formula fed (FF) infants in early life. Two studies investigated ghrelin and leptin levels during the first four months (23, 24). Our group reported differences in ghrelin, PYY and leptin levels between BF and FF infants at age 3 months (25). Human milk macronutrient composition could potentially influence appetite regulating hormone levels in BF infants as we previously found that exclusively BF infants receiving human milk with higher fat and energy content were satiated earlier. This could be a self-regulatory mechanism to prevent intake of excessive macronutrients (26). Associations between ARH and human milk macronutrients and infant appetite until age 6 months, a critical window for adiposity programming as mentioned above, are lacking.

The primary objective of this study was to investigate ghrelin, PYY and leptin levels during the first 6 months of life and their associations with body fat mass development. The other objectives were to investigate ARH in association with infant feeding, human milk macronutrients and appetite. We hypothesized that higher ghrelin and leptin levels would associate with a higher gain in FM% during the first 6 months. Furthermore, we hypothesized that ARH

levels would be different between BF and FF infants and that ghrelin would be lower and PYY and leptin levels higher in infants receiving human milk with a higher fat and energy content.

METHODS

Participants

The study population consisted of healthy, term-born infants, participating in the Sophia Pluto Study, a birth cohort study in Rotterdam area (The Netherlands). All infants fulfilled the following inclusion criteria: term born (≥ 37 weeks of gestation), age < 28 days, uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score < 3 after 5 minutes) and no sepsis or long-term complication of respiratory ventilation. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy, or a significant maternal medical condition, like (gestational) diabetes, that could interfere with the study results. The Sophia Pluto Study obtained approval by the Medical Ethics Committee of Erasmus Medical Center and parental written informed consent for every participant. For present study, we included 297 singleton born infants from whom blood samples were obtained at age 3 months regardless of infant feeding type.

Data Collection and Measures

Outpatient clinic visits were scheduled at the age of 1, 3 and 6 months (Table 1). Pregnancy and birth data were obtained from midwife and hospital records. Measurements and blood collection were performed by trained staff.

Anthropometrics

Weight was measured to the nearest 5 grams by an electronic infant scale (SECA 717, Hamburg, Germany). Length was measured twice by two-person technique to the nearest 0.1 cm with an infantometer (SECA 416). Birthweight standard deviation scores (SDS) were calculated (27) using Growth Analyser (<https://growthanalyser.org/>).

Blood samples

At age 3 months, 297 blood samples were collected by toe prick after the infants had fasted for a minimum of 2 hours. For 184 of these infants, we also collected a blood sample at age 6 months. This number was less than at 3 months because infants were either too distressed to allow a blood collection or had not yet reached age 6 months. Blood samples were collected in EDTA tubes and DPP4 (dipeptidyl peptidase-4) inhibitor, Serine Protease inhibitor and Protease inhibitor (all Merck Chemicals Netherlands – Merck KGaA) were added for stabilizing the appetite regulating hormones. Blood was centrifuged at 4 °C to prepare plasma,

which was quickly frozen and stored at -80°C until analyses. Ghrelin (acylated), PYY and leptin levels in plasma were determined by the MILLIPLEX MAP Human Metabolic Hormone Magnetic Bead Panel, catalog number HMHEMAG-34K (Millipore Corporation, Billerica, MA) using the commercial protocol provided by the supplier. The intra-assay CV was 10%, and the inter-assay CV was 15%. Fasting time was calculated as time of blood collection minus time of last feeding.

Body composition measurements

Body composition was assessed by air-displacement plethysmography (ADP by PEA POD, COSMED, Italy) as described in detail elsewhere (28). The PEA POD was calibrated daily according to standard protocol (29).

Infant feeding

To investigate ARH levels at age 3 months based on exclusive feeding type, infant feeding was classified as exclusive breastfeeding (BF, $n=158$) or exclusive formula feeding (FF, $n=89$) if infants received either BF or FF, respectively, and no mixed feeding during 3 months after birth.

Breastmilk samples

Breastfeeding mothers were instructed to collect hind milk samples, thus after their infants were breastfed, at infant's age of 3 months as described before (26). For 61 exclusively breastfed infants, human milk samples were analyzed for macronutrient composition (fat, energy, carbohydrate and protein).

Baby Eating Behavior Questionnaires (BEBQ)

At age 3 months, mothers were asked to fill out the Baby Eating Behavior Questionnaire (BEBQ) to assess infant appetite (30). Each item was answered using a five-point Likert frequency scale (1 = never, 2 = rarely, 3 = sometimes, 4 = often and 5 = always).

Statistical Analysis

Clinical characteristics are expressed as median and interquartile range [IQR]. Differences in clinical characteristics were determined by independent sample student's t-test or by Mann-Whitney U-test for non-parametric parameters. ARH levels at age 3 and 6 months were analyzed using mixed model analysis. To investigate differences in ARH between boys and girls, we used sex as a covariate in the mixed models. Time was modeled by entering hospital visits at ages 3 and 6 months into the linear mixed models. Linear correlations were determined by Spearman for non-parametric parameters.

Correlations between ARH levels and human milk macronutrients were performed in infants with exclusive breastfeeding. SPSS statistical package version 25 (SPSS Inc. Chicago, Illinois) was used and p-values < 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of the subjects are presented in Table 1. The total group consisted of 53.5% boys. Median (IQR) gestational age was 39.9 (38.9 – 40.7) weeks. Median infant birthweight SDS was -0.15 (-0.89 – 0.48), maternal pre-pregnancy BMI 23.5 (21.4 – 26.2) kg/m² and maternal weight gain during pregnancy 14.0 (10.0 – 18.0) kg.

Table 1. Clinical characteristics of the study population (n=297 infants).

	Boys	Girls	p-value
Birth			
Weight (kg)	3.38 [3.12 – 3.77]	3.27 [2.94 – 3.71]	0.022
Length (cm)	51.0 [49.0 – 52.0]*	50.0 [48.0 – 51.0]*	0.013
FM(%)	NA	NA	
1 month			
Weight (kg)	4.37 [4.02 – 4.73]	4.08 [3.64 – 4.40]	<0.001
Length (cm)	55.0 [53.5 – 56.5]	54.0 [52.1 – 55.4]	0.003
FM(%)	16.1 [13.5 – 18.9]	16.0 [13.6 – 19.4]	0.32
3 months			
Weight (kg)	6.18 [5.73 – 6.64]	5.73 [5.15 – 6.18]	<0.001
Length (cm)	62.0 [60.5 – 63.2]	60.5 [58.9 – 61.5]	0.26
FM(%)	22.1 [19.0 – 24.6]	23.3 [19.6 – 26.5]	0.099
6 months			
Weight (kg)	7.87 [7.34 – 8.45]	7.33 [6.82 – 7.76]	<0.001
Length (cm)	68.9 [67.0 – 70.2]	67.0 [65.1 – 68.3]	<0.001
FM(%)	23.5 [20.0 – 26.7]	25.1 [21.6 – 28.9]	0.036

Data expressed as median [IQR] for boys and girls. * Birth length available for 94 boys and 80 girls. FM: fat mass. NA; not applicable.

Ghrelin, PYY and leptin levels during the first 6 months of life

Ghrelin (acylated) levels increased from age 3 to 6 months ($p<0.001$), while PYY levels decreased ($p<0.001$), resulting in an increase in ghrelin/PYY ratio over time ($p<0.001$). Leptin levels decreased from age 3 to 6 months ($p<0.001$) (Table 2).

Table 2. Ghrelin, PYY, Ghrelin/PYY ratio and leptin levels at age 3 and 6 months in the total group, boys and girls.

	3 months				6 months			
	Total group n=297	Boys n=159	Girls n=138	p-value	Total group n=297	Boys n=159	Girls n=138	p-value
Ghrelin (pg/ml)	67.2 [60.9 – 73.5]	63.4 [54.8 – 71.9]	71.7 [62.4 – 80.9]	0.20	121.9 [107.0 – 136.7]	127.7 [109.3 – 146.2]	108.8 [83.9 – 133.6]	0.23
PYY (pg/ml)	230.8 [219.5 – 242.0]	228.4 [213.0 – 243.9]	233.5 [216.9 – 250.0]	0.66	172.3 [165.0 – 179.6]	173.8 [164.6 – 182.9]	169.4 [157.0 – 181.8]	0.58
Ghrelin/PYY ratio	0.35 [0.31 – 0.39]	0.33 [0.28 – 0.38]	0.37 [0.31 – 0.42]	0.36	0.80 [0.69 – 0.91]	0.83 [0.70 – 0.97]	0.73 [0.55 – 0.92]	0.39
Leptin (pg/ml)	1492.9 [1356.5 – 1629.2]	1306.9 [1123.0 – 1490.9]	1707.1 [1509.6 – 1904.6]	0.004	881.1 [742.3 – 1020.0]	848.1 [673.5 – 1022.7]	892.0 [659.3 – 1124.7]	0.77

Data expressed as estimated marginal means [95% confidence interval] for the total group, boys and girls. P-values are presented for the difference between boys and girls.
N; number, PYY; peptide YY.

Ghrelin and PYY levels and ghrelin/PYY ratio at age 3 and 6 months were not different between boys and girls (Table 2). Leptin levels at age 3 months were higher in girls compared to boys (1707.1 vs 1306.9 pg/ml, $p=0.004$), but similar at age 6 months.

Median fasting time was 3:00 [2:25 – 3:35] hours at age 3 months and 2:45 [2:00 – 3:40] hours at age 6 months. Ghrelin levels at age 6 months correlated with median fasting time ($R=0.205$, $p=0.005$), while PYY levels at age 6 months correlated inversely ($R=-0.211$, $p=0.004$). Ghrelin/PYY ratio at age 6 months correlated with fasting time ($R=0.236$, $p=0.001$), but leptin levels did not correlate. Median fasting time was not different between boys and girls at age 3 and 6 months ($p=0.13$ and 0.14 , respectively).

Neither ghrelin, PYY and ghrelin/PYY ratio, nor leptin levels at age 3 and 6 months correlated with infant birthweight SDS, maternal pre-pregnancy BMI and maternal weight gain during pregnancy.

Correlations between ARH and body fat mass during the first 6 months of life

In the total group, regardless of infant feeding type, leptin at age 3 months correlated with FM% at age 3 and 6 months ($R=0.395$, $p<0.001$ and $R=0.321$, $p<0.001$, respectively.) and with the gain in FM% from 1 to 6 months ($R=0.204$, $p=0.001$) (Table 3). Leptin at age 6 months also correlated with FM% at age 6 months ($R=0.337$, $p<0.001$) and the gain in FM% from 1 to 6 months ($R=0.207$, $p=0.006$). Ghrelin at age 3 months correlated only with FM% at 6 months, while PYY and ghrelin/PYY ratio did not correlate.

In BF infants, leptin, ghrelin and ghrelin/PYY ratio at age 3 months correlated with the gain in FM% from 1 to 6 months (Table 3). In FF infants, however, only leptin at age 6 months correlated with the gain in FM% from 1 to 6 months as well as PYY at age 3 months.

The results in girls and boys were similar to those of the total group, but leptin in girls at age 6 months correlated with the change in FM% from 1 to 6 months ($R=0.379$, $p=0.002$), while it did not correlate in boys ($R=0.093$, $p=0.33$) (Supplemental Table).

ARH and infant feeding at age 3 months

In addition, we investigated ARH at age 3 months in exclusively BF versus FF infants, thus without interference of infants receiving mixed feeding or solid foods, in subgroup analyses. Median duration of breastfeeding in BF infants was 6.87 [4.65 – 10.02] months.

Differences in ARH between BF versus FF infants at age 3 months

Ghrelin levels at age 3 months were lower and PYY levels were higher in BF infants compared to FF infants ($p=0.039$ and <0.001 , respectively) (Table 4). The ghrelin/PYY ratio was lower

Table 3. Correlations between appetite regulating hormones and FM% at age 3 and 6 months and the gain in FM% from 1-6 months in the total group, breastfed infants and formula fed infants.

	Total group n=297				Breastfeeding n=158				Formula feeding n=89			
	FM% 3 months	FM% 6 months	Δ FM% 1-6 months	Δ FM% 1-6 months	FM% 3 months	FM% 6 months	Δ FM% 1-6 months	Δ FM% 1-6 months	FM% 3 months	FM% 6 months	Δ FM% 1-6 months	Δ FM% 1-6 months
3 months												
Ghrelin	0.003, p=0.97	0.122, p=0.043	0.103, p=0.091	-0.072, p=0.37	0.128, p=0.12	0.128, p=0.12	0.172, p=0.037	-0.011, p=0.92	0.034, p=0.77	0.034, p=0.77	-0.033, p=0.78	-0.033, p=0.78
PYY	-0.019, p=0.75	0.064, p=0.29	0.069, p=0.26	-0.083, p=0.30	-0.103, p=0.21	-0.103, p=0.21	-0.104, p=0.21	-0.010, p=0.93	0.214, p=0.057	0.214, p=0.057	0.324, p=0.004	0.324, p=0.004
Ghrelin/PYY ratio	-0.007, p=0.91	0.076, p=0.21	0.059, p=0.34	-0.039, p=0.63	0.154, p=0.059	0.154, p=0.059	0.203, p=0.014	-0.039, p=0.72	-0.060, p=0.60	-0.060, p=0.60	-0.181, p=0.12	-0.181, p=0.12
Leptin	0.395, p<0.001	0.321, p<0.001	0.204, p=0.001	0.354, p<0.001	0.320, p<0.001	0.320, p<0.001	0.224, p=0.007	0.404, p<0.001	0.284, p=0.011	0.284, p=0.011	0.187, p=0.10	0.187, p=0.10
6 months												
Ghrelin		-0.031, p=0.68	-0.017, p=0.82		-0.098, p=0.35	-0.098, p=0.35	-0.078, p=0.47		-0.052, p=0.69	-0.052, p=0.69	0.111, p=0.41	0.111, p=0.41
PYY		-0.032, p=0.66	0.100, p=0.19		-0.130, p=0.22	-0.130, p=0.22	-0.015, p=0.89		0.125, p=0.34	0.125, p=0.34	0.172, p=0.20	0.172, p=0.20
Ghrelin/PYY ratio		-0.019, p=0.80	-0.044, p=0.56		-0.041, p=0.70	-0.041, p=0.70	-0.073, p=0.50		-0.066, p=0.62	-0.066, p=0.62	0.065, p=0.63	0.065, p=0.63
Leptin		0.337, p<0.001	0.207, p=0.006		0.345, p=0.001	0.345, p=0.001	0.195, p=0.070		0.347, p=0.007	0.347, p=0.007	0.280, p=0.033	0.280, p=0.033

Data presented as correlation coefficient (R) with p-values. FM%: fat mass percentage; N; number; PYY; peptide YY.

Table 4. Ghrelin, PYY, ghrelin/PYY ratio and leptin levels at age 3 months in breastfed versus formula fed infants.

	Boys				Girls			
	Breastfeeding	Formula feeding	p-value	n=78	Breastfeeding	Formula feeding	p-value	n=80
	n=158 (78 boys)	n=89 (53 boys)						
Ghrelin (pg/ml)	51.9 [27.7–80.1]	67.0 [30.6–108.0]	0.039	52.4 [31.6–80.0]	50.6 [23.9–81.1]	81.0 [37.3–122.7]	0.036	50.6 [23.9–81.1]
PYY (pg/ml)	231.4 [178.2–314.5]	185.3 [151.0–245.5]	<0.001	231.4 [177.2–306.8]	231.4 [176.6–326.5]	194.0 [150.8–262.8]	0.027	231.4 [176.6–326.5]
Ghrelin/PYY ratio	0.22 [0.10–0.36]	0.34 [0.15–0.61]	0.002	0.22 [0.12–0.39]	0.19 [0.10–0.35]	0.36 [0.14–0.55]	0.015	0.19 [0.10–0.35]
Leptin (pg/ml)	1090.5 [600.5–1776.9]	1417.0 [827.5–2191.7]	0.057	874.4 [457.8–1586.5]	1268.0 [660.0–1960.7]	1441.4 [954.8–2551.2]	0.16	1222.1 [771.8–1964.5]

Data expressed as median [IQR]. N; number, PYY; peptide YY.

in BF infants compared to FF infants ($p=0.002$). Leptin levels at age 3 months tended to be lower in BF infants ($p=0.057$).

Correlations between ARH and human milk macronutrient content at age 3 months

In BF infants, ghrelin, PYY, ghrelin/PYY ratio and leptin levels at age 3 months did not correlate with human milk macronutrients (fat, energy, carbohydrate and protein) at age 3 months.

Correlations between ARH and infant appetite at age 3 months

We investigated ARH levels in relation with infant appetite based on BEBQ scores. In BF infants, none of the ARH levels correlated with infant appetite.

In FF infants, PYY levels correlated inversely with infants getting full up easily ($R=-0.225$, $p=0.039$), indicating that higher PYY levels correlated with less easily getting full up during a feed. Higher ghrelin levels and ghrelin/PYY ratio tended to correlate with infants always demanding a feed ($R=0.205$, $p=0.062$ and $R=0.208$, $p=0.059$, respectively.), indicating that higher ghrelin levels and ghrelin/PYY ratio correlated with less satiety. Leptin levels did not correlate with infant appetite outcomes of the BEBQ.

DISCUSSION

In a large group of healthy, term-born infants, we found that ghrelin levels and ghrelin/PYY ratio increased from 3 to 6 months, while PYY and leptin levels decreased. ARH levels were similar between boys and girls, except for a higher leptin in girls at 3 months. Leptin correlated with FM% at 3 and 6 months and the gain in FM% from 1 to 6 months, a critical window for adiposity programming, in BF and FF infants. In BF infants only, also ghrelin and ghrelin/PYY ratio correlated with the gain in FM% from 1 to 6 months. BF infants had lower ghrelin and higher PYY levels compared to FF infants at age 3 months. ARH levels did not correlate with human milk macronutrients. Regarding appetite, higher PYY levels in FF infants correlated with having more difficulty getting full up during a feed, while a higher ghrelin level and ghrelin/PYY ratio tended to correlate with less satiety.

We present for the first time longitudinal levels of ghrelin, PYY and leptin during the first 6 months of life in healthy infants. These first 6 months after birth are considered a critical window for adiposity programming (9, 10). Ghrelin levels increased significantly during the first 6 months of life, which is in line with a study from birth until 3 months (31), while PYY and leptin levels decreased. Our results complement current knowledge as other studies used cord blood to investigate leptin at birth (16, 17), a single measurement of ghrelin, PYY and leptin at 4 months (24) or a single measurement of ghrelin and leptin between 11 days

and 22 months (23). One study investigated leptin in multiple measurements until age 6 months, but only in a small group (32).

We also present ghrelin/PYY ratios during the first 6 months of life. Ghrelin/PYY might be a marker for orexigenic drive, as studies in subjects with Prader-Willi syndrome reported that subjects with hyperphagia due to PWS have hyperghrelinemia and attenuated PYY response to fat, resulting in a high ghrelin/PYY ratio of 10 (5, 6). We show that ghrelin/PYY ratio in healthy, term-born infants increased from age 3 to 6 months, but remained below 1.0.

Ghrelin levels at age 6 months correlated positively with fasting time, whereas PYY levels correlated inversely with fasting time. This is in line with findings that ghrelin increases pre-prandially and decreases post-prandially and PYY levels act opposite with low levels in fasting state (33).

We investigated several factors that could potentially influence the levels of appetite regulating hormones. Only leptin levels were different between boys and girls, with girls having higher levels at age 3 months, but not at age 6 months. Similar results have been reported for leptin levels at age 1, 4 and 6 months in a small group of infants (32) and at birth (15, 34).

Birthweight SDS did not correlate with ghrelin, PYY and leptin levels at age 3 and 6 months. One previous study reported an association between birthweight and leptin in cord blood with lower cord blood leptin associating with smaller size at birth (13). Maternal pre-pregnancy BMI and weight gain during pregnancy did not correlate with ghrelin, PYY, ghrelin/PYY ratio and leptin levels. This is in contrast to a study showing that infants from mothers with high pre-pregnancy BMI ($> 30 \text{ kg/m}^2$) had higher levels of leptin at age 9 months, but ghrelin and PYY were not investigated (35). The majority of mothers in our cohort had, however, a pre-pregnancy BMI below 25 kg/m^2 and less than 10% had a BMI of $> 30 \text{ kg/m}^2$.

As the first 6 months of life are a critical window for adiposity programming (9, 10), we investigated ARH levels in relation with FM% and the gain in FM% during this period. Leptin at 3 and 6 months correlated with FM% at the same age, and with the gain in FM% from 1 to 6 months in the total group. In BF infants, ghrelin at 3 months did not correlate with FM% at the same age, but did correlate with FM% at 6 months, thus 3 months later, suggesting that potential effects of ghrelin on FM% might reveal later while correlations between leptin levels and FM% are present at the same age. This might be explained by the fact that leptin is secreted by adipose tissue. In BF infants, also ghrelin and ghrelin/PYY ratio at 3 months correlated weakly with the gain in FM% during the critical window, while in FF infants, only PYY at age 3 months correlated with the gain in FM% from 1-6 months.

Studies with one leptin measurement either at birth (36), at age 4 months (24) or leptin measurements during the first 6 months in a small group of infants (37) have shown associations with body composition. Two studies investigated leptin levels at birth or at age 6 months until childhood and associations with FM% and/or BMI in childhood (38, 39), but other ARH levels and measured FM% during the first 6 months were not investigated. We now show that leptin correlates not only with FM% at 3 and 6 months, but also with the gain in FM% in early life, which is of particular interest as we have previously shown that the gain in FM% during the first 6 months is associated with FM% at age 2 years (12).

We show that infants with exclusive breastfeeding versus formula feeding had different levels of ghrelin, PYY and leptin during the first 6 months of life. This is in line with our previous study in a smaller group of infants (25) and two other studies studying the first 4 months of life (23, 40). FF infants had higher ghrelin levels, which stimulates intake, while PYY levels were lower, indicating less satiety. As a result, the ghrelin/PYY ratio was higher in FF infants, supporting a higher orexigenic drive in FF infants.

To the best of our knowledge, present study is the first one to present ghrelin, PYY, ghrelin/PYY ratio and leptin levels in relation to human milk macronutrient composition and infant appetite. In contrast to our hypothesis, ARH levels did not correlate with human milk fat and energy content in BF infants. We investigated human milk macronutrient composition and not the total daily intake and total daily macronutrient intake in BF infants, as it is difficult and laborious to measure the exact intake of human milk by 24 hours infant weighing or deuterium oxide testing in large cohort studies in healthy infants. Future research could investigate if 24-hours macronutrient intake will correlate with levels of ARH.

Infant appetite was investigated by the Baby Eating Behavior Questionnaire (BEBQ), a questionnaire for parents, at infant's age of 3 months (30). In BF infants, ARH did not correlate with infant appetite. In FF infants, however, higher PYY at age 3 months correlated with having more difficulty getting full up during a feed. Furthermore, higher PYY tended to correlate with less satiety. PYY decreases food intake and, as abovementioned, PYY levels were lower in FF infants compared to BF infants, suggesting that they might indeed have less satiety. In addition, PYY correlated with the gain in FM% from 1 to 6 months in FF infants, but not in BF infants, which suggests that early life PYY levels might contribute to the differences in body fat mass development between BF and FF infants.

PYY levels increase rapidly after food intake (2). Our blood samples, however, were collected only in fasting state, which therefore should have lower PYY levels compared to non-fasting state and during feeding (41). Stronger correlations are expected when investigating PYY peak levels in relation to infant appetite. When interpreting our results, one should take into

consideration that the BEBQ is a subjective tool for infant appetite. Our exploratory results, however, emphasize the need for future research on associations of ARH, and specifically the ghrelin/PYY ratio, in relation to infant appetite during and after feeding.

The strength of this study is the availability of longitudinal blood samples during the critical window for adiposity programming in a large group of healthy infants. In addition, we obtained detailed body fat mass measures on the same day that infant blood collection was performed. We did, however, only show fasting ARH levels and were not able to collect samples during and after feeding as we could ethically not take multiple blood samples per infant. We could, therefore, not determine the PYY peak level after food intake.

Conclusions

In conclusion, we present appetite regulating hormone trajectories in a large group of infants during the first 6 months of life, a critical window for adiposity programming. Ghrelin levels increased from 3 to 6 months, while PYY levels decreased. This results in an increase in ghrelin/PYY, suggesting more orexigenic drive over time. Leptin levels decreased in early life. ARH levels were similar between boys and girls, except for higher leptin levels in girls at 3 months. Formula fed infants had higher ghrelin and lower PYY levels, thus a higher ghrelin/PYY ratio, suggesting that formula fed infants have higher orexigenic drive. Higher leptin levels correlated with higher FM% at 3 and 6 months and with a higher gain in FM% during the critical window for adiposity programming. In breastfed infants, ghrelin and ghrelin/PYY ratio also correlated with the gain in FM%, indicating that leptin and ghrelin levels might be involved in adiposity programming during early life.

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Chapter 7

Predictive value of
appetite regulating hormone levels in early life
for later fat mass trajectories

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ABSTRACT

Background The first 1000 days, from conception to age 2 years, are an important window for infant development. Appetite regulating hormones (ARH) coordinate appetite and food intake, and might contribute to adiposity programming. Studies examining ARH trajectories and early fat mass (FM) development do not exist.

Objective To investigate associations between ARH at 3, 6 months and 2 years and FM parameters at 2 years, and associations between ARH and FM trajectories until 2 years.

Methods Birth cohort of 174 healthy infants (Sophia Pluto Cohort). Ghrelin (acylated), peptide YY (PYY), adiponectin and leptin measured at 3, 6 months and 2 years. FM% measured by PEAPOD and DXA scan, abdominal FM by ultrasonography.

Results Ghrelin increased and PYY decreased from 3 months to 2 years ($p < 0.001$), leading to increasing ghrelin/PYY ratio ($p < 0.001$). Adiponectin and leptin decreased ($p < 0.001$). Ghrelin, PYY and ghrelin/PYY ratio at 3 and 6 months did not correlate with FM parameters at 2 years. Adiponectin at 3 and 6 months correlated with FM% at 2 years ($R \geq 0.282, p = 0.001$) while the adiponectin decline correlated inversely. Leptin at 3 and 6 months correlated with FM% at 2 years, leptin at 2 years correlated strongly with FM% at same age ($R = 0.426, p < 0.001$). Ghrelin and ghrelin/PYY ratio trajectories from 3-6 months did not associate with FM% trajectory during that period, but infants with greater increase in ghrelin and ghrelin/PYY ratio had less increase in visceral FM ($p \leq 0.009$). Leptin, not adiponectin, trajectories associated with FM% trajectories until 2 years.

Conclusions Our data show that ghrelin and ghrelin/PYY ratio increased during the first 2 years of life, while PYY, adiponectin and leptin decreased. Ghrelin and ghrelin/PYY ratio had no predictive value for later FM%, but early life adiponectin levels might predict FM% at 2 years, suggesting that adiponectin is involved in early adiposity programming. Leptin levels mainly reflected current FM%, but also had some predictive value for later FM%.

INTRODUCTION

Appetite regulating hormones (ARH) are involved in the regulatory system of appetite, food intake and satiety (1). The hypothalamus plays an important role in controlling glucose and energy homeostasis (1, 2). ARH are secreted from the gastrointestinal tract (ghrelin, PYY) and adipose tissue (leptin, adiponectin) (1, 3). Ghrelin stimulates food intake, which could influence infant growth and body composition, while leptin and PYY reduce food intake and increase metabolic rate. Adiponectin increases fatty acid and carbohydrate uptake (2, 3). Ghrelin/PYY ratio is of interest as an orexigenic drive marker, rather than ghrelin and PYY levels separately (4, 5).

The first 1000 days of life, from conception to age 2 years, is an important period for infant development (6, 7). Within this period, we previously found that the change in fat mass percentage (FM%) during the first 6 months, in contrast to the 6- to 12-month period, is associated with higher FM% and abdominal subcutaneous fat mass (FM) at age 2 years (8). These data support a critical window for adiposity programming in early life (9, 10).

Most studies used cord blood (11-16) or newborn blood spots (17, 18) to investigate ARH at birth and later growth and body composition, or included infants born premature or small-for-gestational age (19, 20). Two studies investigated only leptin and adiponectin levels at birth, 2, 5 and 9 years in a small group of infants or at 6 months, 5.5 and 8 years (3, 21). However, insights in ARH trajectories in association with FM parameters during the first 6 months and the period thereafter until 2 years in term-born infants are still lacking.

Our primary objective was to investigate longitudinal ghrelin, PYY, adiponectin and leptin levels during the first 2 years of life, at 3, 6 months and 2 years, and associate these levels with FM parameters at age 2 years to determine potential predictors for FM development in the total group and in exclusively breastfed versus formula fed infants. Additionally, we investigated associations of ARH trajectories until 6 months and from 6 months to 2 years with trajectories of FM parameters during the same periods. We hypothesized that ghrelin and ghrelin/PYY ratio at 3 and 6 months would predict FM% at 2 years, while adiponectin and leptin would reflect current FM%.

METHODS

Participants

The study population consisted of healthy, term-born infants, participating in the Sophia Pluto Birth Cohort in Rotterdam area (The Netherlands). All participants were term-born (≥ 37

weeks of gestation), age <28 days, uncomplicated neonatal period without signs of severe asphyxia (5-minutes Apgar score <3). Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy or a significant maternal medical condition that could interfere with study results. For present study, we included 174 infants born singleton with blood sampling at 3, 6 months and 2 years. The Sophia Pluto Study obtained approval by the Medical Ethics Committee of Erasmus Medical Center and parental written informed consent for participants.

Data Collection and Measures

Outpatient clinic visits were scheduled at 1, 3, 6 months and 2 years (Table 1). Birth data were obtained from hospital records and anthropometric data were collected (22, 23).

Blood samples

Blood samples at 3, 6 months and 2 years were collected in EDTA tubes by toe prick after infants had fasted ≥ 2 hours. DPP4-inhibitor, Serine-Protease-inhibitor and Protease-inhibitor (Merck Chemicals Netherlands) were added for stabilizing ARH. Blood was centrifuged at 4°C to prepare plasma, which was quickly frozen and stored at -80°C until analyses. Ghrelin (acylated), PYY and leptin were determined by MILLIPLEX MAP Human Metabolic Hormone Magnetic Bead Panel (HMHEMAG-34K) and adiponectin by MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 (HADK1MAG-61K, Millipore Corporation, Billerica, MA), using commercial protocol. Intra-assay and inter-assay CV were 10% and 15%, respectively (both panels). Median (IQR) fasting time (blood collection time minus last feeding time) was $\geq 2:20$ (1:45-2:50) hours at all ages.

Fat mass

Until 6 months, FM was assessed by air-displacement plethysmography (PEA POD, COSMED, Italy) as described in detail elsewhere (22, 24). At 2 years, a Dual Energy X-ray Absorptiometry (DXA) scan was performed (Lunar Prodigy, GE Healthcare, UK, enCORE software version 14.1) (23).

Abdominal fat mass

Subcutaneous and visceral fat thickness (cm) were measured by ultrasound and described in detail elsewhere (22, 25).

Infant feeding

Exclusive breastfeeding (EBF; n=96) or formula feeding (EFF; n=45) was defined as infants receiving only EBF or EFF, respectively, and no mixed feeding for 3 months after birth.

Statistical Analysis

Clinical characteristics are expressed as median (interquartile range (IQR)) in Table 1. Longitudinal ARH levels during the first two years of life were analyzed using linear mixed model analysis. Sex was used as a covariate. Age was modeled as categorical variable indicating hospital visits at 3, 6 months and 2 years.

Spearman correlation coefficient was used to determine correlations between non-parametric parameters. We also investigated associations between ARH and FM parameters with adjustment for sex, but since the main results were similar, we present the unadjusted linear correlations. Data are presented for total group and for EBF and EFF only in case of different results compared to the total group.

Associations between ARH trajectories and trajectories of FM parameters were analyzed by linear mixed models with FM parameters (dependent variable) and sex, interaction of age with sex, change in ARH and interaction of age with change in ARH (covariates). SPSS statistical package version 25 (SPSS Inc. Chicago, Illinois) was used and p-values <0.05 were considered statistically significant.

Table 1. Clinical characteristics of 174 infants.

Age		Birth	1 month	3 months	6 months	2 years
Weight (kg)	M	3.38 [3.09 – 3.77]	4.37 [3.96 – 4.78]	6.24 [5.78 – 6.72]	7.96 [7.41 – 8.66]	13.31 [11.81 – 14.17]
	F	3.21 [2.80 – 3.51]	3.98 [3.52 – 4.33]	5.70 [5.09 – 6.21]	7.37 [6.83 – 7.89]	12.30 [11.44 – 13.10]
Length (cm)	M	51.0 [49.0 – 52.8]	55.0 [53.5 – 56.5]	62.4 [60.5 – 63.4]	69.0 [67.0 – 70.3]	89.9 [87.5 – 91.8]
	F	49.0 [48.0 – 50.5]	53.5 [51.4 – 55.1]	60.0 [58.5 – 61.2]	66.6 [65.2 – 68.1]	87.5 [85.5 – 89.7]
FM (%)	M	NA	15.7 [13.5 – 19.0]	22.8 [19.8 – 25.6]	24.5 [22.2 – 27.4]	17.7 [15.2 – 20.2]
	F	NA	15.1 [11.8 – 19.0]	24.4 [17.9 – 26.5]	25.6 [21.7 – 29.0]	17.8 [15.0 – 21.4]
Abdominal subcutaneous FM (cm)	M	NA	NA	0.41 [0.33 – 0.49]	0.41 [0.35 – 0.52]	0.35 [0.29 – 0.41]
	F	NA	NA	0.37 [0.31 – 0.50]	0.41 [0.33 – 0.51]	0.34 [0.27 – 0.40]
Visceral FM (cm)	M	NA	NA	2.41 [1.89 – 2.87]	2.08 [1.79 – 2.66]	2.02 [1.73 – 2.45]
	F	NA	NA	2.23 [1.94 – 2.70]	2.22 [1.72 – 2.64]	2.19 [1.84 – 2.63]

Data expressed as median [interquartile range] for boys (M) and girls (F). Birth length only available for 64 boys and 37 girls. FM: fat mass.

RESULTS

Clinical characteristics are presented in Table 1, 59.2% was male and 64.4% Caucasian. Median gestational age was 39.9 (39.0-40.9) weeks.

ARH trajectories during the first 2 years of life

Ghrelin (acylated) levels increased from 3 months to 2 years ($p < 0.001$), while PYY levels decreased ($p < 0.001$) (Figure 1), which resulted in an increasing ghrelin/PYY ratio ($p < 0.001$). Ghrelin, PYY and ghrelin/PYY ratio were similar between boys and girls (all $p \geq 0.30$) (Table 2). Adiponectin levels decreased ($p < 0.001$) and trajectories were similar between boys and girls. Leptin levels also decreased ($p < 0.001$). Girls had higher leptin levels at 3 months compared to boys ($p = 0.002$), but similar levels at 2 years.

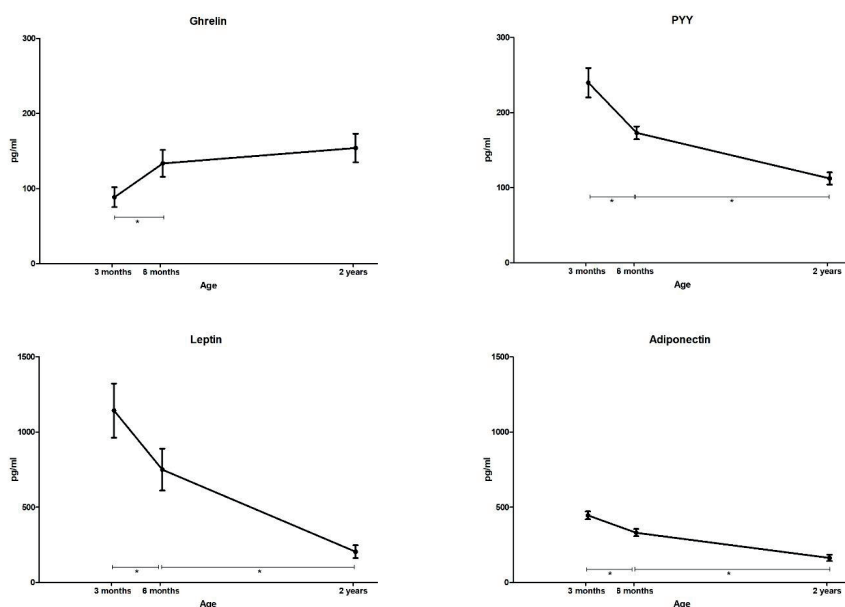


Figure 1. Appetite regulating hormone trajectories during the first 2 years of life.

Data are presented as estimated marginal means (95% confidence interval). * represent a significant change during that period. PYY: peptide YY.

Associations of ARH with FM parameters at 2 years

ARH during the first 6 months of life

First, we investigated if ARH levels at 3 and 6 months and change in ARH between these ages correlated with FM parameters at 2 years (Table 3) to determine potential predictive value. Ghrelin, PYY and ghrelin/PYY ratio at 3, 6 months and change from 3 to 6 months did not correlate with any FM parameter at 2 years.

Table 2. Levels of appetite regulating hormones at age 3 and 6 months and 2 years in boys and girls.

	Boys			Girls		
	3 months	6 months	2 years	3 months	6 months	2 years
Ghrelin (pg/ml)	86.5 [68.6 – 104.3]	144.8 [121.5 – 168.2]	152.3 [127.6 – 177.1]	91.1 [71.4 – 110.8]	118.1 [90.9 – 145.4]	156.5 [126.2 – 186.8]
PYY (pg/ml)	245.2 [218.5 – 271.9]	176.6 [165.3 – 187.9]	110.8 [100.3 – 121.3]	233.4 [204.4 – 262.4]	167.9 [154.7 – 181.1]	114.5 [101.7 – 127.4]
Ghrelin/PYY ratio	0.43 [0.31 – 0.54]	0.90 [0.72 – 1.07]	1.75 [1.43 – 2.06]	0.47 [0.35 – 0.60]	0.81 [0.61 – 1.00]	1.63 [1.24 – 2.02]
Adiponectin (pg/ml)	436.4 [400.8 – 472.1]	329.3 [297.5 – 361.1]	166.9 [138.8 – 194.9]	457.5 [418.0 – 497.1]	333.2 [296.4 – 368.9]	158.2 [124.8 – 191.6]
Leptin (pg/ml)	864.7 [632.0 – 1097.4]	659.4 [476.7 – 842.0]	196.0 [142.2 – 249.8]	1471.0 [1218.3 – 1723.8]	869.7 [655.2 – 1084.1]	217.9 [151.9 – 283.9]

Data presented as estimated marginal means [95% confidence interval], based on mixed model analysis with samples at 3 months (n=102), 6 months (n=156) and 2 years (n=158). * p-value for the change in ARH from 3 months to 2 years in boys versus girls. PYY; peptide YY.

Table 3. Correlations between (change in) appetite regulating hormones and FM%, abdominal visceral and subcutaneous FM at the age of 2 years.

	FM% 2 years		Visceral FM 2 years		Subcutaneous FM 2 years	
	R	p-value	R	p-value	R	p-value
3 months						
Ghrelin	0.057	0.60	0.030	0.77	0.063	0.54
PYY	0.029	0.79	0.019	0.86	0.053	0.60
Ghrelin/PYY ratio	0.024	0.83	0.004	0.97	0.041	0.69
Adiponectin	0.335	0.001	0.013	0.90	0.144	0.15
Leptin	0.214	0.047	-0.025	0.81	0.177	0.08
6 months						
Ghrelin	0.095	0.27	0.096	0.24	-0.025	0.76
PYY	-0.004	0.96	0.002	0.98	0.074	0.37
Ghrelin/PYY ratio	0.076	0.38	0.098	0.24	-0.065	0.43
Adiponectin	0.282	0.001	0.021	0.80	0.081	0.33
Leptin	0.286	0.001	0.042	0.61	0.305	<0.001
2 years						
Ghrelin	-0.072	0.39	-0.076	0.35	0.026	0.75
PYY	-0.013	0.88	0.105	0.20	0.005	0.95
Ghrelin/PYY ratio	-0.077	0.37	-0.087	0.29	0.008	0.92
Adiponectin	0.124	0.15	0.038	0.65	0.019	0.82
Leptin	0.426	<0.001	0.024	0.77	0.244	0.002
Δ 3-6 months						
Ghrelin	-0.022	0.85	0.082	0.45	-0.105	0.32
PYY	-0.078	0.49	-0.080	0.45	-0.032	0.76
Ghrelin/PYY ratio	-0.022	0.85	0.125	0.24	-0.105	0.32
Adiponectin	-0.271	0.015	-0.072	0.50	-0.064	0.55
Leptin	-0.081	0.47	0.014	0.90	0.069	0.51
Δ 6 months – 2 years						
Ghrelin	-0.127	0.16	-0.144	0.09	0.061	0.48
PYY	0.050	0.58	0.041	0.64	-0.057	0.50
Ghrelin/PYY ratio	-0.153	0.087	-0.136	0.11	0.027	0.75
Adiponectin	-0.218	0.016	-0.073	0.41	-0.057	0.51
Leptin	-0.129	0.15	-0.038	0.66	-0.214	0.012

Data expressed as correlation coefficient (R) with corresponding p-values. FM%; fat mass percentage, FM; fat mass, PYY: peptide YY.

Adiponectin at 3 and 6 months correlated with FM% at 2 years ($R=0.335$ and $R=0.282$, $p=0.001$) and change in adiponectin from 3 to 6 months correlated inversely ($R=-0.271$, $p<0.001$), indicating that a greater decline in adiponectin correlated with higher FM% at 2 years. Adiponectin at 3 months correlated inversely with change in adiponectin from 3 to 6 months ($R=-0.413$, $p<0.001$). Adiponectin at 3 and 6 months and change in adiponectin did not correlate with abdominal visceral and subcutaneous FM at 2 years.

Leptin at 3 months and 6 months correlated with FM% at 2 years ($R=0.214$, $p=0.047$ and $R=0.286$, $p=0.001$, resp.), but change in leptin from 3 to 6 months did not. Leptin did not correlate with visceral FM, but leptin at 6 months correlated with abdominal subcutaneous FM at 2 years ($R=0.244$, $p=0.002$).

ARH from 6 months until 2 years

Secondly, we investigated if ARH levels at 2 years and change in ARH between 6 months and 2 years correlated with FM parameters at 2 years.

Neither ghrelin and ghrelin/PYY ratio at 2 years and change from 6 months to 2 years, nor adiponectin at 2 years correlated with FM parameters. Change in adiponectin from 6 months to 2 years, however, correlated inversely with FM% at 2 years ($R=-0.218$, $p=0.016$), indicating that a greater decline in adiponectin correlated with higher FM%, in line with the period from 3 to 6 months. Adiponectin at 6 months correlated inversely with change in adiponectin from 6 months to 2 years ($R=-0.599$, $p<0.001$). Leptin at 2 years correlated with FM% at 2 years ($R=0.426$, $p<0.001$), while the change from 6 months until 2 years did not. Leptin at 2 years and change in leptin from 6 months until 2 years did correlate with abdominal subcutaneous FM at 2 years ($R=-0.214$, $p=0.012$, $R=0.244$, $p=0.002$, resp.).

In EFF infants, ghrelin and ghrelin/PYY ratio at 2 years correlated inversely with visceral FM at 2 years ($R=-0.519$, -0.356 , resp., $p\leq 0.026$), indicating that higher ghrelin and ghrelin/PYY ratio correlated with lower visceral FM. The increase in ghrelin and ghrelin/PYY ratio from 6 months to 2 years correlated also inversely with visceral FM at 2 years in EFF ($R=-0.386$, -0.436 , resp., $p\leq 0.026$) and EBF infants ($R=-0.228$, -0.227 , resp., $p\leq 0.046$).

Associations between ARH trajectories and FM trajectories during the first 2 years of life

In addition, we investigated if ARH trajectories were associated with the trajectories of FM parameters during the first 2 years of life. As FM% increases during the first 6 months of life and subsequently decreases until 2 years (23), we investigated trajectories of FM parameters from 3 to 6 months and from 6 months to 2 years separately (Table 4).

Table 4. Associations between appetite regulating hormone trajectories and trajectories of FM%, abdominal visceral and subcutaneous FM from 3 to 6 months and from 6 months to 2 years.

	Change in FM%		Change in visceral FM		Change in subcutaneous FM	
	Estimate	p-value	Estimate	p-value	Estimate	p-value
3 – 6 months						
Δ Ghrelin (pg/ml)	0.078	0.82	-0.191	0.009	-0.000	0.98
Δ PYY (pg/ml)	0.380	0.32	0.107	0.17	-0.028	0.024
Δ Ghrelin/PYY ratio	0.172	0.71	-0.276	0.003	0.008	0.61
Δ Adiponectin (pg/ml)	0.093	0.80	0.004	0.96	-0.008	0.54
Δ Leptin (pg/ml)	0.121	0.001	-0.006	0.45	0.003	0.009
6 months – 2 years						
Δ Ghrelin (pg/ml)	-0.307	0.28	-0.082	0.08	0.006	0.38
Δ PYY (pg/ml)	0.539	0.35	0.055	0.56	-0.007	0.63
Δ Ghrelin/PYY ratio	-0.395	0.12	-0.035	0.39	-0.003	0.62
Δ Adiponectin (pg/ml)	-0.009	0.98	-0.042	0.41	0.004	0.60
Δ Leptin (pg/ml)	0.165	<0.001	0.002	0.79	-0.000	0.80

Associations presented as Estimate per 100pg/ml change in ARH (except for ghrelin/PYY ratio), with p-value. FM%; fat mass percentage, FM; fat mass, PYY; peptide YY.

Trajectories during the first 6 months of life

The trajectories of ghrelin, PYY and ghrelin/PYY ratio from 3 to 6 months did not associate with the FM% trajectory during the same period. However, the ghrelin and ghrelin/PYY ratio trajectories from 3 to 6 months were inversely associated with the visceral FM trajectory during the same period ($p=0.009$ and 0.003 , resp.), indicating that infants with greater increase in ghrelin and ghrelin/PYY ratio had less increase in visceral FM. The ghrelin and ghrelin/PYY ratio trajectories did not associate with the abdominal subcutaneous FM trajectory. The PYY trajectory from 3 to 6 months was inversely associated with the abdominal subcutaneous FM trajectory during the same period ($p=0.024$), indicating that infants with greater decline in PYY had a greater increase in abdominal subcutaneous FM.

The adiponectin trajectory from 3 to 6 months did not associate with FM%, visceral and subcutaneous FM trajectories during this period. The leptin trajectory from 3 to 6 months did associate with FM% and subcutaneous FM trajectories during the same period ($p=0.001$ and 0.009 , resp.), indicating that infants with less decline in leptin had greater increase in FM% and abdominal subcutaneous FM.

Trajectories from 6 months until 2 years

In contrast to the period of 3 to 6 months, there were no associations between ghrelin, PYY and ghrelin/PYY ratio trajectories from 6 months to 2 years and any trajectory of FM parameters. The adiponectin trajectory did also not associate with any trajectories of FM parameters.

The leptin trajectory from 6 months to 2 years, however, associated with the FM% and abdominal subcutaneous FM trajectories during the same period ($p < 0.001$), indicating that infants with less decline in leptin had less decline in FM%.

DISCUSSION

We present for the first time ARH trajectories during the first 2 years of life and associations with longitudinally measured FM parameters in healthy infants. Ghrelin and ghrelin/PYY ratio, representing orexigenic drive, increased from 3 months until 2 years, while PYY, adiponectin and leptin decreased. When investigating the potential predictive value of ARH for adiposity development, we observed that adiponectin at 3 and 6 months, a greater decline in adiponectin during the first 2 years and leptin at all ages correlated with higher FM% at 2 years. When investigating ARH trajectories, ghrelin and ghrelin/PYY ratio trajectories from 3-6 months only associated with the visceral FM trajectory during the same period. The leptin trajectory associated with the FM% trajectory until 2 years. Our findings suggest that ghrelin and ghrelin/PYY ratio in early life are, in contrast to our hypothesis, no predictors for later FM%, but adiponectin during the first 6 months, and not thereafter, might predict FM% at 2 years. Leptin mainly reflects current FM%, but has also some predictive value for later FM%.

The first 2 years of life are an important period for infant development (6, 7), but early life longitudinal studies with multiple ARH measurements are very limited. Most studies used cord blood to investigate ARH at birth (12, 15, 16) or one sample during infancy (26, 27). One study showed lower adiponectin and leptin at 2 years compared to levels at birth, but had no samples between those ages (3). We now show that ghrelin increased during the first 2 years of life, while PYY, adiponectin and leptin decreased. The changes in ghrelin and PYY until 2 years result in an increase in ghrelin/PYY ratio over time. As this ratio is considered a marker for orexigenic drive (4, 5), the increase in ghrelin/PYY ratio in infants might indicate increasing orexigenic drive, leading to more appetite stimulation over time.

ARH trajectories were similar in boys and girls, but girls had higher leptin levels compared to boys until 2 years. This difference started with significantly higher leptin levels in girls at 3 months, which is in line with literature (13, 28, 29).

The first 6 months of life are considered a critical window for adiposity programming (9, 10). FM% increases during this period (23, 30, 31) and decreases thereafter (23). We have previously shown that only the change in FM% until 6 months and not in the 6 months thereafter associated with greater adiposity at 2 years (8). We, therefore, investigated whether ARH levels during a critical window for adiposity programming and thereafter, from 6 months until 2 years, were associated with FM parameters at 2 years. In addition, we investigated if ARH trajectories from 3 to 6 months and from 6 months to 2 years associated with trajectories of FM parameters in the same periods.

In contrast to our hypothesis, ghrelin and ghrelin/PYY ratio at 3 and 6 months were not associated with any FM parameter at 2 years, thus not predictive for FM% at 2 years. Ghrelin and ghrelin/PYY ratio trajectories during the first 6 months of life did also not associate with the FM% trajectory during the same period. However, greater increase in ghrelin and ghrelin/PYY ratio during the first 6 months associated with less increase in visceral FM during the same period. These findings are in line with literature describing that visceral FM inversely correlated with ghrelin levels (32) and studies reporting lower ghrelin levels in obese children and adults (33, 34). In subjects with Prader-Willi syndrome, characterized by hyperphagia and excessive weight, higher ghrelin levels (34, 35) and reduced visceral adiposity compared to obese controls have been described (35, 36).

In EFF infants, a higher ghrelin and ghrelin/PYY ratio at 2 years correlated with lower visceral FM at 2 years, while a greater increase in ghrelin and ghrelin/PYY ratio from 6 months to 2 years correlated with lower visceral FM at 2 years in both EFF and EBF infants. Our data, therefore, suggest that ghrelin and ghrelin/PYY ratio trajectories during a critical window for adiposity programming might contribute specifically to visceral FM development instead of total FM development. This could be an important finding as specifically increased visceral FM has been associated with unfavorable metabolic health profiles in childhood and later life (37, 38). Future studies with longer-term follow-up are required for confirmation.

A greater decline in PYY during the first 6 months was only associated with greater increase in subcutaneous FM in the same period, which is in line with mice studies showing that PYY-deficient mice had increased subcutaneous adiposity (39).

Higher adiponectin at 3 and 6 months and greater decline in adiponectin until 2 years resulted in higher FM% at 2 years. This suggests that, in contrast to our hypothesis, the actual adiponectin levels during a critical window for adiposity programming might predict FM% at 2 years, while also the decline in adiponectin between 3 months and 2 years associated with FM% at 2 years. The latter finding could be explained by the fact that infants with greater decline in adiponectin from 3 months to 2 years had higher adiponectin levels at 3

and 6 months. Our findings are in line with literature showing lower adiponectin in adults with overweight or obesity (40-42) and complement a study investigating adiponectin and leptin at birth, 2, 5 and 9 years (3). That study, however, did not investigate adiponectin between birth and 2 years. Factors affecting adiponectin and consequences of its changes during early infancy were therefore not investigated (3). We now show that specifically the adiponectin levels during the first 6 months as well as the decline in adiponectin from 3 months to 2 years might be involved in adiposity programming.

Leptin at 3 and 6 months correlated with FM% at 2 years, but leptin at 2 years showed the strongest correlation with FM% and abdominal subcutaneous FM at the same age. The leptin trajectory from 3 months to 2 years also associated with FM% and abdominal subcutaneous FM trajectories during this period. We have previously also shown that leptin at 3 and 6 months correlated with FM% at the same ages (43). Altogether, these findings indicate that leptin mainly reflects current FM%. This is in line with the abovementioned study showing that leptin closely reflects body size (3). We now add that also the leptin trajectory during the first 2 years of life corresponds with the FM% trajectory during the same period and that early leptin levels have some predictive value for later FM%.

We had expected that ARH during the period from 6 months until 2 years would have shown more associations with FM parameters at 2 years, since this period is closer to the study endpoint. Our findings, however, show that mainly ARH levels during the period from 3 to 6 months associated with FM parameters at 2 years, thereby supporting that the first 6 months of life are a critical window for adiposity programming in which particularly adiponectin and leptin could play a role in FM development.

Conclusions

In conclusion, ghrelin levels increase during the first 2 years of life and PYY levels decrease, resulting in an increase in ghrelin/PYY ratio which represents an increase in orexigenic drive. Leptin and adiponectin levels decrease. Our data show that ghrelin and ghrelin/PYY ratio in early life had, in contrast to our hypothesis, no predictive value for later FM%, but early life adiponectin levels and its trajectories might predict FM% at 2 years, suggesting that adiponectin is involved in early adiposity programming. Leptin levels mainly reflect current FM%, but have also some predictive value for later FM%. Our findings can potentially be used for personalized screening tools for obesity prevention starting at early age.

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Chapter 8

General discussion

GENERAL DISCUSSION

This thesis presents the results of 6 studies in healthy term-born infants participating in the Sophia Pluto Study.

We addressed the use of a vacuum cushion during Dual Energy X-ray Absorptiometry (DXA) scans for obtaining accurate measurements of body composition in order to construct longitudinal values of body composition. We investigated the influence of a rapid increase in fat mass in early life on body composition during the first 2 years of life. In addition, we explored the influence of human milk macronutrients, infant appetite, leukocyte telomere length and appetite regulating hormones on body composition development during the first 2 years of life.

In this chapter, the results of the studies are discussed in light of current literature. In addition, the clinical implications of our findings and the directions for future research are addressed.

Body composition assessment in early life

Chapter 2 describes the reliability of the use of a vacuum cushion during Dual Energy X-ray Absorptiometry (DXA) scans to prevent movement artifacts in infants between age 6 months and 2 years. It also describes the comparability between body composition measurements by air-displacement plethysmography (ADP) up to age 6 months and DXA measurements from age 6 months. In addition, this chapter presents longitudinal sex-specific reference values for body composition until age 2 years.

We show that the use of a vacuum cushion during DXA measurements limited movement artifacts and resulted in reliable measurements of fat mass percentage (FM%) up to the age of 2 years. The measurements of FM% by ADP and DXA with vacuum cushion were comparable at the transition point of both measurement techniques at age 6 months, whereas the measurements of ADP and DXA without vacuum cushion were not. These findings allowed us to use a vacuum cushion during DXA scans in order to construct for the first time longitudinal body composition charts from 1 month until 2 years, which are important for clinical and research use. Our reference charts show that FM% increased until age 6 months and declined from 6 months to 2 years. Girls had higher FM% compared to boys.

DXA measurements are difficult to perform in young infants due to movement artifacts, resulting in high percentages of unsuccessful measurements (1, 2). For the first time we show that the use of a vacuum cushion resulted in a high number of successful DXA measurements by limiting movement artifacts. The use of a vacuum cushion also resulted in reliable FM% measurements. A recent review of body composition measurements from birth until age 5 years stated that swaddling of children older than age 6 months during DXA scan was ineffective at preventing movement (3) and that it is important to avoid infants and children to be separated from their parent during body composition measurements, because it may

cause distress (3). Our approach of measuring FM% by ADP until 6 months, with the infant being able to see its parent during a short duration of the measurement, followed by DXA with vacuum cushion from age 6 months, with the parent being close to the infant, proved to be effective in preventing distress and movement during DXA.

FM% measurements by ADP and DXA with vacuum cushion were comparable at the transition point at age 6 months, with Bland-Altman plot showing no potential bias. These results are in contrast to FM% measurements of ADP and DXA without vacuum cushion, which showed potential bias. Measurements of body composition by DXA without vacuum cushion at this young age are, therefore, not suitable. Two earlier studies compared FM% by ADP and DXA (4, 5), but showed conflicting results, probably due to the small group of infants. In addition, both studies did not use a vacuum cushion during DXA scan. One study showed higher FM% measured by ADP compared to DXA (5), while another study showed higher FM% by DXA without cushion compared to ADP (4).

Our abovementioned findings allowed us to use a vacuum cushion during DXA scans to prevent movement artifacts and to subsequently construct longitudinal body composition charts from age 1 month until 2 years for the first time. These longitudinal charts are particularly important for clinical and research use. Earlier studies were based on anthropometric outcomes, for example weight-for-length SDS and BMI (6), but we have previously shown that infants with a similar weight and weight-for-length SDS may have a different FM% (7). Until now, assessment of longitudinal body composition from infancy into childhood was challenging as the various methodologies have barriers and limitations (8-10). A very recent study compared body composition by ADP, DXA and quantitative nuclear magnetic resonance and computed the 4C model in infants and children until age 6 years with completed study visits at the age range of 14 days until 74 months (8). In infants, ADP was the best method for assessment of individual FM, whereas DXA was best in estimating individual and group FM in children (8). Bland-Altman plots in infants revealed bias in all methods compared with the 4C model, with DXA having the lowest agreement in infants. That study, however, did not use a vacuum cushion during DXA scan. The use of a vacuum cushion resolves this low agreement between measurements.

We conclude that the use of a vacuum cushion to prevent movements during DXA scan provides reliable measurements of body composition that are comparable to ADP measurements, allowing the construction of longitudinal body composition charts. Our longitudinal measurements show that FM% increases until age 6 months and decreases thereafter until age 2 years and that girls have higher FM% compared to boys.

Fat mass in early life and later body composition

In Chapter 3, associations between gain in FM% SDS in the first and second 6 months of life with body composition at age 2 years were assessed. In addition, we investigated whether a

rapid increase in FM% in the first 6 months of life was associated with higher trajectories of body fat mass during the first 2 years of life.

We show that only the change in FM% in the first 6 months of life, and not in the 6 months thereafter, was associated with more adiposity at age 2 years. Infants with a rapid increase in FM% in these first 6 months had higher trajectories of FM% and FM index during the first 2 years of life.

The first 2 years of life are important for infant and adiposity development (11, 12). Accelerated weight gain, defined as a change in weight-for-age SDS of >0.67 between two time points (13, 14), has been associated with overweight and obesity in childhood and adulthood (15). Accelerated weight gain from birth to 2 years has been associated with more fat and more central fat distribution at the age of 5 years (14), and with an increase in visceral fat, abdominal subcutaneous fat and total adiposity in adulthood, at a mean age of 46.5 years (16).

Our research group previously reported that particularly rapid catch-up in weight in the first months of life, instead of the first 2 years, resulted in a higher FM%, more central adiposity and reduced insulin sensitivity in young adults (17). Others also reported a contribution of rapid weight gain during the first months to the risk of adiposity in infants (18, 19), not only in infants with low birthweight or born small-for-gestational age (20-22). The first 6 months of life are, therefore, considered a critical window for adiposity programming (17, 23).

For the first time we now show the associations of a rapid increase in FM%, instead of weight-for-age SDS, during the first year of life with later FM trajectories. FM measurements were used as we previously found differences in FM% in neonates with a similar weight (7). The change in FM% in the first 6 months of life, and not the 6 months thereafter, positively associated with adiposity at age 2 years. In addition, we found that infants with a rapid increase in FM% during the first 6 months of life had higher trajectories of FM% and FM index during the first 2 years of life, resulting in a higher FM% and FM index at age 2 years. Our findings convincingly show that there is a critical window for adiposity programming during the first 6 months of life. This knowledge is important for future research and particularly for the development of primary health care guidelines to prevent adiposity programming in the first months after birth.

Our study shows that the change in FM% during the first 6 months of life associates with more adiposity at the age of 2 years and that infants with a rapid increase in FM% during this period have higher trajectories of FM% until the age of 2 years. Our results support a critical window for adiposity programming in early life.

Human milk macronutrients, body composition and infant appetite

Chapter 4 describes longitudinal human hind milk macronutrient composition collected at infant's age of 1 and 3 months and associations with infant body composition until age 2 years and appetite during early life, in exclusively breastfed infants.

In 133 exclusively breastfed infants we show that human milk protein and energy content decreased between age 1 and 3 months, while fat and carbohydrate content tended to decrease. Human milk macronutrient composition was remarkably different between mothers, particularly in fat content. Higher milk fat and energy at age 3 months were associated with higher FM% at 6 months and higher gain in FM% between age 1 and 6 months. Furthermore, infants receiving higher caloric human milk satiated earlier and finished feeding faster. Human milk composition studies are very difficult to compare due to large variations in sampling and measurements. A review from 2018 by Eriksen et al, highlighted the importance of using standardized sampling and measurements to obtain high quality studies with comparable results (24). We instructed the mothers to collect human milk samples according to our study protocol and analyzed all samples by one human milk analyzer (Miris, Uppsala, Sweden), according to a standardized protocol. One investigator handled all samples (KdF) in order to limit measurement variations, which strengthened our results.

Human milk protein and energy content decreased, and fat and carbohydrate content tended to decrease over time, in line with literature (25-29). In addition, there was a wide variation in human milk macronutrient composition between mothers, particularly in fat content. We show for the first time that higher milk fat and energy at 3 months associate with higher FM% at 6 months and higher gain in FM% between age 1 and 6 months, in a large group of exclusively breastfed infants. Studies investigating human milk macronutrients in association with longitudinally measured FM% were very scarce (24). These studies comprised only small groups of infants (30, 31) or used skinfold measurements in the newborn, at 3 and 12 months to estimate adiposity (32).

In breastfed infants, it is difficult to determine the exact amount of daily milk intake (33) and thus the daily macronutrient intake. For the first time we show that infants receiving human milk with higher fat and energy content satiated earlier and finished feeding faster, which could be an important self-regulatory mechanism. This mechanism could prevent the intake of excessive macronutrients and subsequent abnormal adiposity programming. The difference in caloric value of human milk between mothers might also explain why some infants drink for a longer time than others. This is an important finding for parents, caretakers, researchers and health care professionals.

Our study shows that human milk fat and energy content at age 3 months associate with FM% at age 6 months and with the gain in FM% from age 1 to 6 months, the critical window for adiposity programming. Exclusively breastfed infants receiving higher caloric human milk satiate earlier and finish feeding faster.

Telomere length and body composition

Chapter 5 describes the results of longitudinal measures of leukocyte telomere length (LTL) during the first two years of life and the associations with potential stressors of LTL and body composition until age 2 years.

Our findings show that LTL shortened considerably (8.5%) between age 3 months and 2 years. More shortening in LTL from 3 months to 2 years was associated with a higher FM%, FM index and visceral FM at age 2 years. LTL shortening during the first 2 years of life tended to associate with the gain in FM% from 3 to 6 months.

Not only the change in LTL from age 3 months to 2 years was investigated, but we also compared our data to a previously published study by our research group with LTL measurements at age 21 years. These subjects met the same inclusion criteria of healthy, term-born infants of the Sophia Pluto Cohort and LTL measurements were performed at the same laboratory using the same quantitative PCR-based technique (34). LTL decreased significantly more during the first 2 years of life compared to the period from age 2 years to 21 years.

LTL is one of the markers of biological aging since shortening occurs over time (35, 36). Shortening in LTL is influenced by factors such as inflammation, stress, radiation and obesity (37). Furthermore, shorter LTL has been linked to an increased risk of cardiovascular diseases in later life (36).

Until now, only one other study has investigated LTL longitudinally during infancy (38), which also presents an impressive decline in LTL during the first 2 years of life. This study, however, started measuring LTL at an older age (mean age of 8.6 months) and did not investigate LTL in association with body composition. It is challenging to obtain longitudinal blood samples of healthy infants in early life, which could explain the lack of longitudinal studies. We were able to collect 2 samples during infancy, at age 3 months and 2 years. These samples were collected by toe prick to limit the discomfort, resulting in parents giving permission for these blood collections.

Several methods exist for measuring telomere length (39), but it remains impossible to compare LTL between different cell types, such as blood cells, buccal cells and fibroblasts. We used the quantitative PCR-based technique to measure LTL (35, 40). This technique has the advantage that smaller DNA amounts are sufficient (39) and is therefore suitable for studying infant DNA.

Obesity has been associated with shorter LTL in adults (37, 41-43) and in children at the age of 8 years (44). It is for the first time that we present associations between LTL and body composition measurements during the first 2 years of life, which is an important window for infant development (11, 12). Our first LTL measurements took place at infant's age of 3 months, within the critical window for adiposity programming in the first 6 months after birth.

In conclusion, LTL decreases considerably during the first 2 years of life. More shortening in LTL from age 3 months to 2 years associates with higher FM%, FM index and visceral FM, and tends to associate with the gain in FM% from 3 to 6 months, suggesting that adverse adiposity programming in early life could contribute to more LTL shortening.

Appetite regulating hormones and body composition during the first 6 months

Chapter 6 presents appetite regulating hormone (ARH) levels during the first 6 months of life, a critical window for adiposity programming. We investigated fasting serum levels of ghrelin, peptide YY (PYY) and leptin at age 3 and 6 months and the associations of these hormones with body composition development until age 6 months.

For this study, we investigated three hormones; ghrelin stimulating food intake and PYY and leptin inhibiting appetite and increasing metabolic rate (45, 46). These hormones are involved in the regulation of food intake through specific brain centers (45, 47) and might thus contribute to adiposity programming early in life. However, studies investigating ARH during these first 6 months were very limited. We also determined the ghrelin/PYY ratio, as this ratio is a marker of orexigenic drive rather than both hormones separately (48, 49). Infant blood collection and body composition measurements were performed on the same day, at age of 3 and 6 months.

Our findings show that that ghrelin levels and ghrelin/PYY ratio increased from 3 to 6 months, while PYY and leptin levels decreased. Girls had a higher leptin at 3 months than boys, but other ARH levels were similar between boys and girls. Leptin correlated with FM% at 3 and 6 months and the gain in FM% from 1 to 6 months in exclusively breastfed (BF) and formula fed (FF) infants. In BF infants only, ghrelin and ghrelin/PYY ratio correlated also with the gain in FM% from 1 to 6 months. BF infants had lower ghrelin and higher PYY levels at age 3 months compared to FF infants. ARH levels did not correlate with human milk macronutrients. Regarding appetite, higher PYY levels in FF infants correlated with infants having more difficulty getting full up during a feed, while a higher ghrelin level and ghrelin/PYY ratio tended to correlate with less satiety.

We found that higher ghrelin and leptin, but not PYY, were associated with more FM development during the first 6 months of life, suggesting that they are involved in early adiposity programming. Leptin has been associated with adiposity, but most studies in infants or children used cord blood or neonatal blood spots instead of longitudinal ARH values in the first 6 months of life (50-56). Studies with one leptin measurement either at birth (57), at age 4 months (58) or leptin measurements during the first 6 months in a small group of infants (59) showed associations with body composition, which is in line with our study.

We found differences between ARH levels in BF and FF infants at age 3 months. Only few studies (58, 60), including a previous study of our own research group (61), investigated differences in early ARH levels of BF and FF infants. These differences might contribute to BF and FF infants having different body composition (62, 63).

Exclusively BF infants had lower ghrelin, higher PYY and as a result a lower ghrelin/PYY ratio than FF infants. This suggests that exclusively BF infants have more satiety, which is in line with a study describing that breastfeeding in the first year of life promotes satiety responsiveness in infants between age 18 and 24 months (64).

We now show that, in FF infants, a higher PYY at age 3 months correlated with having more difficulty getting full up during a feed. PYY decreases food intake and the finding of lower PYY levels in FF infants compared to BF infants therefore suggests that FF infants might indeed have less satiety. In addition, PYY correlated with the gain in FM% from 1 to 6 months in FF infants, but not in BF infants, which suggests that early life PYY levels might indeed contribute to the differences in body fat mass development between BF and FF infants.

Our study shows increasing ghrelin and decreasing PYY levels until age 6 months, resulting in an increasing ghrelin/PYY ratio. This suggests an increasing orexigenic drive until the age of 6 months. Ghrelin and leptin, but not PYY, associate with more FM development during the first 6 months, suggesting that they might be involved in early adiposity programming. Appetite regulating hormones at the age of 3 months are different between BF and FF infants, with lower ghrelin and higher PYY levels in BF infants.

Appetite regulating hormones and body composition during the first 2 years

Chapter 7 describes longitudinal appetite regulating hormone levels during the first 2 years of life. We investigated fasting serum levels of ghrelin, PYY, adiponectin and leptin at age 3 and 6 months and age 2 years and associated these levels with FM parameters measured at age 2 years to determine if ARH levels at 3 and 6 months are predictive for later FM. In addition, we investigated the associations of appetite regulating hormone trajectories until age 6 months and from 6 months to 2 years with trajectories of FM parameters during the same periods.

Our findings in 174 healthy infants show that ghrelin and ghrelin/PYY ratio increased and PYY, adiponectin and leptin decreased during the first 2 years of life. When investigating the potential predictive value of ARH for adiposity development, adiponectin levels at 3 and 6 months and a greater decline in adiponectin during the first 2 years as well as leptin levels at all ages correlated with higher FM% at 2 years. When investigating ARH trajectories, ghrelin and ghrelin/PYY ratio trajectories from 3 to 6 months were associated with the visceral FM trajectory during the same period. The leptin trajectory was associated with the FM% trajectory until age 2 years.

For the first time we show ARH trajectories in association with measured FM parameters. Studies on multiple ARH trajectories in early life were very scarce. Most studies used cord blood (50-54, 65) or newborn blood spots (55, 56) to investigate ARH at birth in association with later body composition. Two studies investigated ARH trajectories at birth or 6 months until childhood, but they did not investigate ARH in multiple blood samples during the first 2 years of life (46, 66).

Ghrelin increases food intake, while PYY reduces intake (45, 47). The ghrelin/PYY ratio is of interest as a marker of orexigenic drive rather than both levels separately (48, 49). Ghrelin and ghrelin/PYY ratio at age 3 and 6 months were, in contrast to our hypothesis, not predictive for FM% at age 2 years. A greater increase in ghrelin and ghrelin/PYY ratio during the

first 6 months of life, however, associated with less increase in visceral FM during the same period in the total group. These findings are in line with literature showing higher ghrelin levels and less visceral adiposity in subjects with Prader-Willi syndrome, characterized by hyperphagia and excessive weight, compared to obese controls (67-69). In addition, a higher ghrelin and ghrelin/PYY ratio at 2 years associated with lower visceral FM at age 2 years in exclusively FF infants, while also a greater increase in ghrelin and ghrelin/PYY ratio from 6 months to 2 years associated with lower visceral FM at 2 years.

Our data show that ghrelin and ghrelin/PYY ratio trajectories during the critical window for adiposity programming could contribute to visceral FM development instead of FM development. This is an important finding, because specifically increased visceral FM has been associated with unfavorable metabolic health during childhood and later on (70, 71).

Higher adiponectin levels at 3 and 6 months were significantly associated with higher FM% at age 2 years. Also a greater decline in adiponectin until age 2 years was associated with a higher FM% at 2 years, which could be explained by the fact that infants with a greater decline had higher levels at age 3 and 6 months. These findings can contribute to the understanding why lower adiponectin levels were found in adults with overweight and obesity (72-74), as we now show that specifically adiponectin during the first 6 months might be involved in the adiposity programming.

Leptin levels mainly reflected current FM%, which is in line with literature (46), but we showed that early leptin levels had also some predictive value for later FM%. In addition, the leptin trajectory during the first two years of life corresponded with the FM% trajectory during the same period.

In conclusion, ghrelin and ghrelin/PYY ratio increase during the first 2 years of life, while PYY, adiponectin and leptin decrease. Ghrelin levels and ghrelin/PYY ratio during the first 6 months of life have no predictive value for later FM%, but might be involved in visceral FM development. Early adiponectin levels might predict FM% at 2 years, suggesting that adiponectin is involved in early adiposity programming. Leptin levels mainly reflect current FM%, but have also some predictive value for later FM%. Our findings can potentially be used for the development of personalized screening tools for obesity prevention in early infancy.

GENERAL CONCLUSIONS, CLINICAL IMPLICATIONS AND FUTURE RESEARCH DIRECTIONS

General conclusions

Based on our findings presented in this thesis, we can conclude that there is a critical window for adiposity programming during the first 6 months of life. We found multiple determinants involved in early adiposity programming, such as rapid gain in fat mass during the first months, fat and energy content of human hind milk and satiety. In addition, early appetite

regulating hormone trajectories are involved in early life body composition development. Lastly, the shortening of leukocyte telomere length is also associated with body composition development.

Our findings show that an accelerated increase in weight and particularly in fat mass in the first 6 months should be avoided. For that reason, it is very important to closely monitor infants during the first months after birth. Our findings can potentially be used for the development of personalized screening tools in order to prevent obesity development at an early age. It is very important to prevent obesity in early life, as it is extremely difficult to effectively treat obesity once it has developed (75).

Clinical implications of this thesis

Our detailed body composition charts, using a combination of ADP and DXA with vacuum cushion, in healthy term-born infants from birth until age 2 years are of great importance for clinical and research use. The use of a vacuum cushion contributes to obtaining successful FM% measurements by DXA and allowed us to show the longitudinal changes in FM% during the first 2 years of life. Our body composition data can be used as reference values for our center, but the raw data cannot be just copied one to one if different DXA machines, vacuum cushions or software are being used. Each center should validate their own vacuum cushion and DXA machine.

Our findings show that the first 6 months of life are a critical window for adiposity programming, hence an optimal time for obesity prevention in early life. These findings point out that health care professionals and researchers should closely monitor infants in early life and should be aware of risk factors for developing obesity. If it is not possible to measure fat mass, it is necessary to monitor a proxy for fat mass in early infancy such as gain in weight-for-length and to prevent crossing of SD lines on the weight-for-length SDS charts.

Early appetite hormone levels are involved in adiposity programming in the first months after birth. Adiponectin levels at age 3 and 6 months might predict FM development. These hormones can potentially be used for the development of personalized screening tools for obesity prevention during early infancy. Leptin levels closely reflect FM and have some predictive value for later FM development. Early ghrelin levels and ghrelin/PYY ratio have some predictive value for the gain in FM% in the first 6 months of life, but not for later FM%, while both are involved in visceral FM development.

Human milk macronutrient levels showed wide variations between mothers. Exclusively breastfed infants receiving higher caloric human milk satiated earlier and finished feeding faster. These findings could explain the differences in duration of feeding time between infants. Therefore, presumed that infants have a healthy growth pattern, the duration of drinking could not always be considered a consequence or determinant of underlying problems, because we found that infants receiving higher caloric human milk finished their feeding earlier. This could be a mechanism to protect them from excess adiposity programming

by preventing an abnormally high intake of macronutrients, knowing that higher human milk fat and energy content at 3 months were associated with higher FM% at age 6 months and with more gain in FM% during the first 6 months of life.

Future research directions

Longer-term follow-up of body composition development in healthy term-born infants is warranted, particularly in infants with a rapid increase in fat mass during the first months of life in order to investigate whether they will also have more adiposity and specifically visceral fat mass after the age of 2 years. This could be important for targeted prevention strategies. Future research should also focus on elucidating the differences between hind milk and fore milk samples and their correlations with body composition and appetite. Furthermore, the effects of exclusive breastfeeding versus formula feeding during early life on later body composition need more investigation. The daily food intake, appetite regulating hormone levels and body composition later in life could be different between subjects who received exclusive breastfeeding versus formula feeding during infancy.

More research is required to investigate which additional factors might influence LTL during the first 2 years of life.

Potential effects of early adiposity programming on later cognitive functioning and behavior are presently unknown and need more investigation.

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Chapter 9

Summary
Samenvatting

SUMMARY

Chapter 1

Chapter 1 gives a general introduction about the influence of early life growth on adult health and the importance of measuring body composition in infants. Different techniques for measuring body composition are discussed, as well as potential determinants of early body composition development. The studies described in this thesis are embedded in the Sophia Pluto Cohort and the study population is described in this chapter. Finally, the aims of the studies and the outline of this thesis are presented.

Chapter 2

In chapter 2 we describe the difficulty of obtaining longitudinal measurements of body composition during infancy because of different measurement techniques and unsuccessful measurements due to infant movement. We investigated the reliability of the use of a vacuum cushion during Dual Energy X-ray Absorptiometry (DXA) scans and the comparability between body composition measurements by air-displacement plethysmography (ADP) and DXA. We showed that the use of a vacuum cushion limited movement artifacts and resulted in reliable measurements of fat mass percentage (FM%). In 278 infants, we showed that measurements of FM% by ADP and DXA with vacuum cushion are comparable, in contrast to the measurements of ADP and DXA without cushion. Using a combination of ADP until age 6 months and DXA with cushion from 6 months to 2 years allowed us for the first time to construct longitudinal body composition charts during the first 2 years of life. We showed that FM% increased between age 1 and 6 months and decreased thereafter until age 2 years and that girls had higher FM% than boys.

In conclusion, the use of a vacuum cushion to prevent infant movement during DXA scans provides reliable measurements of body composition, which are also comparable with ADP measurements. This allowed us to use both techniques in order to construct longitudinal body composition charts, which are essential for clinical and research use.

Chapter 3

Chapter 3 describes that only a higher change in FM% during the first 6 months of life, and not the 6 months thereafter, was associated with a higher FM% at age 2 years. Infants with a higher gain in FM% in the first 6 months of life had higher trajectories of FM%, fat mass index (FMI) and abdominal subcutaneous FM until age 2 years, resulting in a higher FM%, FMI and more abdominal subcutaneous FM at age 2 years.

In conclusion, our study convincingly shows that the change in FM% during the first 6 months is associated with more adiposity at age 2 years and that infants with a rapid increase in FM% have higher FM% trajectories during early life, supporting a critical window for adiposity programming in early life.

Chapter 4

In the study presented in chapter 4, we investigated longitudinal human milk macronutrient composition (fat, carbohydrate, protein and energy) from age 1 to 3 months in exclusively breastfed infants. Protein and energy content decreased, while fat and carbohydrate tended to decrease. Human milk macronutrient composition, particularly fat content, differed considerably between mothers. Fat and energy at infant's age 3 months associated with the gain in FM% from age 1 to 6 months, suggesting that higher fat and energy content associate with higher gain in FM% during the critical window for adiposity programming. Infants receiving higher caloric breastfeeding satiated earlier, which could be a self-regulatory mechanism to prevent the intake of excessive amounts of macronutrients.

In conclusion, we show that human milk fat and energy content at age 3 months associate with early body composition development and that exclusively breastfed infants receiving higher caloric human milk satiate earlier and finish feeding faster.

Chapter 5

Leukocyte telomere length (LTL) is a marker of biological aging, because LTL shortening occurs over time. In chapter 5, longitudinal LTL values are presented from age 3 months until 2 years. We showed that LTL had already shortened considerably by 8.5% during the first 2 years of life. Boys tended to have a shorter LTL at age 2 years. More shortening in LTL during the first 2 years of life was associated with higher FM%, FMI and abdominal visceral fat mass at age 2 years.

In conclusion, we show a remarkable decline in LTL during the first 2 years of life and our findings suggest that adverse adiposity programming in early life could contribute to more LTL shortening.

Chapter 6

Appetite regulating hormones (ARH) are involved in food intake regulation and might therefore play a role in adiposity programming. Chapter 6 presents longitudinal serum ARH levels during the first 6 months of life, the critical window for adiposity programming. We showed that ghrelin levels and ghrelin/PYY ratio increased from 3 to 6 months, while PYY and leptin levels decreased. Leptin correlated with FM% at 3 and 6 months and the gain in FM% from 1 to 6 months in exclusively breastfed (BF) and formula fed (FF) infants. In exclusively BF infants only, ghrelin and ghrelin/PYY ratio correlated also with the gain in FM% from 1 to 6 months. Exclusively BF infants had lower ghrelin and higher PYY levels at age 3 months compared to FF infants, resulting in a lower ghrelin/PYY ratio which suggested more satiety. ARH levels did not correlate with human milk macronutrient composition. As for appetite, higher PYY levels in FF infants correlated with infants having more difficulty getting full up during a feed, while a higher ghrelin level and ghrelin/PYY ratio tended to correlate with less satiety.

In conclusion, our findings suggest an increasing orexigenic drive until the age of 6 months and ghrelin and leptin being involved in early FM development.

Chapter 7

In chapter 7 we describe longitudinal serum appetite regulating hormone (ARH) levels during the first 2 years of life, at age 3, 6 months and 2 years, and their associations with body composition development during the same period. We showed that ghrelin levels and ghrelin/PYY ratio increased and PYY, adiponectin and leptin levels decreased during the first 2 years of life. Adiponectin levels at 3 and 6 months and a greater decline in adiponectin during the first 2 years, but also leptin levels at all ages correlated with higher FM% at 2 years. When investigating ARH trajectories, ghrelin and ghrelin/PYY ratio trajectories from 3 to 6 months did not associate with the FM% trajectory during the same period, but were associated with the visceral FM trajectory from 3 to 6 months. The leptin trajectory associated with the FM% trajectory until age 2 years.

In conclusion, early adiponectin levels might predict FM% at 2 years, suggesting that adiponectin is involved in early adiposity programming. Leptin levels also have some predictive value, be it to a lesser extent, and mainly associate with actual FM%. Early ghrelin levels and ghrelin/PYY ratio are not predictive for later FM%, but might be involved in visceral FM development.

Chapter 8

In this chapter, the general discussion, the most important findings are discussed in a broader context. We describe the clinical implications and provide future research directions.

SAMENVATTING

Hoofdstuk 1

Hoofdstuk 1 beschrijft een algemene inleiding over de invloed van groei en gewichtsontwikkeling in het vroege leven op de gezondheid van volwassenen, alsmede over het belang van het longitudinaal meten van de lichaamssamenstelling van jonge kinderen. Verschillende methoden om de lichaamssamenstelling van jonge kinderen te meten worden besproken en er wordt uiteengezet welke factoren op zeer jonge leeftijd van invloed zouden kunnen zijn op de ontwikkeling van de lichaamssamenstelling. De studies die worden beschreven in dit proefschrift zijn onderdeel van het Sophia Pluto Cohort en de studiepopulatie wordt in dit hoofdstuk beschreven. Tot slot worden de doelstellingen van de studies en de opzet van dit proefschrift uiteengezet.

Hoofdstuk 2

In hoofdstuk 2 wordt besproken dat longitudinale metingen van de lichaamssamenstelling vanaf de geboorte tot de leeftijd van 2 jaar belangrijk zijn voor de gezondheidszorg en voor onderzoeksdoeleinden. Het verkrijgen van deze gegevens is echter lastig door beperkte meettechnieken en doordat metingen onsuccesvol kunnen zijn doordat jonge kinderen vaak niet stil liggen.

We onderzochten of het gebruik van een vacuüm kussen tijdens een Dual Energy X-ray Absorptiometry (DXA) scan de bewegingen van jonge kinderen tijdens metingen kan verminderen en we op deze manier betrouwbaarder kunnen meten. Aanvullend onderzochten we of de uitslagen van metingen met een vacuüm kussen vergelijkbaar zijn aan de uitslagen van metingen middels air-displacement plethysmography (ADP). In 278 kinderen laten we zien dat de metingen van het vetpercentage tussen ADP en DXA scan met vacuüm kussen goed overeen komen, in tegenstelling tot de metingen tussen ADP en DXA scan zonder kussen. We tonen aan dat het vacuüm kussen inderdaad beweging tijdens DXA scan beperkt en dat het vetpercentage op deze manier betrouwbaar kan worden gemeten, en ook vergelijkbaar met ADP metingen. Daardoor hebben we voor het eerst longitudinale waarden van de lichaamssamenstelling gedurende de eerste twee levensjaren kunnen beschrijven. Het vetpercentage stijgt tussen de leeftijd van 1 en 6 maanden en daalt daarna in de leeftijd van 6 maanden tot 2 jaar. Meisjes hebben een hoger vetpercentage vergeleken met jongens.

Concluderend, het gebruik van een vacuüm kussen tijdens DXA scans bij jonge kinderen resulteert in betrouwbare metingen van de lichaamssamenstelling, vergelijkbaar met metingen middels ADP. We presenteren grafieken met data over de ontwikkeling van de lichaamssamenstelling, die van belang zijn voor de gezondheidszorg en voor onderzoek.

Hoofdstuk 3

In hoofdstuk 3 wordt beschreven dat alleen de verandering in het vetpercentage in de eerste 6 maanden van het leven, en niet in de 6 maanden daarna, geassocieerd is met een hoger vetpercentage op de leeftijd van 2 jaar. Kinderen met een snelle toename van het vetpercentage in de eerste 6 maanden hadden een hogere curve van het vetpercentage, de vetmassa index (vet in kilogram gedeeld door lengte in het kwadraat) en het subcutane buikvet gedurende de eerste 2 levensjaren. Dit resulteerde in een hoger vetpercentage en vetmassa index alsmede meer subcutaan buikvet op de leeftijd van 2 jaar.

Concluderend, onze studie toont aan dat de veranderingen in het vetpercentage in de eerste 6 maanden geassocieerd zijn met meer vetmassa op de leeftijd van 2 jaar en dat kinderen met een snelle toename in vetpercentage in de eerste 6 maanden een hogere curve van het vetpercentage hebben in het vroege leven. Onze data laten zien dat de eerste 6 maanden van het leven een belangrijke periode is waarin de lichaamssamenstelling en (over)gewicht geprogrammeerd worden.

Hoofdstuk 4

Hoofdstuk 4 beschrijft de samenstelling van macronutriënten (vet, koolhydraat, eiwit, energie) in borstvoeding op de leeftijd van 1 en 3 maanden, in kinderen die exclusief borst gevoed werden. Melk eiwit en energie daalden van leeftijd 1 tot 3 maanden en melk vet en koolhydraten lieten een trend van daling zien. De samenstelling van borstvoeding toonde grote verschillen tussen moeders, met name in melk vet. Hoger vet en energie in borstvoeding als kinderen 3 maanden oud waren, waren geassocieerd met een grotere stijging in het vetpercentage van het kind tussen de leeftijd van 1 en 6 maanden, hetgeen wordt gezien als een belangrijke periode voor de ontwikkeling en programmering van de lichaamssamenstelling en (over)gewicht. Kinderen die hoog-calorische borstvoeding kregen, dus met hoog vet en energie, waren eerder verzadigd en stopten eerder met drinken. Dit kan een zelfregulerend mechanisme zijn om te voorkomen dat kinderen te veel macronutriënten binnen krijgen.

Concluderend, vet en energie in borstmelk op de leeftijd van 3 maanden zijn geassocieerd met de ontwikkeling van de lichaamssamenstelling en kinderen die exclusief borstvoeding krijgen met hoger vet en energie zijn eerder verzadigd en drinken korter.

Hoofdstuk 5

Telomeerlengte is een van de markers van biologische veroudering, omdat verkorting van telomeren optreedt als het individu ouder wordt. In hoofdstuk 5 worden longitudinale waarden van telomeerlengte gepresenteerd van de leeftijd van 3 maanden tot 2 jaar. We laten zien dat de telomeerlengte al met 8.5% afnam gedurende de eerste 2 levensjaren. Jongens hadden een trend voor een kortere telomeerlengte vergeleken met meisjes. Meer

verkorting van de telomeerlengte in de eerste 2 levensjaren was geassocieerd met een hoger vetpercentage, vetmassa index en meer visceraal vet op de leeftijd van 2 jaar.

Concluderend, er vindt een opmerkelijke daling van de telomeerlengte plaats in de eerste twee levensjaren. Onze resultaten suggereren dat een ongunstige vetprogrammering in het vroege leeftijd kan bijdragen aan meer verkorting van de telomeerlengte.

Hoofdstuk 6

In hoofdstuk 6 wordt beschreven dat eetlust regulerende hormonen betrokken zijn bij de regulatie van de voedselinname en daardoor mogelijk betrokken kunnen zijn bij de ontwikkeling van de lichaamssamenstelling in de eerste 6 levensmaanden. We laten zien dat serum ghrelin stijgt en peptide YY (PYY) daalt, wat leidt tot een stijging in de ghrelin/PYY ratio, en dat leptine daalt in de eerste 6 maanden van het leven. Leptine was gecorreleerd met het vetpercentage op de leeftijd van 3 en 6 maanden en ook met de stijging in het vetpercentage tussen de leeftijd van 1 en 6 maanden. In exclusief borst gevoede kinderen waren ook ghrelin en de ghrelin/PYY ratio gecorreleerd met de stijging in het vetpercentage tussen de leeftijd van 1 en 6 maanden. Eetlust regulerende hormonen waren verschillend tussen kinderen die borstvoeding versus flesvoeding kregen, waarbij borst gevoede kinderen lagere ghrelin en hogere PYY spiegels hadden in vergelijking met fles gevoede kinderen. Dit resulteerde in een lagere ghrelin/PYY ratio, hetgeen suggereerde dat borst gevoede kinderen meer verzadigd zijn. Er werden geen correlaties met de macronutriënten samenstelling in de borstvoeding gevonden. De hogere PYY levels in fles gevoede kinderen correleerde met het moeilijker verzadigd raken en hun hogere ghrelin spiegels en ghrelin/PYY ratio lieten een trend zien voor minder verzadiging.

Concluderend, onze bevindingen laten zien dat er een stijging in eetluststimulans is tot de leeftijd van 6 maanden en dat ghrelin en leptine, maar niet PYY, betrokken zijn bij de vroege ontwikkeling van het vetpercentage in de eerste 6 levensmaanden.

Hoofdstuk 7

Hoofdstuk 7 beschrijft de longitudinale bloedspiegels van eetlust regulerende hormonen gedurende de eerste 2 jaar van het leven (leeftijd van 3, 6 maanden en 2 jaar), en de associaties met de ontwikkeling van de lichaamssamenstelling in dezelfde periode. We laten zien dat ghrelin spiegels en de ghrelin/PYY ratio stijgen en PYY, adiponectine en leptine spiegels dalen in de eerste 2 jaar. Hogere adiponectine spiegels op de leeftijd van 3 en 6 maanden en een grotere daling in adiponectine in de eerste 2 jaar, alsmede leptine spiegels op alle leeftijden waren gecorreleerd met een hoger vetpercentage op de leeftijd van 2 jaar. Het beloop van ghrelin en ghrelin/PYY ratio van 3 tot 6 maanden was niet geassocieerd met het beloop van het totale vetpercentage tot 2 jaar, maar wel met het beloop van het visceraal vet van 3 tot 6 maanden. Het beloop van leptine over de tijd liep gelijk aan het beloop van het vetpercentage tot de leeftijd van 2 jaar.

Concluderend, vroege adiponectine spiegels kunnen mogelijk het vetpercentage op de leeftijd van 2 jaar voorspellen, wat suggereert dat adiponectine betrokken is bij de vroege programmering van de lichaamssamenstelling. Leptine spiegels hebben in mindere mate ook een voorspellende waarde voor het vetpercentage op 2 jaar, maar zijn vooral geassocieerd met het actuele vetpercentage. Ghrelin en ghrelin/PYY ratio zijn niet predictief voor het totale vetpercentage na de leeftijd van 6 maanden, maar zijn mogelijk betrokken bij de ontwikkeling van het viscerale vet.

Hoofdstuk 8

In hoofdstuk 8 worden de belangrijkste resultaten van alle studies, die gepresenteerd zijn in dit proefschrift, in een bredere context besproken. Afsluitend worden algemene overwegingen en suggesties voor toekomstig onderzoek beschreven.



Chapter 10

Abbreviations

Co-authors and affiliations

Publications

PhD portfolio

Acknowledgements

About the author

LIST OF ABBREVIATIONS

ADP	air displacement plethysmography
ARH	appetite regulating hormones
BEBQ	baby eating behavior questionnaires
BF	breastfeeding
BMI	body mass index
CI	confidence interval
CV	coefficient of variation
CVD	cardiovascular disease
DXA	dual-energy X-ray absorptiometry
EBF	exclusive breastfeeding
EFF	exclusive formula feeding
FF	formula feeding
FFM	fat-free mass
FFMI	fat-free mass index
FFM%	fat-free mass percentage
FM	fat mass
FMI	fat mass index
FM%	fat mass percentage
HMA	human milk analyzer
IQR	interquartile range
LTL	leukocyte telomere length
PYY	peptide YY
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SGA	short for gestational age
SDS	standard deviation score
SPSS	Statistical Package for Social Sciences
T/S ratio	Telomere to single-gene copy ratio

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de Fluiter KS, van Beijsterveldt IALP, Goedegebuure WJ, Breij LM, Spaans AMJ, Acton D, Hokken-Koelega ACS. Longitudinal body composition assessment in healthy term-born infants until 2 years of age using ADP and DXA with vacuum cushion. *European Journal of Clinical Nutrition* 2020; 74(4):642-50.

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PHD PORTFOLIO

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Summary of PhD training	Year	ECTS
<u>1. General courses</u>		
Good Clinical Practice (BROK), Erasmus MC	2016	1.5
Biostatistical Methods I, NIHES, Erasmus MC	2017	5.7
Research Integrity, Erasmus MC	2019	0.3
<u>2. Specific courses</u>		
Radiation protection 5A, Zorgacademie, Erasmus MC	2015	1.0
Basic Introduction to SPSS, Molmed, Erasmus MC	2016	1.0
Access, Molmed, Erasmus MC	2016	0.3
Excel, Molmed, Erasmus MC	2016	0.3
Limesurvey Basic, Erasmus MC	2016	-
Open Clinica, Erasmus MC	2018	-
Epigenetic regulation in health and disease	2018	0.8
Basic and translational endocrinology	2019	2.0
<u>3. Seminars and workshops</u>		
Weekly research meeting, Pediatric Endocrinology, Erasmus MC	2015-2020	4.0
Annual PhD day, Erasmus MC	2015-2019	1.8
Annual Sophia Research Day, Sophia Children's Hospital, Erasmus MC	2015-2019	1.2
<u>4. International and national conferences</u>		
55 th Meeting of the European Society of Pediatric Endocrinology (ESPE), Paris, France	2016	1.0
10 th International Joint Meeting of Pediatric Endocrinology (IMPE), Washington, USA (poster presentation)	2017	1.0
4 th International conference Nutrition&Growth, Amsterdam, the Netherlands (poster presentation)	2017	1.0
57 th Meeting of the European Society of Pediatric Endocrinology (ESPE), Athens, Greece (poster presentation)	2018	1.0

5 th International conference Nutrition&Growth, Paris, France (oral presentation)	2018	1.0
58 th Meeting of the European Society of Pediatric Endocrinology (ESPE), Vienna, Austria (poster presentation)	2018	1.0
6 th International conference Nutrition&Growth, Valencia, Spain (poster presentation)	2019	1.0
7 th International conference Nutrition&Growth, Online due to covid-19 (poster presentation)	2020	1.0

5. Lecturing

Lecturing medical students	2016	0.3
Annual IMC Weekendschool 'Growth and Development', Rotterdam	2016-2019	1.0

6. Research proposals

Postnatal determinants of metabolic health during the critical window for later obesity – the Sophia Pluto Study, follow-up study	2016	5.0
Study on infant Adiposity development To Understand the Role of early life Nutrition – the Sophia Saturn Study	2017	

7. Other activities

Radiation protection supervisor	2016-2020	2.0
Organizing committee Sophia Research day	2017	2.0
Organizing committee IMC weekendschool	2017-2019	2.0
10 th International Meeting Pediatric Endocrinology (ESPE), Washington, USA. Travel Award	2018	
Meetings Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Amersfoort, the Netherlands (2 oral presentations)	2018	1.0

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