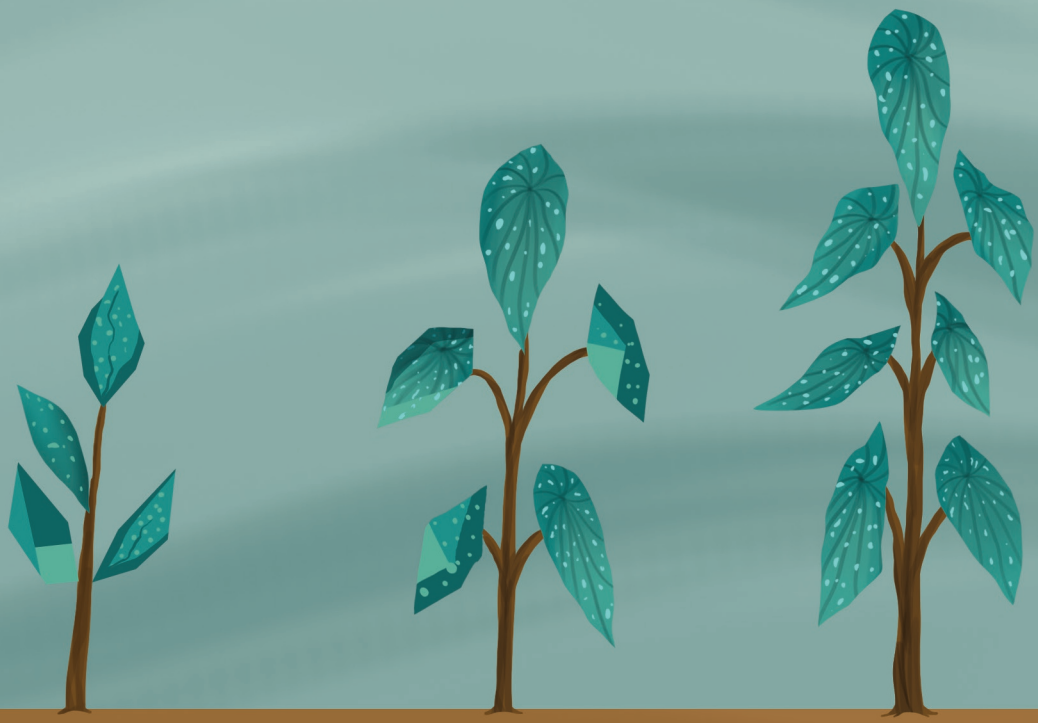
Body composition in young children

UNRAVELING FAT MYSTERIES



Inge van Beijsterveldt

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Body composition in young children

UNRAVELING FAT MYSTERIES

Lichaamssamenstelling in jonge kinderen
ontrafelen van vette mysteries

Proefschrift

Ter verkrijging van de graad van Doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

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"Wonder is the seed of all knowledge"

F. Bacon

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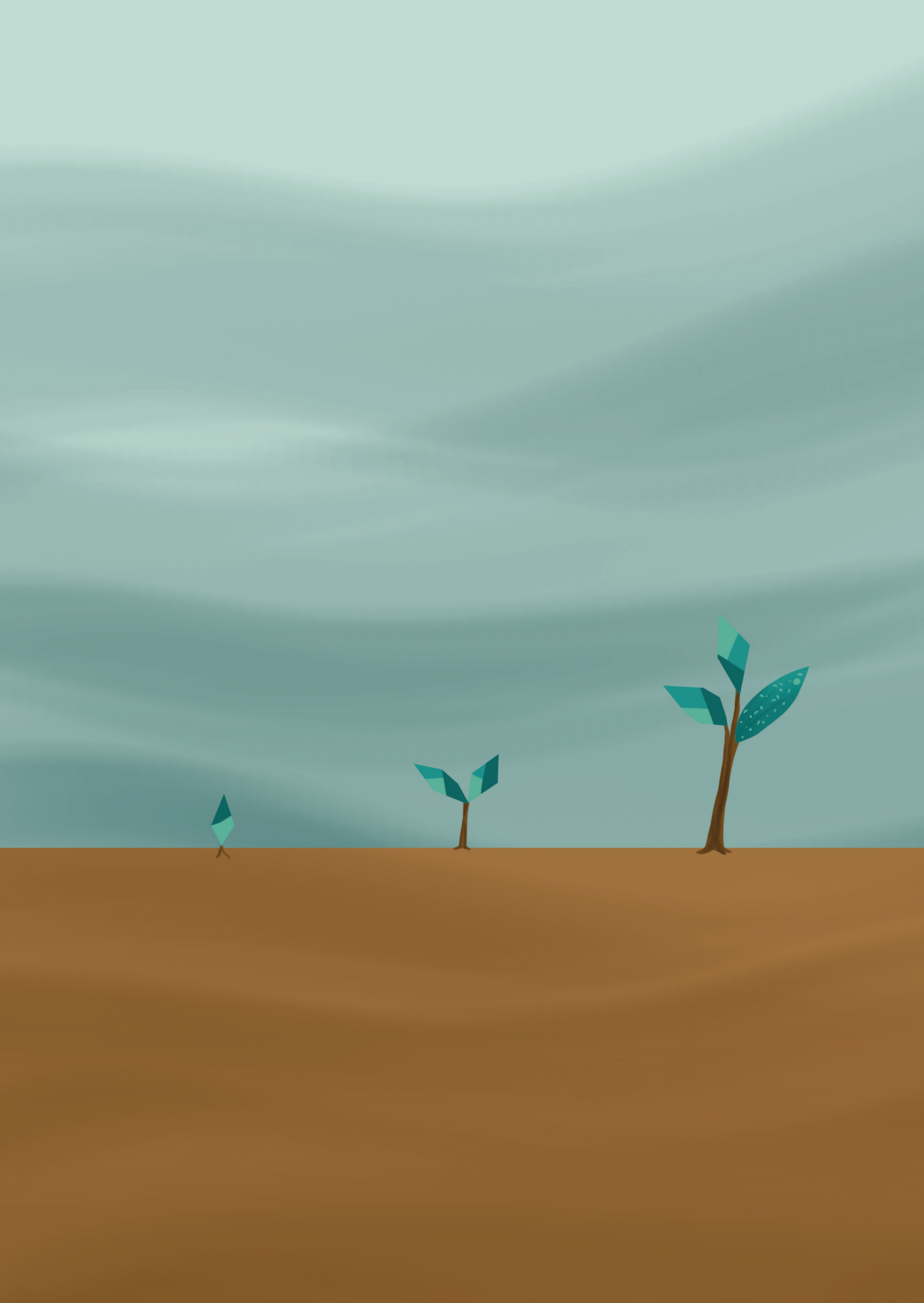

Chapter 1

General introduction

Introduction

Childhood obesity is a global public health threat, with an alarming rising incidence. Globally, 39 million children under the age of 5 years were overweight or obese in 2020 [1]. Obesity at a young age has direct adverse consequences as well as long-term morbidity, like non-communicable diseases such as obesity, cardiovascular disease, insulin resistance and type 2 diabetes mellitus [1].

Influence of early life on adult health

The origin of obesity is thought to be multi-factorial and especially the first months of life seem to be determinative. Over time, several hypothesis have been postulated to explain potential underlying mechanisms.

The first hypothesis dates from the 1990s. Barker *et al.* hypothesized that *in utero* malnutrition could result in permanent physiologic, endocrine and metabolic changes in the fetus. Barker *et al*, also assumed an inverse association between birth weight and adult disease. The fetus would, at first, benefit from the endocrine and metabolic changes, since it would increase survival during a period of fetal malnutrition. However, eventually the re-programming would increase the risk for non-communicable diseases in adulthood [2, 3].

In early 2000, it was added that weight acceleration during early childhood may lead to higher risk of coronary heart disease in adulthood. Accelerated weight gain in childhood would lead to a disproportionately high fat mass and lower muscle mass. Subsequently causing insulin resistance and disturbance in lipid metabolism and blood coagulation, eventually leading to cardiovascular diseases in adulthood [4, 5].

Our research group added that especially rapid gain in weight-for-height in the first few months of life was associated with higher body fat percentage, higher blood pressure and unfavorable metabolic health e.g. increased serum levels of total cholesterol, triglycerides and low density lipoprotein (LDL) cholesterol in young adults [6], suggesting the presence of a critical window for adiposity programming in the first months of life, with rapid fat accumulation in early life as important determinant of adult outcome. This was underlined in 2020, when we found that infants with rapid rise in fat mass during the first 6 months of life, had higher fat mass trajectories until 2 years of age [7].

Body composition assessment

To identify children who are at risk for developing excess adiposity, it is important to monitor the body composition during infancy and childhood. In general, body mass index (BMI) is mostly used to determine adiposity, but BMI is poorly associated with true body composition and BMI SD-scores have low specificity to identify changes in fat mass percentage (FM%) [8, 9]. Other reliable methods to longitudinally assess body composition were, therefore, needed. However, with the available tools it was not possible to use one single method longitudinally from birth to adolescence. As a result, several methods have been used to determine body composition in infancy and childhood (Figure 1), but longitudinal reference values were not yet available.

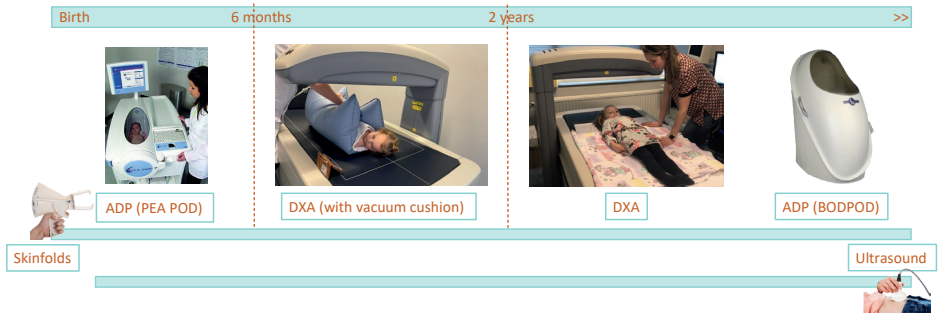

Figure 1. Several methods to determine body composition during infancy and childhood*. *Pictures are displayed with parental permission . Abbreviations: ADP = Air Displacement Plethysmography, DXA = Dual energy X-ray Absorptiometry.*

Skinfold measurements are described to be better correlated with fat mass compared to BMI [10, 11]. They are useful as stand-alone measurements to identify children with extremely low or high fat mass [11, 12] and have also been used to estimate body fat using anthropometry based equations [13, 14]. However, these equations are often developed using very young infants or older children and adolescents [13-15]. Existing equations were, therefore, biased and had random and systematic errors in prepubertal children [16, 17].

Air displacement plethysmography (ADP) is a relatively new method, that can be used to determine body composition. It was developed in the mid 1990's and is advanced to a non-invasive, relative fast technique. It calculates fat and fat free mass based on body mass and volume. Body volume is determined using

* Pictures are displayed with parental permission

the inverse pressure-volume relation, based on the change in air pressure in a secluded space with a known volume [18]. ADP by PEA POD can be used in infants until approximately age 6 months or 8 kilograms of body weight [19, 20]. ADP by BODPOD was initially only usable from the age of 6 years. Since the development of specialized hard- and software for young children in 2011, it can now be used in children aged ≥ 2 years and ≥ 12 kilograms [21]. However, a gap between age 6 months and 2 years remains.

ADP has multiple limitations. It uses a 2-component technique and determines only fat mass and fat free mass. BODPOD requires the child to sit alone in a secluded space, which can cause separation anxiety in young children and makes it particularly difficult to use for children aged < 3 years. Because the technique is based on volume change in a sealed chamber the child is not allowed to cry, as that would influence volume change and, therefore, the results. Importantly, ADP uses multiple assumptions to calculate body composition parameters, including estimates for fat free mass density. These estimates are based on small studies, conducted in the 1980's, in which results of still born infants, older children and adults were extrapolated to children aged <8 years, in 2-year age ranges[22, 23].

Dual energy X-ray Absorptiometry (DXA) is the most used method to determine body composition in children and is often used as a reference method [24]. This technique was initially developed to measure bone mineral density, but DXA can also determine body composition [25]. With use of specialized software, the quantity of fat mass, lean body mass and bone mineral content can be determined based on the difference in X-ray attenuation by the different body tissues [26]. Especially in children, DXA has some limitations. It uses a small dose of radiation (approximately 0.0002 mSv). It assumes that fat free mass hydration is a constant value, while it is known that the hydration state in young children decreases over time [27] and results differ between different machines [28], modes [29] and software [30]. So, it is crucial to use the same machine and software for comparison and longitudinal measurements.

Lastly, it requires the subject to lay still, which is especially challenging in very young children. Our research group found that a vacuum cushion can be used to avoid movement artifacts. We found that body composition results measured by ADP using PEA POD and DXA with vacuum cushion are comparable in children aged 6 months and that DXA scanning, with a vacuum cushion, provides accurate measurements until the age of 2 years [31]. We constructed longitudinal values from

birth to age 2 years, by combining ADP by PEA POD until age 6 months and DXA with vacuum cushion from age 6 months to 2 years, which partly removed the gap of data, but the lack of longitudinal reference values for body composition between age 2 and 5 years remained.

Not only fat mass, but also its distribution in the body plays an important role in the development of unfavorable metabolic outcomes [32, 33]. Especially excessive truncal and visceral fat accumulation compared to peripheral fat is associated with an unfavorable metabolic profile [34]. Abdominal ultrasound measurements of subcutaneous and visceral fat mass thickness are found to be reliable and reproducible estimates of abdominal subcutaneous fat mass and intra-abdominal visceral fat [35, 36].

Potential determinants of adiposity programming

Several factors might influence adiposity programming and body composition and in early life (Figure 2). Unraveling these factors and the identification of infants at risk of excess adiposity at an early stage, will provide the opportunity to develop more targeted preventative strategies in the battle against (childhood) obesity.

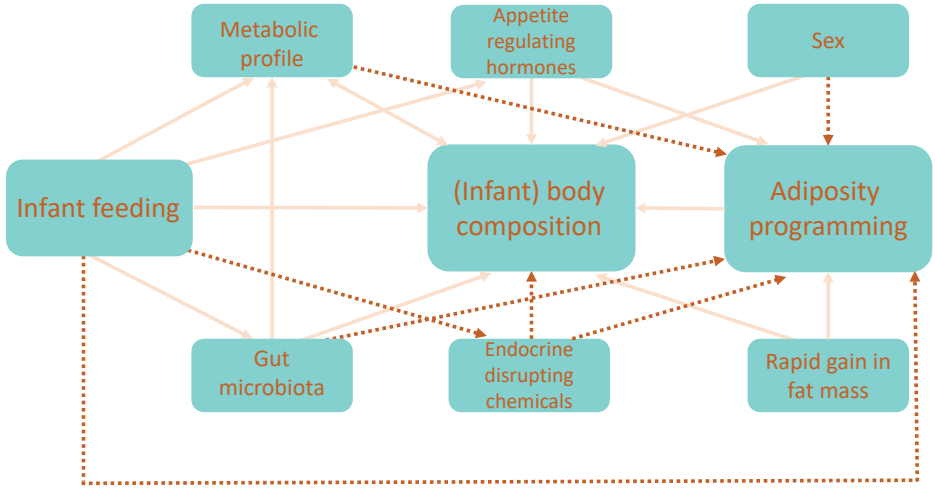

Figure 2. Visual summary of factors (potentially) influencing body composition development.

Continues lines represent previously described associations and dashed lines represent hypotheses about potential associations.

Sex

Already at an early age, body composition is different between boys and girls, with girls having higher fat mass and lower fat free mass from infancy onwards [31, 37, 38]. These differences in body composition are likely caused by different hormone levels, including endogenous testosterone production in boys and estradiol in girls in the first months of life [38]. This could potentially cause sex-difference in adiposity programming. However, such differences in adiposity programming have not yet been described.

Plasma metabolites and lipids

An unfavorable body composition with excessive body fat and more visceral fat is associated with an adverse lipoprotein profile in children and adults, especially with high LDL cholesterol levels, which increases the risk of cardiovascular disease [39, 40]. However, not only the standard lipoproteins, but several hundred lipid species are present in plasma [41]. Branched chain amino acids (BCAAs) concentrations were reported to be higher in children with obesity [42]. In infants, the metabolic profile is also associated with growth patterns. At 3 months of age, plasma levels of phosphatidylcholine (PC) (20:4/18:0), PC plasmalogen (36:4) and sphingomyelin (d18:1/16:0) were associated with poor weight gain, while plasma levels of PC (18:1/16:0) and PC plasmalogen (34:1) were associated with accelerated weight gain [43, 44]. Besides, metabolic profile can internally be influenced, for example by the gut microbiota, which produce short chain fatty acids that can potentially influence metabolic pathways and energy regulation throughout the body [45, 46] and also contribute to the development of obesity.

These changes in metabolic profile and phospholipid composition could be associated with adiposity programming and metabolic health, and could, therefore, potentially serve as early biomarkers for unfavorable metabolic outcomes.

Infant feeding

Exclusive breastfeeding during the first 6 months of life is recommended by the World Health Organization, due to its health benefits, in terms of growth outcomes, mother and child bonding, and protection against obesity, infections, asthma, eczema and allergies [47-49].

There is evidence that infant feeding influences body composition directly. It was reported that already in the first months of life, infants with exclusive breastfeeding have different growth trajectories compared to formula fed infants, with higher body fat mass percentage, predominantly due to more

subcutaneous instead of visceral fat accumulation [50, 51]. Infant feeding could also influence adiposity programming indirectly, via other determinants, e.g. appetite regulating hormones, plasma metabolites and lipids, gut microbiota and endocrine disrupters.

Appetite regulating hormone levels are different between breast- and formula fed infants in early life [51, 52]. It has been reported that exclusively breastfed infants had lower ghrelin and higher PYY, resulting in a lower ghrelin/PYY ratio compared to exclusive formula fed infants [53]. A lower ghrelin/PYY ratio promotes the sense of satiety with decreasing food intake and increasing metabolic rate and energy expenditure [54], with the potential of eventually resulting in less adiposity.

Plasma lipids and metabolic profile in early life have also been reported to differ between breast- and formula fed infants [43, 44, 55]. In breastfed infants, total phosphatidylcholine levels are higher and linoleic acid is less incorporated in palmitate into the phospholipid fraction as compared to that of formula fed infants [44, 56]. In formula fed infants, even the amount of formula did influence the metabolic profile [56].

Lastly, it has been reported that gut microbiota of children with exclusive breastfeeding have lower diversity and contains more *Lactobacillus, Staphylococcus* and *Bifidobacterium* compared to children with formula feeding [57]. In contrast, the gut microbiota of children with exclusive formula feeding contains more microbes that are associated with low grade inflammation, which can potentially lead to the development of metabolic disorders and obesity [57].

Endocrine disrupting chemicals

Endocrine disrupting chemicals (EDCs) are non-degradable chemicals, which can accumulate in humans. They interfere with endocrine processes and can cause adverse effects on perinatal, neurodevelopmental, metabolic and reproductive outcomes. Especially when exposure occurs during susceptible periods of human development, such as early life [58]. Also, the exposure of endocrine disrupting chemicals can be different between breast- and formula fed infants. The majority of endocrine disrupting chemicals are lipophilic, meaning that they are mainly stored in adipose tissue in the human body. Since the mammary glands consist mostly of adipose tissue, endocrine disrupting chemicals can be stored in the breasts and can be excreted into human milk [59]. Therefore, breastfed infants are potentially exposed to higher levels of endocrine disrupting chemicals compared to formula fed infants. Multiple EDCs have been thoroughly studied, such as Bisphenol A and

phthalates. These are associated with obesity and impaired glucose tolerance and diabetes mellitus [58]. However, about the exposure of infants of another class of EDCs, per- and polyfluoroalkyl substances (PFAS), data were lacking.

PFAS are a group of > 3000 man-made chemicals, produced since the 1950's. They are used in a variety of consumer and industrial products. Because of their water-, dirt- and grease-repellent quality, they are used in food-packing materials and non-stick coating in pans, among other things. PFAS can migrate easily into the environment. They are not degradable and can easily be spread through the air and water. PFAS can be taken up by plants, animals and humans. Because of their very long elimination half-life up to 8.5 years, they have a tendency to accumulate in humans [60, 61]. Rodent studies show concerning effects in offspring that was exposed to high levels of PFAS during pregnancy or in early life. These adverse effects consist of a wide range of developmental effects, such as growth restriction, altered behavioral patterns and endocrine disruption [61, 62]. Unfortunately human studies to confirm or refute these findings are very scarce and have conflicting conclusions, partly because of small study populations and short or even lack of follow-up periods [63-65]. Especially the potential association between PFAS exposure and adiposity programming and body composition is of interest, since the period in which childhood obesity has become an increasing problem overlaps with the production of PFAS and their increasing presence in the environment.

Hypotheses

We hypothesized that anthropometry based equations, ADP and DXA can all be used to determine body composition in infants and young children, but that DXA is the most reliable tool for longitudinal use until the age of 5 years. Furthermore, we hypothesized that plasma metabolic profile in early life is associated with body composition at age 2 years and that fat mass in early life is strongly associated with body composition at age 4 years. Lastly, we hypothesized that metabolic profile and serum PFAS levels are strongly associated with infant feeding type.

Sophia Pluto study cohort

The studies in this thesis were embedded in the Sophia Pluto study; a prospective birth cohort study to determine early growth and body composition trajectories and identify determinants of adult non-communicable diseases in early life. The Sophia Pluto study was initiated in 2012 based on the outcomes of the PROGRAM study, which showed that especially the first months of life were important for metabolic health and body composition outcomes in young adulthood.

Currently, a total of 1011 healthy term-born children are included in the Sophia Pluto study. All were recruited from maternity wards and children's healthcare centers and fulfilled the same inclusion criteria: (1) healthy and term-born infants (gestational age ≥ 37 weeks) and (2) age at enrollment less than 28 days. Exclusion criteria were (1) complicated neonatal period with severe asphyxia (defined as an Apgar-score <3 after 5 minutes) or serious disease such as sepsis, long-term mechanical ventilation and oxygen supply, bronchopulmonary dysplasia or other lung disease, (2) known congenital or postnatal diseases that could interfere with body composition development, confirmed viral intrauterine infection and (3) any significant maternal medical condition or medication use that could interfere with infant's growth and body composition development (e.g. corticosteroids or insulin).

Birth data were taken from hospital and midwife records. Parental characteristics and dietary information from mother and child were obtained by standardized interviews and questionnaires.

Study visits took place at age 1, 3, 6, 9, 12 and 18 months and annually between 2 and 5 years of age. At every visit, anthropometrics were measured, as well as several body composition measurements by skinfolds, abdominal ultrasound, ADP by PEA POD until 6 months of age, by ADP by BODPOD from 3 years onwards and by DXA during all visits from age 6 months. Lastly, many different samples were collected at each visit, such as blood, stool and human milk.

Aims of this studies

This thesis describes the results of 8 studies in healthy term-born children aged 0-5 years, who participated in the Sophia Pluto study.

Assessing body composition by DXA, ADP and skinfolds
To develop sex-specific reference values and charts for body composition and bone mineral density for children age 2-5 years using DXA, based on almost 600 measurements in healthy term-born children. In addition, to compare results of ADP by BODPOD with DXA in children born full-term and very preterm, aged 3-5 years and improve the default density model used in BODPOD software for young children. Lastly, to determine and validate anthropometry based equations in infants age 0-6 months to predict fat mass based on skinfold measurements.

Tracking of body composition
To investigate the tracking of high fat mass percentage and abdominal fat distribution, i.e. high abdominal subcutaneous and visceral fat, from age 1, 3 and 6 months until the age of 4 years, and to determine if high fat free mass index will track from infancy to childhood. In addition, to investigate if this tracking will be different between boys and girls or between types of infant feeding.

Early life metabolite profile and body composition
To investigate if plasma metabolites at age 3 months are associated with, and even could predict specific body composition outcomes at age 2 years in a cohort of healthy infants, and to investigate if this metabolite profile model at age 3 months is different between boys and girls and between infants with exclusive breastfeeding and those with exclusive formula feeding. Also, to evaluate if plasma metabolites at age 3 months associate similarly with body composition outcomes at age 2 years in breastfed and formula fed infants.

PFAS
To determine longitudinal plasma PFOS, PFOA, PFHxS, PFNA and PFDA levels in a large cohort of healthy infants at 3 months and 2 years of age in the Rotterdam area, and to investigate which child- and maternal characteristics are associated with infant PFAS plasma levels and if infant feeding type associates with PFAS plasma levels. Furthermore, to evaluate longitudinal PFAS levels in human milk and to determine which determinants are associated with daily PFAS intake through infant feeding. Lastly, to investigate the correlation between daily PFAS intake through infant feeding with PFAS plasma levels throughout infancy until age 2 years.

Outline of this thesis

Chapter 1 gives an introduction in the topics described in this thesis. **Chapter 2** presents sex-specific reference values for body composition and bone mineral density in children aged 2-5 years. **Chapter 3** reports the comparison of body composition measurements by ADP with DXA, in full-term and very preterm born children aged 3-5 years. **Chapter 4** presents anthropometry based equations to predict fat mass in infants. **Chapter 5** describes tracking of body composition from early infancy to age 4 years. **Chapter 6** reports plasma metabolites in early life and their association with skinfold ratio at age 2 years. **Chapter 7** reports distinct infant feeding specific plasma metabolites in early life and their association with body fat and visceral fat at age 2 years. **Chapter 8** describes longitudinal plasma levels of poly- and perfluoroalkyl substances in infancy. **Chapter 9** describes the exposure of poly- and perfluoroalkyl substances through infant feeding in the first months of life. **Chapter 10** provides a discussion of the results of the studies in relation to the current literature, thereby also providing conclusions, clinical implications and directions for future research. **Chapter 11** summarizes the study findings in English and Dutch. **Chapter 12** contains a list of abbreviations, a list of publications, co-authors, and the PhD portfolio, acknowledgments and information about the author.

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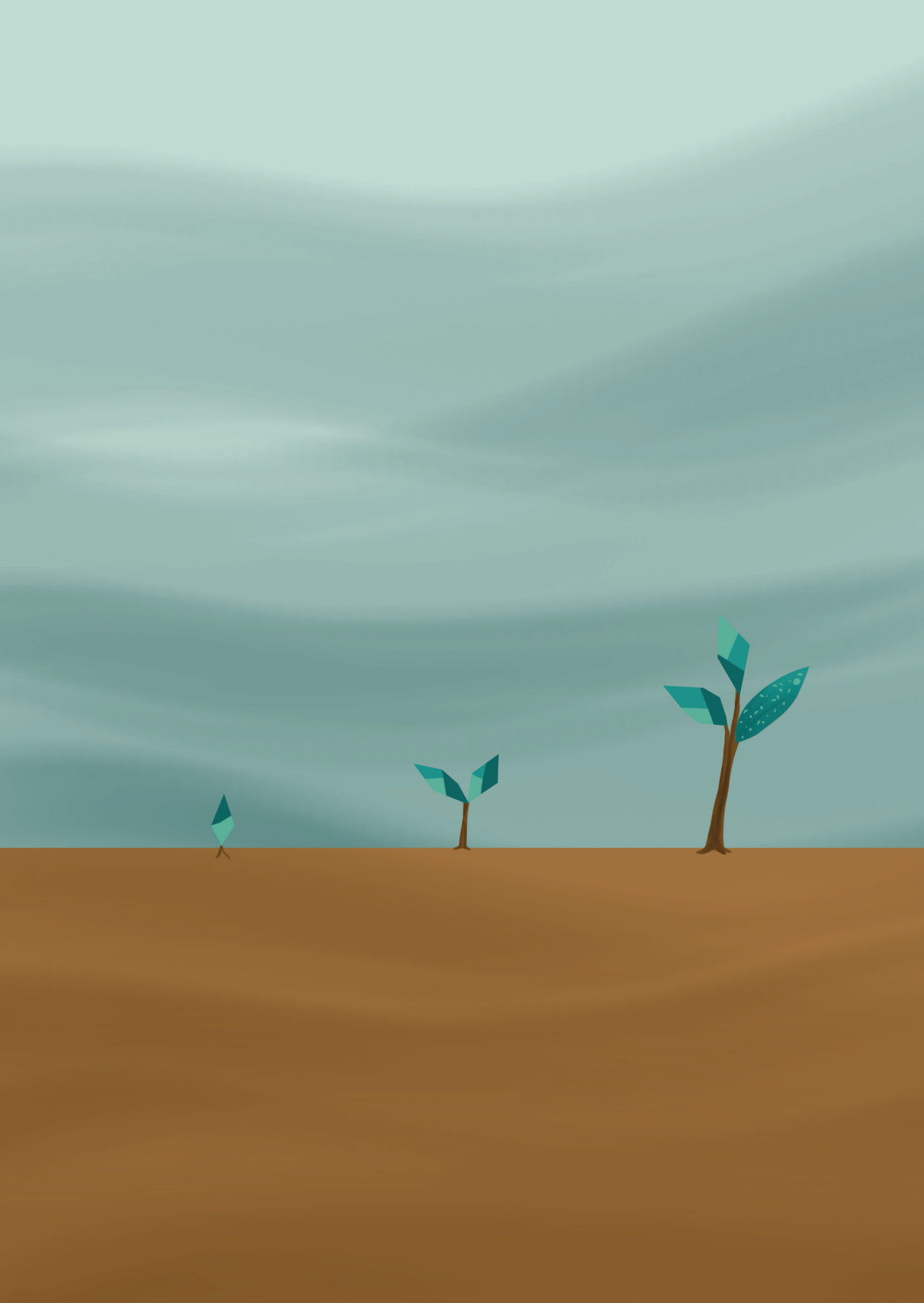
Part I

Assessing longitudinal body composition in infancy and early childhood



Chapter 2

Body composition and bone mineral density by Dual energy X-ray Absorptiometry: Reference values for young children

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Abstract

Background: Childhood obesity is a global public health threat, with an alarming rise in incidence. Obesity at young age has short-term and long-term morbidity. It is, therefore, important to accurately assess body composition throughout infancy and childhood to identify excess adiposity. However, reference values for age 2-5 years, needed to interpret measurements and identify young children at risk, are lacking. Our primary objective was to fill the current gap in reference values by constructing sex-specific body composition reference values and charts for fat mass (FM), fat mass percentage (FM%), fat mass index (FMI), lean body mass (LBM), lean body mass index (LBMI) and total body less head bone mineral density (BMD_{TBLH}) for children aged 2-5 years using Dual-Energy X-ray Absorptiometry (DXA).

Methods: We performed 599 accurate DXA-measurements in 340 term-born children aged 2 – 5 years, using Lunar Prodigy with Encore software (V14.1). Using GAMLSS, sex-specific reference values and charts were created for FM, FM%, FMI, LBM, LBMI and BMD_{TBLH}.

Results: Sex-specific body composition reference values and charts for age 2-5 years were constructed. In boys and girls, FM and LBM increased from age 2 to 5 years (all, $p \leq 0.001$), but body size-corrected FM% and FMI decreased (all, $p \leq 0.023$). LBMI remained similar between 2 and 5 years of age. Girls had higher FM, FM% and FMI and lower LBM and LBMI compared to boys. BMC and BMD_{TBLH} increased with age between 2-5 years of age (all, $p < 0.001$) and were similar for boys and girls.

Conclusions: We present sex-specific reference values and charts for body composition and total body bone mineral density measured by DXA, based on a large cohort of healthy children aged 2-5 years. These longitudinal references can be used for clinical practice and research purposes to monitor body composition and bone mineral density development and identify children at risk for excess adiposity.

Introduction

Childhood obesity is a global public health threat [1]. Excess adiposity at young age has not only short-term, but particularly long-term morbidity, such as cardiovascular disease, insulin resistance and type 2 diabetes [1, 2]. In general, BMI is mostly used to determine adiposity. However, BMI is poorly associated with true body composition and BMI SD-scores have low specificity to identify changes in fat mass percentage (FM%) [3, 4]. It is important to monitor true body composition longitudinally during childhood to identify children who are at risk for developing excess adiposity.

Several methods can be used to determine body composition in infants and young children. Until the age of 6 months and/or weight of 8 kg, air displacement plethysmography (ADP) by PEA POD can be used to determine body composition [5, 6]. Dual-Energy X-ray Absorptiometry (DXA) can also be used in infancy and is often used to determine body composition in older children [7]. DXA is reported to be accurate and has a high sensitivity and specificity to detect high body fat [3]. Results, however, can differ between various machines [8], modes [9] and software [10]. A major limitation of DXA is that it requires the child to lay still [11], which is a great challenge and leads to a high rate of unsuccessful measurements in infants and young children [12].

An advantage of DXA measurements is that it uses the three component method, which allows measurements of body composition as well as bone mineral content and density [7]. In early adulthood, bones reach their maximum density and strength, the peak bone mass [13]. Decreased bone mineral density in childhood and adolescence, results in lower peak bone density and has long-term consequences, such as increased osteoporosis risk [14]. High fat mass and especially high truncal fat is associated with lower bone mineral density (BMD_{TBLH}) in mid-childhood [15].

Reference values for body composition in young children are needed to identify children with excess adiposity. For BMD_{TBLH}, reference values are needed to monitor skeletal health in children. There are reference values available for body composition measured by ADP until 6 months of age and thereafter by DXA for children until age 2 years [12]. However, reference values for DXA measurements of body composition and BMD_{TBLH} for young children aged ≥ 2 years are very scarce and the limited available reference values for children aged 2-5 years are based on small populations [16, 17].

We, therefore, performed longitudinal DXA measurements in a large cohort of healthy term-born children aged 2 - 5 years. The aim of this study was to construct sex- and age specific reference data and SD-scores of fat mass (FM), fat mass percentage (FM%), fat mass index (FMI), lean body mass (LBM) and lean body mass index (LBMI), as well as for total body less head bone mineral density (BMD_{TBLH}) and bone mineral content (BMC) for children aged 2-5 years. Our final objective was that these DXA references could be used in clinical practice and research to monitor body composition and bone mineral density development in young children and to identify children at risk for excess adiposity.

Material and Methods

Study setting and subjects

This study was embedded in the Sophia Pluto study, a birth cohort study of healthy infants, aiming to provide detailed data on trajectories of growth and body composition in early childhood [18, 19]. Infants were recruited before age 28 days, from several maternity wards in Rotterdam, the second largest city in The Netherlands. All participants met the following inclusion criteria: singleton, term birth (≥37 weeks of gestation), an uncomplicated neonatal period and no severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation and at least one accurate DXA measurement between age 2 and 5 years. Exclusion criteria were maternal disease or medication that could interfere with the child's growth and development, like maternal use of corticosteroids or insulin, known congenital or postnatal disease and intrauterine infection. The Medical Ethics Committee of Erasmus Medical Center approved the Sophia Pluto study (MEC-2012-164). The study was conducted according to the ethical principles stated in the Helsinki declaration[20]. We obtained written informed consent of all parents or caregivers with parental authority.

Data collection and measurements

For this study, outpatient clinic visits were scheduled at 2, 3, 4 and 5 years. The same trained staff carried out the measurements according to standard procedures. Birth data were taken from hospital and midwife records.

Body composition and BMD were measured at every visit with the same DXA machine (Lunar Prodigy Advance R00279 (GE Healthcare, UK)), using Encore v14.1 software. The DXA machine was calibrated daily, according to the supplier's manual. During DXA scan, children wore only a diaper or underwear. BMC and BMC results are presented for total body less head (TBLH)[21].

Of the 340 children, 205 and 89 had accurate DXA measurements during at least 2 and 3 study visits, resp. Accurate DXA measurements could be conducted in 193, 204 and 179 measurements at 3, 4 and 5 years of age, resp. At the age of 2 years, we had only 23 accurate measurements, due to resistance of the toddlers and movement artifacts, but children aged ≥ 3 years were cooperative after thorough explanation and could lay still during the DXA measurement (accurate DXA measurements in 84%).

To test reliability, a random sample of 16 children was measured twice, with repositioning between measurements. Intra-class correlation coefficient (ICC) and percent coefficient of variance (%CV) for FM, FM%, LBM and BMD_{TBLH} were calculated. ICC was 0.991, 0.985, 0.993 and 0.980, resp. (all, $p < 0.001$) and %CV was 3.5%, 3.2%, 1.5% and 2.0%, resp.

Weight was measured to the nearest 5 grams by an electronic infant scale (Seca 717, Hamburg, Germany) at 2 years and by a flat scale (Seca 876) until age 5 years. Length was measured twice in supine position to the nearest 0.1 cm by an infantometer (Seca 416) at 2 years and in upright position by a stadiometer (Seca 213) until 5 years.

Fat mass index (FMI) was calculated as FM (kg) divided by length (m) squared. Lean body mass index (LBMI) was calculated as LBM (kg) divided by length (m) squared.

Statistical analysis

SD scores for length and weight corrected for sex and age were calculated at birth and for every visit, by Growth Analyser RCT software (https://www.growthanalyser.org) using Dutch references [22]. Child characteristics are presented as mean (standard deviation (SD)). Gestational age was non-normally distributed and is, therefore, expressed as median (interquartile range [IQR]). Differences between boys and girls were determined with an independent student's t-test.

To create sex-specific charts for FM, FM%, FMI, LBM, LBMI and BMD_{TBLH} plotted against age, generalized additive models for location, scale and shape (GAMLSS) were used [23, 24]. To construct the charts, Box-Cox Cole and Green distribution (BCCG) was applied to fit the three parameters of mu (μ), sigma (σ) and nu (ν) [25]. The distribution expresses the mean (μ), variance (σ) and skewness (ν) that change as a function of age presented as z-scores [25]. To improve readability for clinicians, the median, ±1 and ±2 SD-scores (or z-scores) were plotted against age for FM (kg), FM%, FMI (kg/m^2), LBM (kg), LBMI (kg/m^2), and BMD_{TBLH} (g/cm^2) against age. In addition, tables with median, ±1 and ±2 SD-scores for children aged 2- 5 years were made with 6 months intervals.

To test continuity of the results of this study with the results of our previous study in children aged ≤ 2 years [12], we measured a random sample of 8 extremely cooperative children aged 2 years twice, with and without vacuum cushion. Comparability was tested using Bland-Altman analyses. Raw data of the measurements with and without vacuum cushion had a fixed bias (all, $p < 0.001$), with mean differences

and [95% confidence interval] for FM, FM% and fat free mass of 1.81 [1.28-2.34] kg, 13.40 [9.07-17.73]% and -1.52 [-1.95 to -1.09] kg. However, when SD-scores were calculated, using the age-appropriate reference values (reference values of our previous study [12] for DXA measurement with vacuum cushion up to age 2 years, and current reference values for DXA measurement without vacuum cushion), there was no fixed bias. We found SD-scores to be similar, with mean differences [95% confidence interval] for FM, FM% and fat free mass SDS of -0.15 [-0.64-0.34], -0.13 [-0.89-0.63] and 0.05 [-0.64-0.74], resp.

Statistical tests were performed with R including GAMLSS-package v.5.2.0 (V4.0.0) and SPSS (V25.0.0.1). Results were regarded statistically significant at $p < 0.05$.

Results

The study group consisted of 340 healthy children aged 2-5 years. Child characteristics are shown in Table 1. Fifty-four percent of the group was male and 70% Caucasian. Length-for-age and weight-for-length SD-scores were not different between boys and girls.

Reference values for body composition in children aged 2-5 years

Sex-specific body composition reference values for age 2-5 years were constructed. The median, ±1 and ±2 SD-scores for FM, FM%, FMI, LBM and LBMI for boys and girls aged 2-5 years are presented in Table 2. Figure 1 shows the sex-specific reference charts for FM, FM%, FMI. In girls, median FM increased from 4.07 kg at 2 years to 5.46 kg at 5 years of age ($p < 0.001$). In boys, median FM increased from 3.93 kg to 4.80 kg during the same period ($p = 0.001$). Median FM% decreased from 32.26% to 27.86% in girls and from 30.86% to 26.60% in boys ($p = 0.010$ and $p = 0.019$, resp.). Median FMI decreased from 5.21 kg/m^2 to 4.38 kg/m^2 in girls and from 4.81 kg/m^2 to 3.83 kg/m^2 in boys ($p = 0.013$ and $p=0.023$, resp.).

Figure 2 shows the sex-specific reference charts for LBM and LBMI. In girls, median LBM increased from 7.77 kg at 2 years to 13.13 kg at 5 years and in boys from 8.78 kg to 14.14 kg (all, $p < 0.001$). LBMI remained stable from 2 to 5 years of age. In girls, LBMI was 10.40 kg/m^2 and 10.70 kg/m^2 at 2 and 5 years of age, resp. ($p = 0.447$) and in boys 11.07 kg/m^2 and 11.19 kg/m^2, resp. ($p = 0.386$).

Girls had higher median FM% at ages 3 - 5 years ($p < 0.001$, $p = 0.005$ and $p < 0.001$, resp.), higher median FM at 5 years of age ($p = 0.009$) and lower median LBM at ages 2 - 5 years ($p = 0.036$, $p = 0.001$, $p < 0.001$ and $p < 0.001$, resp.), compared to boys. Median FMI was, compared to boys, also significantly higher in girls at age 4 and 5 years ($p = 0.011$ and $p = 0.001$) and median LBMI was lower at ages 3 - 5 years ($p < 0.001$, $p < 0.001$ and $p = 0.002$, resp.).

Reference values for bone mineral density and bone mineral content in children aged 2-5 years

Sex-specific reference values for BMD_{TBLH} were constructed for children aged 2-5 years (Table 2). Figure 3 shows the sex-specific reference charts for BMD_{TBLH}. BMD_{TBLH} increased with age and similarly in boys and girls. Median BMD_{TBLH} in girls increased between age 2-5 years from 0.394g/cm^2 to 0.518g/cm^2 and in boys from 0.398 g/cm^2 to 0.516g/cm^2 (both $p < 0.001$).

Table 1. Child characteristics

	Boys	Girls	*p*-value
Birth			
N	183	157	
Gestational age*	39.71 [38.86 – 40.71]	39.86 [39.00-40.80]	0.452
Birth weight SDS	0.30 (1.10)	0.15 (1.11)	0.196
Birth length SDS^	0.75 (1.18)	0.56 (1.13)	0.256
Ethnicity *(N (%))*			0.088
Caucasian	128 (69.9%)	101 (64.3%)	
Black	6 (3.3%)	16 (10.2)	
Asian	1 (0.5%)	2 (1.3%)	
Latin-American	1 (0.5%)	0	
Mixed	40 (21.9%)	32(20.4%)	
Missing	7 (3.8%)	6 (3.8%)	
Age 2 years			
N#	9	14	
Length SDS	0.13 (0.61)	0.19 (0.91)	0.869
Weight-for-length SDS	-0.63 (0.68)	-0.09 (1.32)	0.274
Age 3 years			
N	102	91	
Length SDS	-0.24 (0.87)	-0.38 (1.01)	0.309
Weight-for-length SDS	0.086 (1.00)	0.18 (1.00)	0.538
Age 4 years			
N	111	93	
Length SDS	-0.20 (0.89)	-0.29 (0.94)	0.482
Weight-for-length SDS	0.17 (1.08)	0.23 (0.95)	0.702
Age 5 years			
N	101	78	
Length SDS	0.17 (1.09)	-0.30 (0.97)	0.637
Weight-for-length SDS	-0.23 (0.90)	0.32 (0.96)	0.340

*Data expressed as mean (SD), except for data indicated with *, which are expressed as median [IQR]. ^112 boys and 86 girls. # 93% of measurements failed, due to movement artifacts. Abbreviations: N=number of subjects, SDS= standard deviation score.*

Table 2. Sex- specific reference values for body composition and bone mineral density and content

	Boys					Girls				
	-2 SD	**-1 SD**	**0 SD**	**+ 1 SD**	**+2 SD**	**- 2 SD**	**-1 SD**	**0 SD**	**+ 1 SD**	**+2 SD**
Body composition										
FM (kg)										
2 years	2.63	3.24	3.93	4.70	5.55	2.38	3.22	4.07	4.94	5.83
2.5 years	2.77	3.41	4.14	4.98	5.92	2.62	3.42	4.30	5.26	6.28
3 years	2.90	3.56	4.34	5.24	6.27	2.84	3.63	4.54	5.57	6.72
3.5 years	3.00	3.68	4.50	5.46	6.59	3.02	3.82	4.77	5.87	7.16
4 years	3.08	3.78	4.63	5.64	6.87	3.20	4.02	5.00	6.18	7.57
4.5 years	3.14	3.85	4.72	5.79	7.11	3.38	4.21	5.23	6.48	8.01
5 years	3.19	3.90	4.80	5.92	7.34	3.56	4.40	5.46	6.79	8.46
5.5 years	3.24	3.96	4.87	6.05	7.58	3.70	4.56	5.66	7.05	8.83
FM%										
2 years	21.82	26.87	30.86	34.24	37.20	23.18	27.60	32.26	37.14	42.24
2.5 years	21.35	25.92	29.82	33.27	36.41	23.17	27.12	31.53	36.42	41.84
3 years	20.84	24.98	28.77	32.32	35.67	23.00	26.60	30.79	35.68	41.39
3.5 years	20.29	24.04	27.73	31.37	34.97	22.71	26.05	30.06	34.92	40.86
4 years	19.70	23.11	26.69	30.43	34.33	22.36	25.48	29.33	34.14	40.28
4.5 years	19.08	22.18	25.65	29.50	33.78	22.00	24.91	28.59	33.36	39.76
5 years	18.44	21.26	24.60	28.59	33.36	21.58	24.32	27.86	32.57	39.15
5.5 years	17.78	20.34	23.56	27.69	33.14	21.18	23.82	27.25	31.88	38.51
FMI (kg/m²)										
2 years	3.24	4.01	4.81	5.65	6.51	3.35	4.24	5.21	6.25	7.37
2.5 years	3.26	3.98	4.76	5.58	6.44	3.33	4.15	5.08	6.12	7.29
3 years	3.22	3.90	4.65	5.47	6.35	3.29	4.05	4.94	5.99	7.21
3.5 years	3.06	3.72	4.47	5.32	6.28	3.25	3.95	4.81	5.85	7.14
4 years	2.90	3.53	4.29	5.20	6.29	3.19	3.84	4.66	5.71	7.06
4.5 years	2.73	3.32	4.05	4.98	6.16	3.14	3.74	4.53	5.57	7.01
5 years	2.67	3.17	3.83	4.72	5.98	3.07	3.63	4.38	5.43	6.96
5.5 years	2.61	3.03	3.61	4.44	5.73	3.01	3.53	4.26	5.30	6.93
LBM (kg)										
2 years	7.11	7.89	8.78	9.80	10.97	6.24	6.93	7.77	8.81	10.12
2.5 years	7.70	8.58	9.57	10.66	11.89	6.98	7.75	8.66	9.77	11.14
3 years	8.31	9.30	10.38	11.55	12.83	7.73	8.56	9.55	10.73	12.15
3.5 years	8.94	10.05	11.23	12.48	13.82	8.46	9.38	10.45	11.70	13.18
4 years	9.61	10.85	12.14	13.48	14.88	9.19	10.19	11.34	12.67	14.21

Table 2. Continued

	Boys					Girls				
	-2 SD	**-1 SD**	**0 SD**	**+ 1 SD**	**+2 SD**	**- 2 SD**	**-1 SD**	**0 SD**	**+ 1 SD**	**+2 SD**
4.5 years	10.33	11.72	13.14	14.58	16.03	9.90	10.99	12.23	13.65	15.26
5 years	11.04	12.60	14.14	15.67	17.18	10.60	11.79	13.13	14.63	16.32
5.5 years	11.75	13.50	15.17	16.79	18.37	11.18	12.45	13.87	15.44	17.19
LBMI (kg/m²)										
2 years	9.76	10.35	11.07	11.94	13.05	8.87	9.60	10.40	11.26	12.20
2.5 years	9.71	10.31	11.01	11.86	12.91	8.99	9.68	10.43	11.26	12.16
3 years	9.68	10.28	10.98	11.80	12.80	9.09	9.74	10.47	11.27	12.15
3.5 years	9.68	10.28	10.97	11.77	12.72	9.16	9.80	10.51	11.30	12.19
4 years	9.71	10.32	11.00	11.79	12.70	9.21	9.85	10.56	11.36	12.28
4.5 years	9.78	10.39	11.08	11.85	12.73	9.28	9.91	10.63	11.44	12.38
5 years	9.88	10.50	11.19	11.95	12.80	9.36	9.98	10.70	11.53	12.49
5.5 years	10.08	10.71	11.40	12.16	13.00	9.43	10.05	10.77	11.60	12.59
Bone mineral density and content										
BMD_{TBLH} (g/cm²)										
2 years	0.347	0.370	0.398	0.436	0.488	0.340	0.367	0.394	0.419	0.444
2.5 years	0.363	0.388	0.418	0.456	0.504	0.359	0.387	0.415	0.442	0.470
3 years	0.378	0.406	0.438	0.475	0.521	0.378	0.407	0.436	0.466	0.497
3.5 years	0.393	0.423	0.457	0.495	0.538	0.397	0.426	0.457	0.489	0.523
4 years	0.409	0.441	0.477	0.515	0.556	0.416	0.445	0.477	0.512	0.550
4.5 years	0.424	0.459	0.496	0.535	0.575	0.435	0.465	0.498	0.535	0.578
5 years	0.439	0.487	0.516	0.555	0.594	0.453	0.484	0.518	0.559	0.606
5.5 years	0.454	0.496	0.536	0.575	0.614	0.472	0.503	0.539	0.582	0.636
BMC (g)										
2 years	167.4	188.9	216.4	253.1	304.2	148.5	177.0	207.8	240.7	275.8
2.5 years	192.0	220.4	254.2	294.6	343.3	179.3	210.2	244.9	283.5	326.4
3 years	214.0	251.4	292.0	336.0	383.3	210.9	243.8	282.0	326.2	377.4
3.5 years	233.9	282.0	329.8	377.4	424.7	243.0	277.7	319.1	368.8	429.0
4 years	256.1	313.3	367.6	419.6	469.7	275.7	311.9	356.2	411.3	481.2
4.5 years	286.3	346.5	405.4	463.3	520.4	308.8	346.3	393.3	453.7	534.0
5 years	321.2	380.8	443.2	508.2	575.6	342.2	380.9	430.4	496.0	587.5
5.5 years	356.2	415.4	481.0	553.6	663.4	376.0	415.8	467.5	538.1	641.9

Data expressed as median, ±1 and ±2SD scores for FM (fat mass), FM% (fat mass percentage), FMI (fat mass index), LBM (lean body mass) and LBMI (lean body mass index) and for BMC (bone mineral content) and BMD_{TBLH} (total body less head bone mineral density) for boys and girls aged 2-5 years, obtained with DXA (Lunar Prodigy).

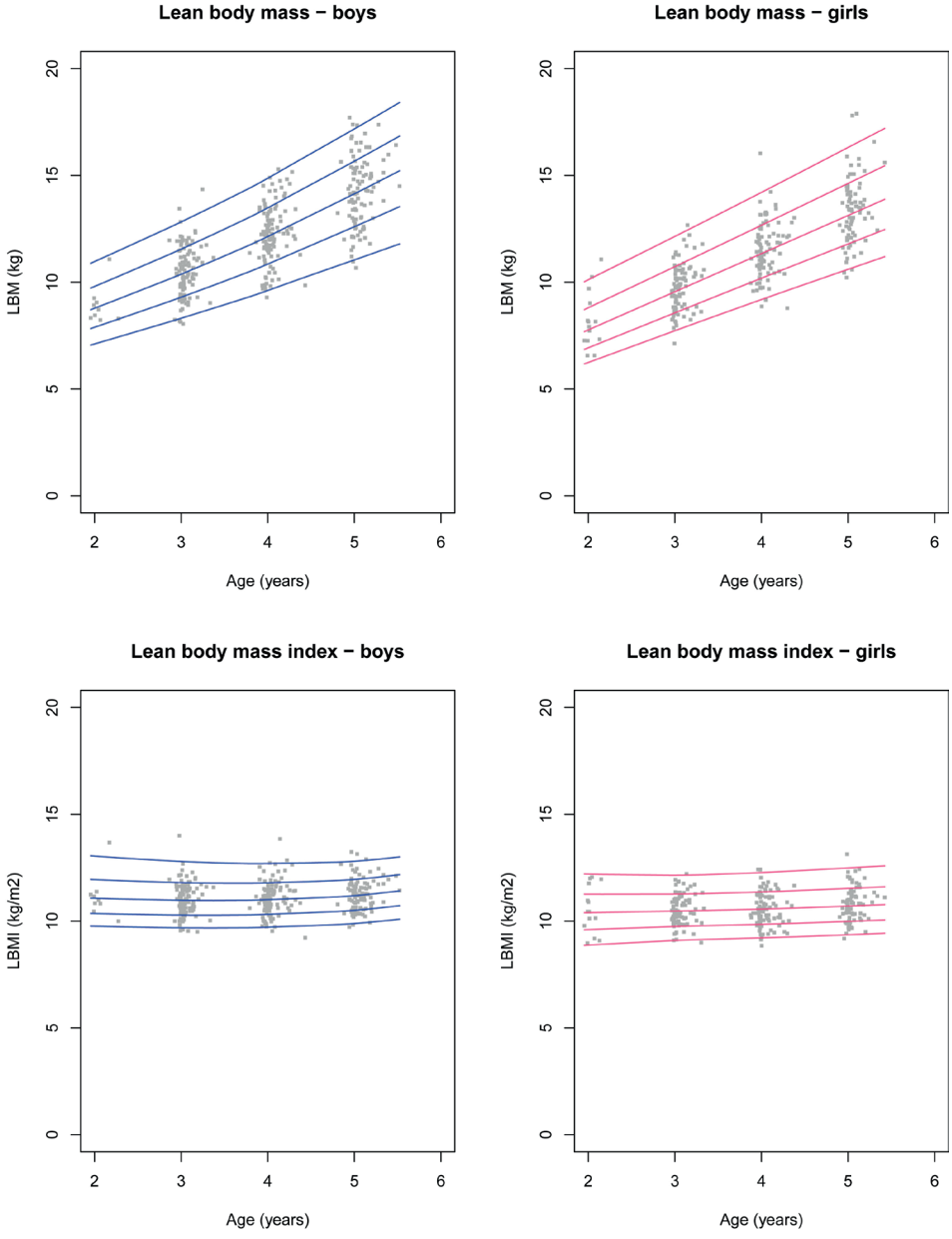

Figure 1. Sex-specific reference charts of FM, FM% and FMI for boys and girls measured by DXA. *Data are expressed as median and ±1 and ±2 SD scores obtained with DXA (Lunar Prodigy). Sex-specific reference charts for boys are presented in blue and for girls in pink.*

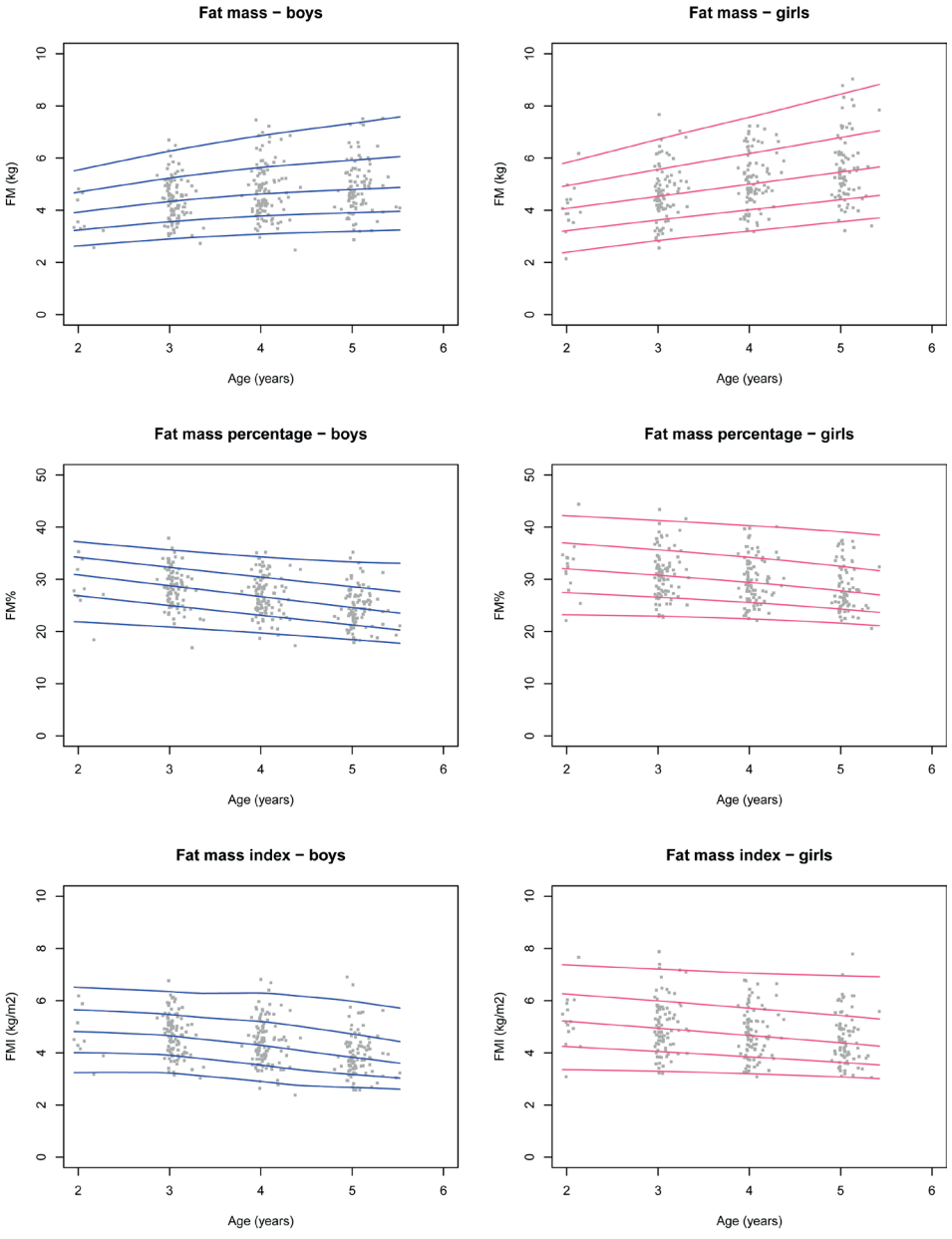

Figure 2. Sex-specific reference charts for LBM and LBMI for boys and girls measured by DXA.

Data are expressed as median and ±1 and ±2 SD scores obtained with DXA (Lunar Prodigy). Sex-specific reference charts for boys are presented in blue and for girls in pink.

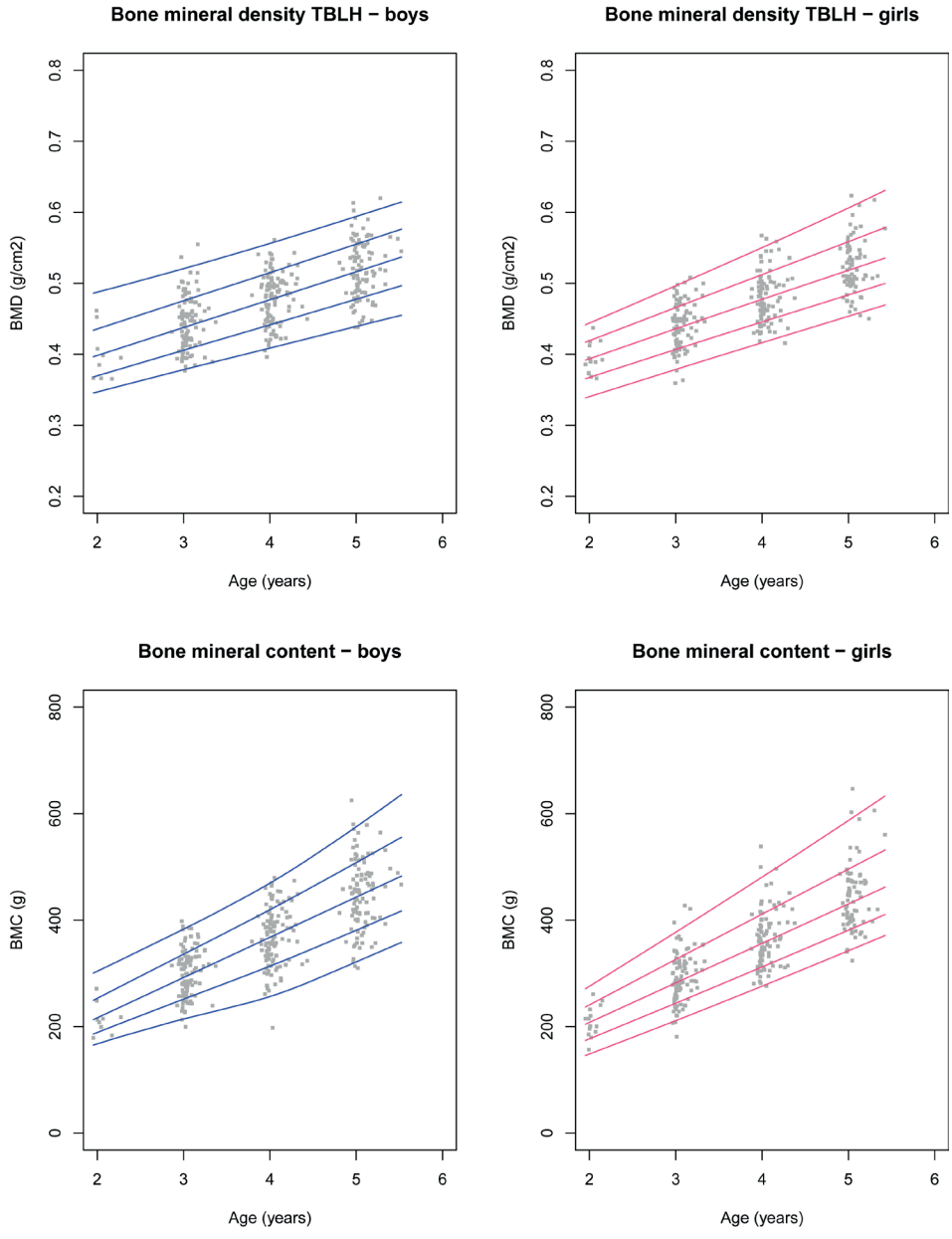

Figure 3. Sex-specific reference charts for BMD_{TBLH} and BMC for boys and girls measured by DXA. *Data are expressed as median and ±1 and ±2 SD scores obtained with DXA (Lunar Prodigy), measurements total body less head (TBLH). Sex-specific reference charts for boys are presented in blue and for girls in pink.*

Discussion

We present sex-specific reference values and charts for fat mass and lean body mass parameters as well as bone mineral density based on a large group of healthy children aged 2-5 years.

Reference values for body composition and bone mineral density measured by DXA are very scarce in young children aged 2-5 years and the limited reference values were based on small groups [16, 17]. We now present, sex-specific reference values and charts for young children aged 2-5 years which can be used for clinical practice and research, provided that the same equipment and software is used (DXA by Lunar Prodigy with Encore software (V14.1)).

Between 2 and 5 years of age, FM and LBM increased in boys and girls. Since body size and length increase during infancy and childhood, monitoring longitudinal body composition solely by FM and LBM in kg is misleading [26]. In clinical practice and for research purposes, it is, therefore, preferable to use FM% or FMI and LBMI to monitor body composition [27]. We found that FM% and FMI decreased between the age of 2 and 5 years, while LBMI remained similar, in both boys and girls. Our results are in line with our previous findings during infancy until age 2 years and in children older than age 5 years [12, 16, 28]. We now present reference values and charts for FM, LBM as well as FM%, FMI and LBMI in children aged 2-5 years, thereby filling the current gap in reference values.

We found differences in body composition between boys and girls. At age 3-5 years, girls had significantly higher FM% and FMI and lower LBMI compared to boys. The higher FM% in girls compared to boys is in line with another study [17]. We found previously similar sex differences in body composition during infancy [12, 29] and others described such differences in children aged ≥4 years [16]. These differences in body composition are likely caused by different endogenous testosterone production in girls versus boys [30]. It is, therefore, important to use sex-specific reference values from an early age onwards to monitor body composition.

We also present reference data for BMD_{TBLH} for children aged 2-5 years. We found no differences between boys and girls, which is in line with three other research groups who did not find sex differences in BMD in prepubertal children aged 5 years and older [16, 31, 32]. Median BMD_{TBLH} increased with age during this period, in boys and girls alike. This is in line with another study [17]. Our data show that, unlike for body composition, sex differences in BMD_{TBLH} are not present in early childhood.

If we compare our data to the limited literature, our reference data are higher for FM% [17] and lower for LBM in kg [16] and BMD_{TBLH} [17] in both boys and girls at age 4 and 5 years. However, both previous studies had smaller study populations and older ages compared to ours. Lifshitz *et al* performed 58 measurements in boys and 68 in girls aged 4-5 years [17] and van der Sluis *et al* in only 11 boys and 11 girls at age 4-5 years [16], while our data are based on measurements of 340 children aged 2-5 years, of which 211 measurements in boys and 171 in girls at age 4-5 years. Furthermore, their studies used the Lunar DPX-L. We used the newer Lunar Prodigy with Encore software V14.1. Body composition measurements and BMD_{TBLH} by DXA are known to differ between various types of DXA machines [8], modes [9] and software [10]. The difference in DXA machines and software might be another explanation why our reference values differ from values reported earlier.

The strength of this study is the large number of detailed and longitudinal measurements of fat mass, fat mass percentage, lean body mass and bone mineral density in a large cohort of healthy term-born children. The number of DXA measurements at age 2 years was small, which is a limitation of the study, The main reason for that is the fact that it is extremely difficult to obtain accurate DXA measurements in infants aged ≤ 2 years due to movement artifacts. We have previously shown that accurate DXA measurements can be obtained by using a vacuum cushion to avoid movement artifacts in this age group [12]. From 3 years onwards, most children were cooperative, leading to 84% accurate DXA measurements in children aged ≥ 3 years. Because of the different methods to assess accurate DXA measurements before and after the age of 2 years, we constructed reference values in 2 age segments (birth – 2 years [12] and 2 years – 5 years (present study)). We found that the SD-scores are similar for the reference data from birth – 2 years and from 2-5 years. Thus provided that age-appropriate reference data are used, these references and charts can be used longitudinally to monitor DXA measurements and identify children at risk for excess adiposity.

In conclusion, we present novel sex-specific reference values and charts for body composition and total body bone mineral density measured by DXA for children aged 2-5 years, based on a large cohort of healthy children. These longitudinal DXA references for young children can be used for clinical practice and research purposes to monitor body composition and bone mineral density development and to identify children at risk for excess adiposity.

Acknowledgements

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Author contributions AHK was in charge of designing the study. IvB, KdF and AHK were in charge of the cohort, design, and collecting of the data. IvB and SS did the statistical analysis. Drafting the manuscript was primarily done by IvB under supervision of AHK. All authors were involved in writing the manuscript and had final approval of the submitted version.

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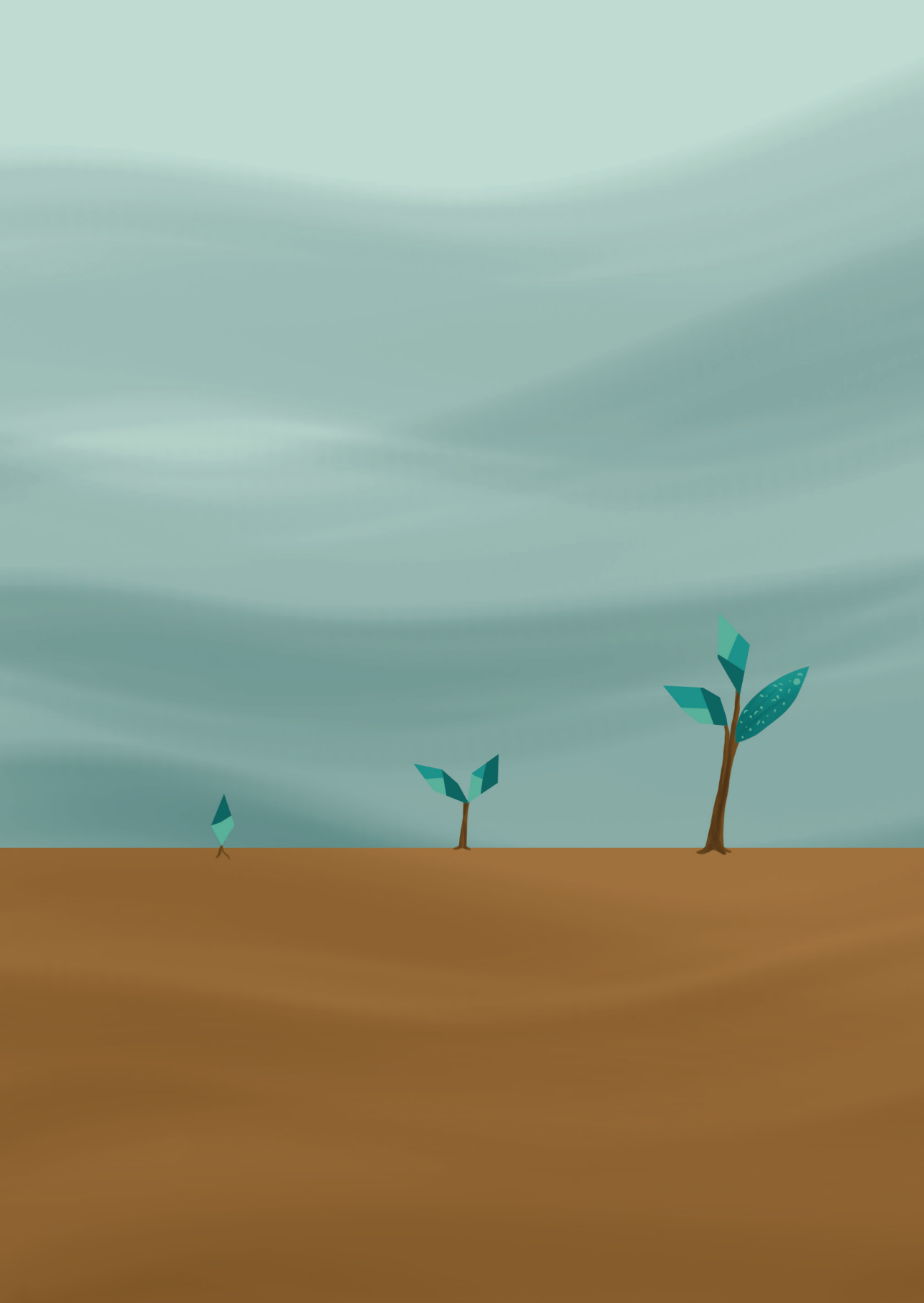

Chapter 3

Body composition assessment by Air Displacement Plethysmography compared to Dual energy X-ray absorptiometry in full-term and preterm aged three to five years

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Abstract

Background: It is important to monitor body composition longitudinally, especially in children with atypical body composition trajectories. Dual energy X-ray absorptiometry (DXA) can be used and reference values are available. Air displacement plethysmography (ADP) is a relatively new technique, but reference values are lacking. In addition, estimates of fat free mass density (Dffm), needed in ADP calculations, are based on children aged >8 years and may not be valid for younger children. We, therefore, aimed to investigate whether DXA and ADP results were comparable in young children aged 3–5 years, either born full-term or preterm, and if Dffm estimates in the ADP algorithm could be improved.

Methods: In 154 healthy children born full-term and 67 born <30 weeks, aged 3–5 years, body composition was measured using ADP (BODPOD, with default Lohman Dffm estimates) and DXA (Lunar Prodigy). We compared fat mass (FM), fat mass percentage (FM%) and fat free mass (FFM), between ADP and DXA using Bland–Altman analyses, in both groups. Using a 3-compartment model as reference method, we revised the Dffm estimates for ADP.

Results: In full-term born children, Bland–Altman analyses showed considerable fixed and proportional bias for FM, FM%, and FFM. After revising the Dffm estimates, agreement between ADP and DXA improved, with mean differences [LoA] for FM, FM%, and FFM of -0.67 kg (-2.38; 1.04), -3.54% (-13.44; 6.36), and 0.5 kg (-1.30; 2.30), respectively, but a small fixed and proportional bias remained. The differences between ADP and DXA were larger in preterm born children, even after revising Dffm estimates.

Conclusions: Despite revised and improved sex and age-specific Dffm estimates, results of ADP and DXA remained not comparable and should not be used interchangeably in the longitudinal assessment of body composition in children aged 3-5 years, and especially not in very preterm born children of that age.

Introduction

Childhood obesity tracks into adulthood and has been linked to both short-term and long-term morbidity [1, 2]. Consequently, it is important to identify children at risk of excess adiposity early in childhood in order to start preventive and therapeutic strategies as early as possible. Body composition is a more adequate indicator of adiposity than standard anthropometric measures such as weight or body mass index (BMI), especially in infants and young children [3, 4]. Therefore, reliable methods to longitudinally assess body composition from early childhood onwards are needed. Specific attention should be given to children at risk of altered adiposity trajectories, such as children born preterm [5].

Multiple tools are available for measuring body composition during childhood, with Dual energy X-ray Absorptiometry (DXA) and Air Displacement Plethysmography (ADP) being most frequently used [6, 7]. DXA is often used as a reference method to determine body composition in research and clinical practice [7]. Longitudinal reference values are available for infants and young children from birth until age 5 years, showing slightly higher fat mass in girls as compared to boys [8, 9]. However, DXA uses a very small dose of radiation (0.0002 mSv). ADP calculates body composition by measuring body volume, using the inverse pressure-volume-relation [10] and can be applied in infants ≤6 months old and/or ≤8 kg using PEAPOD [11, 12], and in children ≥2 years and ≥12 kg using BODPOD [13]. ADP is, however, currently more costly than DXA and requires cooperation of the child, as movement and crying influence results [13]. It is our experience that BODPOD is feasible in children ≥3 years of age. Importantly, ADP uses multiple assumptions to calculate body composition parameters from measured body volume. These assumptions include estimates for fat free mass (FFM) density (Dffm). The default estimates in BODPOD software are based on outdated, small studies in which results of healthy older children and adults were extrapolated to children aged <8 years, per 2-year-intervals [14, 15]. These Dffm-estimates may, therefore, not be valid in young children. In fact, especially in young children with deviant body composition, such as preterm born children, we noticed that ADP results are often clinically questionable (e.g. extremely low values of fat mass percentage (FM%), <5%). Wells *et al* developed novel Dffm-estimates for healthy children aged ≥5 years [16], which have been reported to be superior to the default estimates in ADP for children aged 5 years [17]. However, improved Dffm-estimates for children aged <5 years are not yet available.

In order to monitor body composition longitudinally in infants and young children, it would be favorable if ADP and DXA could be used interchangeably. Our research group reported that results from ADP (PEA POD) were comparable with DXA in infants aged 6 months [8]. Studies in healthy school-children, adolescents and adults, however, showed conflicting results on comparability [10, 18-20]. In young children, aged 3-5 years, comparison between ADP and DXA has not yet been described.

The primary aim of our study was to compare fat mass (FM), FM% and FFM results assessed by ADP with DXA, in a cohort of healthy full-term born children aged 3 to 5 years. Secondly, we aimed to explore potential improvements to the default Dffm-estimates in the ADP algorithm for full-term born children in this age category. Furthermore, we evaluated body composition based on the default and revised Dffm-estimates in a group of very preterm born children aged 3-5 years. We hypothesized that ADP and DXA are both reliable methods to estimate body composition in young children, but may not be used interchangeably, especially not in very preterm born children aged 3-5 years.

Material and Methods

Study setting and subjects

The current cross-sectional study included subjects of two ongoing prospective birth cohort studies on growth and body composition which started from 2012 at the Erasmus MC Sophia Children's Hospital in Rotterdam, The Netherlands. The Sophia Pluto study included healthy full-term born infants [21, 22], whereas the BOND study included infants born very preterm (<30 weeks gestation) [23]. The full-term born infants (≥37 weeks) were recruited from all seven maternity wards in Rotterdam and experienced an uncomplicated neonatal period. Infants with a complicated perinatal or neonatal period were excluded: in case of maternal disease or medication that could interfere with growth and development, perinatal asphyxia, neonatal sepsis, neonatal respiratory ventilation and significant congenital or intrauterine disease [21]. The very preterm born children were admitted to our level IV neonatal intensive care unit within 48 hours after birth. Exclusion criteria for this group included congenital and chromosomal anomalies that could interfere with growth, severe brain injury, congenital infection, or perinatal asphyxia [23].

The present analyses were based on a subgroup of children from both cohorts whose body composition was, per study protocol, measured by both ADP and DXA at the age of 3-5 years, between April 2019 and November 2021. All participants of both cohorts who were in this age range within this timeframe were eligible. The Medical Ethics Committee of the Erasmus MC approved both studies (MEC-2012-164 and MEC-2014-379). We obtained written informed consent of all parents/caregivers.

Data collection and measurements

For full-term born subjects, outpatient clinic visits were scheduled at 3, 4 and 5 years, and for preterm born subjects at 3 and 5.5 years corrected age. Data on child ethnicity were derived from parental questionnaires.

Anthropometrics

Weight was measured without heavy clothing to the nearest 5 grams using a flat scale (Seca, Hamburg, Germany). Height was measured to the nearest 0.1 cm in upright position by a stadiometer (Seca). Age and sex-corrected SD-scores for weight and length at birth and at 3-5 years were calculated using Dutch reference values, and Fenton charts at preterm birth [24, 25].

Body composition

In 154 healthy full-term born children and 67 very preterm children, body composition was measured by ADP and DXA within one hour. For ADP, we used BODPOD (COSMED) with pediatric hardware and software, including the default Lohman density model [14]. Children wore tight underwear (without diaper) and a Lycra cap covering all scalp hair [14]. The DXA (Lunar Prodigy, GE Healthcare) was used with Encore v14.1 software. During DXA-scan, children wore light clothing. FFM was calculated as the sum of lean body mass and bone mineral content.

The same ADP and DXA devices were used during the entire study period. Both devices were calibrated daily, and used and maintained according to the supplier's manuals [13]. During measurements, children were instructed not to move. We excluded measurements if the supplier's terms of use were not met, or when the child cried. To test reliability, a random sample of full-term born children was measured twice, after repositioning, with the same device (13 with ADP and 16 with DXA). Intra-class correlation coefficients for FM, FM% and FFM for ADP were 0.980, 0.978 and 0.994, and for DXA 0.991, 0.985 and 0.994 (all, $p < 0.001$), respectively.

The BODPOD calculates FM% using two constants (C1 and C2), derived from the programmed, sex-specific density models for Dffm and FM density (Dfm), and measured body density (BD (kg/L)), as expressed in Formula (1) [14]:

$$C1 = (Dffm * Dfm)/(Dffm - Dfm)$$
$$C2 = Dfm/(Dffm - Dfm)$$

$$FM\% = (C1/BD - C2) * 100\% \quad (1)$$

The standard algorithm in the BODPOD software follows the assumption that Dfm remains stable during life at 0.9007 kg/L[14-16]. Consequently, Formula (1) can be rewritten as Formula (2):

$$Dffm = ((0.9007 * FM\% - 90.07) * BD) / (FM\% * BD - 90.07) \quad (2)$$

Statistical analysis

Children born full-term and preterm were analyzed as separate groups. Independent sample t-tests were used to compare group characteristics. Paired sample t-tests were used to compare ADP and DXA results for each group at all ages. Bland-Altman analyses were used to test agreement between ADP and

DXA results. Fixed bias was determined by one sample t-test, and proportional bias by linear regression. As body composition, like anthropometrics, differs per sex, we analyzed boys and girls separately [8, 9].

As the current algorithms used in ADP are based on Dffm's of children aged > 8 years, which are extrapolated for younger ages [14], we re-calculated Dffm for each included full-term born child. We used Formula (2) with body density (BD) as measured by ADP, and FM% as derived from the 3-compartment model [26]. For the 3 compartments, we entered body volume (BV) measured by ADP (BODPOD), bone mineral content (BMC) measured by DXA and body weight (BW) measured by scale, as follows:

$$FM\% = \frac{(6.386 * BV + 3.961 * BMC - 6.09 * BW)}{BW} * 100\%$$

We used the re-calculated Dffm-values to create sex-specific curves by age, using generalized additive models for location, scale, and shape (GAMLSS) [27, 28]. Box-Cox-Cole and Green distribution (BCCG) was applied to fit the three parameters of mu (μ), sigma (σ) and nu (ν). The distribution expresses the mean (μ), variance (σ) and skewness (ν) that change as a function of age. Median Dffm was then assessed for ages between 3 and 5 years, using 0.25-year time-intervals. Based on these new sex- and age-specific median Dffm-estimates, we re-calculated FM% for each ADP-measurement using Formula (1). For children with age >5 years, which was above the modelled age range, we used Wells *et al* Dffm-estimates [17].

A 2-tailed *p*-value <0.05 was considered statistically significant. Analyses were performed using SPSS-package 25.0 (IBM SPSS Statistics, Armonk, NY) and R with GAMLSS-package v.5.2.0 (V 4.0.0 for MacOS, R Core Team, Vienna, Austria).

Results

Clinical characteristics of the full-term and preterm born children are shown in Table 1. SD-scores for weight-for-height and height were lower in the preterm compared to the full-term group at 3 and 5 years corrected age. Body composition parameters assessed by ADP and DXA are presented in Table 2. FM and FM%, assessed by DXA, were higher in full-term compared to preterm born children at each time point (all, $p \leq 0.001$). In both groups, FM and FFM increased with age and body-size corrected FM% decreased with age (Supplement Table 1).

Comparison between ADP and DXA in full-term born children

Absolute results of FM, FM% and FFM by ADP and DXA were significantly different (all, $p < 0.001$) (Table 2). Bland-Altman analyses (Figure 1) showed that mean differences [limits of agreement [LoA]] for FM, FM% and FFM between ADP and DXA were -1.08 [-2.92 - 0.76], -5.78% [-16.2 - 4.69] and 0.90 kg [-1.00 - 2.80], respectively. For all three parameters, a fixed bias (all, $p<0.001$) and a proportional bias for FM (β: 0.135, $p = 0.014$), FM% (β: 0.396, $p < 0.001$) and FFM (β: 0.109, $p = 0.002$) were observed. Proportional bias indicates that the difference between ADP and DXA increased when the result deviated more from the mean.

Revised FFM density model

Table 3 presents the revised, sex-specific estimates for Dffm for full-term children aged 3-5 years. Dffm increased between age 3 and 5 years. Compared to the default Lohman Dffm model, the revised Dffm estimates are higher at all ages. At age 5 years, they are in the range of the Wells *et al* [16] estimates (Figure 2).

The agreement with DXA improved when using the revised Dffm-estimates for ADP, with mean differences [LoA] for FM, FM% and FFM of -0.67kg [-2.38-1.04], -3.54% [-13.44-6.36] and 0.50 kg [-1.30-2.30], respectively (Table 2). Although smaller, a fixed (all, $p < 0.001$) and proportional bias remained, for FM (β: 0.135, $p = 0.010$), FM% (β: 0.374, $p < 0.001$) and FFM (β: 0.106, $p = 0.002$).

Comparison between ADP and DXA in very preterm born children

Using the default Dffm-estimates in children born very preterm, absolute results of ADP and DXA were very different (all, $p < 0.001$) (Table 2). In fact, differences in FM, FM% and FFM results between both methods were significantly larger in preterm compared to full-term born children (all, $p < 0.001$), with mean differences [LoA] of -1.89 kg [-4.10-0.32] for FM, -9.79% [-20.92-1.34] for FM%

and 1.64 kg [-0.63-3.91] for FFM (Figure 1). Similar to the full-term group, a fixed bias (p <0.001) was observed for all three parameters, and a proportional bias for FM% (β: 0.575, p <0.001) and FFM (β: 0.264, p = 0.001), but not for FM (β: 0.080, p = 0.504).

Table 1. Clinical characteristics

	Full-term born		Very preterm born		*p*-value
	Boys	**Girls**	**Boys**	**Girls**	
Birth	N=79	N=75	N=39	N=28	
Gestational age (weeks)	39.47 (1.29)	39.77 (1.24)	27.50 (1.55)	27.44 (1.55)	**<0.001**
Birth weight SDS	0.39 (1.00)	0.19 (1.09)	0.27 (0.68)	0.05 (0.76)	0.416
BPD (%)	*NA*	*NA*	12 (30.8%)	5 (17.9%)	
Ethnicity (%)					**<0.020**
Caucasian	54 (68.4%)	45 (60.0%)	30 (76.9%)	23 (82.1%)	
Non-Caucasian	25 (31.6%)	30 (40.0%)	9 (23.1%)	5 (17.9%)	
All visits, total group					
Weight-for-height SDS	0.07 (1.11)	0.40 (0.91)	−0.55 (1.10)	−0.51 (1.13)	**<0.001**
Height SDS	−0.26 (0.79)	−0.20 (1.02)	−0.87 (0.76)	−0.70 (1.11)	**<0.001**
Age 3 years	N=18	N=24	N=13	N=10	
Age (years)	3.06 (0.11)	3.08 (0.10)	3.44 (0.15)	3.46 (0.18)	**<0.001**
Weight-for-height SDS	0.31 (1.08)	0.51 (1.02)	-0.43 (0.94)	-0.47 (0.95)	**0.001**
Height SDS	-0.13 (0.72)	0.09 (0.93)	-0.69 (0.65)	-0.58 (1.34)	**0.008**
Age 4 years	N=33	N=24			
Age (years)	4.11 (0.13)	4.15 (0.15)	*NA*	*NA*	
Weight-for-height SDS	-0.13 (1.21)	0.45 (0.83)	*NA*	*NA*	
Height SDS	-0.32 (0.89)	0.02 (1.08)	*NA*	*NA*	
Age 5 years	N=46	N=41	N=26	N=18	
Age (years)	5.11 (0.14)	5.08 (0.13)	5.97 (0.17)	5.94 (0.12)	**<0.001**
Weight-for-height SDS	0.13 (1.05)	0.31 (0.14)	-0.61 (1.18)	-0.53 (1.25)	**<0.001**
Height SDS	-0.27 (0.75)	-0.51 (0.98)	-0.96 (0.80)	-0.77 (1.00)	**0.002**

Data are expressed as absolute numbers (percentage) or mean (SD). P-values represent the differences between full-term and very preterm born children (both sexes combined), analyzed with independent t-test. Significant p-values are boldfaced. Abbreviations: n= number; SDS=standard deviation score, NA=not applicable; BPD=bronchopulmonary dysplasia.

Table 2. Body composition parameters assessed by ADP and DXA

	Full-term N=186	Very preterm N=67	*p*-value
FM (kg)			
DXA	5.16 (1.26)	4.43 (1.26)	**<0.001**
ADP default	4.09 (1.45)	2.54 (1.35)	**<0.001**
ADP revised	4.47 (1.40)	2.98 (1.73)	**<0.001**
Mean difference [LoA] $_{\text{ADP default –DXA}}$	-1.08 * [-2.92-0.76]	-1.89 * [-4.10-0.32]	**< 0.001**
Mean difference [LoA] $_{\text{ADP revised –DXA}}$	-0.67 * [-2.38-1.04]	-1.45 * [-3.53-0.63]	**<0.001**
FM%			
DXA	28.26 (4.88)	24.39 (4.76)	**<0.001**
ADP default	22.47 (6.91)	14.60 (7.88)	**<0.001**
ADP revised	24.90 (6.64)	17.07 (7.93)	**<0.001**
Mean difference [LoA] $_{\text{ADP default –DXA}}$	-5.78 * [-16.25-4.69]	-9.79 * [-20.92-1.34]	**<0.001**
Mean difference [LoA] $_{\text{ADP revised –DXA}}$	-3.54 * [-13.44-6.36]	-7.32 * [-18.26-3.62]	**<0.001**
FFM (kg)			
DXA	13.06 (2.01)	13.72 (2.59)	0.064
ADP default	13.96 (2.25)	15.36 (3.36)	**0.002**
ADP revised	13.41 (2.13)	14.91 (3.28)	**0.001**
Mean difference [LoA] $_{\text{ADP default –DXA}}$	0.90 * [-1.00-2.80]	1.64 * [-0.63-3.91]	**<0.001**
Mean difference [LoA] $_{\text{ADP revised –DXA}}$	0.50 * [-1.30-2.30]	1.20 * [-0.92-3.32]	**<0.001**

*Data are expressed as mean (SD). P-value term vs. preterm is difference between mean difference in term and very preterm born children. * indicates differences between ADP and DXA p<0.001. Abbreviations: ADP=air displacement plethysmography; DXA=dual energy X-ray absorptiometry; FM= fat mass; FM% = fat mass percentage; FFM=fat free mass, LoA=limits of agreement (95% CI).*

When using the revised Dffm-estimates, comparison of ADP and DXA showed smaller fixed bias, with mean differences [LoA] for FM: -1.45 kg [-3.53-0.63], FM%: -7.32% [-18.26-3.62], FFM: 1.20kg [-0.92-3.32] (Table 2), but the proportional bias remained similar.

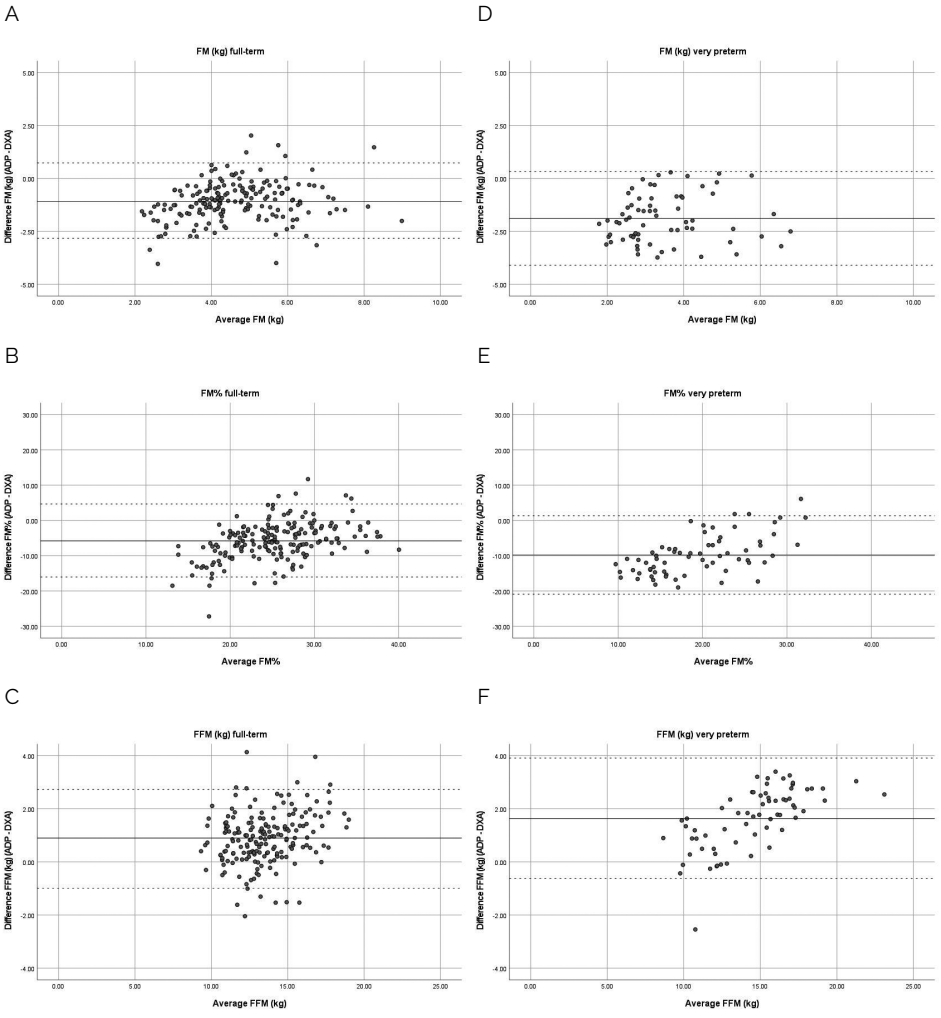


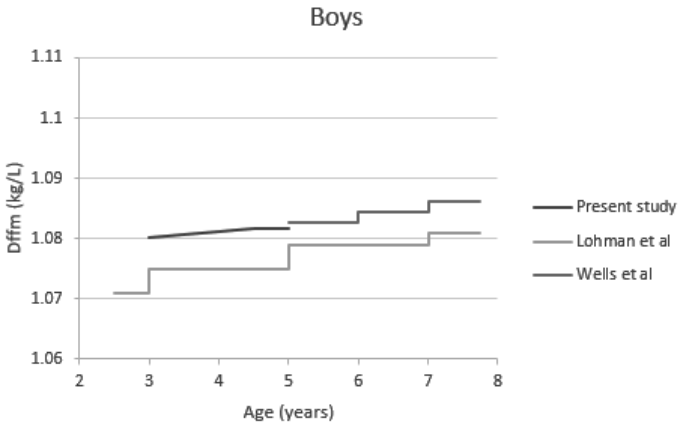
Figure 1. Bland-Altman plots for FM, FM% and FFM measured by ADP and DXA in full-term (A, B, C) and very preterm born children (D, E and F) aged 3-5 years. *Continuous line represents the mean difference between ADP and DXA. The dashed lines represent the limits of agreement. Abbreviations: FM=fat mass, FM%= fat mass percentage, FFM=fat free mass; kg= kilograms; DXA=dual energy X-ray absorptiometry; ADP=air displacement plethysmography*

Table 3. Revised fat free mass density models for children aged 3-5 years

Age (years)	Boys			Girls		
	C1	C2	Dffm	C1	C2	Dffm
2.75	5.432	5.031	1.0797	5.449	5.050	1.0790
3	5.424	5.022	1.0801	5.426	5.025	1.0800
3.25	5.416	5.013	1.0804	5.405	5.001	1.0808
3.5	5.409	5.005	1.0807	5.393	4.987	1.0813
3.75	5.402	4.998	1.0809	5.386	4.980	1.0816
4	5.395	4.990	1.0812	5.384	4.978	1.0816
4.25	5.390	4.984	1.0814	5.384	4.978	1.0816
4.5	5.386	4.980	1.0816	5.384	4.978	1.0816
4.75	5.384	4.978	1.0817	5.384	4.978	1.0816
5	5.384	4.977	1.0817	5.384	4.978	1.0816

*Median Dffm and C1 and C2 predicted in 0.25-year intervals for children aged 3-5 years. C1 = (Dffm * Dfm)/ (Dffm-Dfm). C2 = Dfm/ (Dffm-Dfm)) Abbreviations: Dffm= fat free mass density. Dfm= fat mass density = 0.9007 kg/L.*

A



B

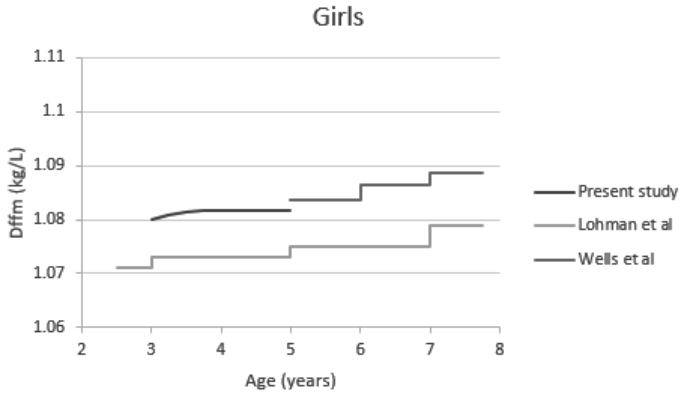


Figure 3. Dffm-estimates plotted against age for boys and girls separately.

Presented are the revised Dffm-estimates from present study and those of Lohman *et al* [14] and Wells *et al* [16].

Discussion

To our knowledge, this is the first study to compare ADP with DXA in a relatively large group of young children aged 3–5 years who underwent both ADP and DXA. We observed significant differences in FM, FM% and FFM results derived with both techniques. Based on our cohort of healthy full-term born children, we provide a revised Dffm-model to be used with ADP in children aged 3-5 years. Furthermore, differences between ADP, using default or revised Dffm-estimates, and DXA were significantly larger in very preterm compared to full-term born children. Although our revised Dffm-estimates improved agreement between ADP and DXA, we have to conclude that results of both techniques are not comparable and should thus not be used interchangeably in the longitudinal assessment of body composition in children aged 3-5 years.

Literature on comparison of ADP and DXA in the pediatric population is limited, but shows similarities with our findings. Two studies in infants aged 0-6 months observed that both methods generated highly correlated but significantly different absolute results[29, 30]. In particular, FM and FM% estimates by ADP were significantly lower compared to DXA, while FFM results were higher; as also observed in present study. In adolescence, ADP and DXA results were reported to be strongly correlated[20, 31]. However, FM% results were not comparable in subjects with more deviant body composition, such as individuals with severe under- or overweight[20, 31]. These findings correspond with the observed proportional bias, as well as the larger inter-method differences in very preterm born children. We extend the previous literature by adding data on ADP and DXA comparison in young children aged 3–5 years, in whom comparative studies were lacking.

Although DXA and ADP have been validated against 4-component models in small samples of healthy children with normal weight[13], both machines use different techniques with limitations that could explain the observed differences. ADP has several limitations. It measures body volume using the inverse pressure-volume-relation, which is sensitive for environmental factors that influence air pressure and density, such as crying of the subject or fluctuations in room temperature[10, 13]. Besides, in order to calculate body composition parameters based on body volume, it uses density models that are based on multiple assumptions[10]. First, FM is thought to contain no water and have a constant density throughout life, whereas Dffm is considered to increase with age, as FFM hydration decreases throughout life[14-16]. Other assumptions include the content of bone mineral constituents and the amount of fat in the bones, as well as lung volume[10]. The Lohman Dffm-model, used

as default in the ADP-software, was extrapolated from data of small populations of subjects aged 0-1 and 8-30 years measured in the 1980's[14-16]. Dffm-estimates were then extrapolated to other pediatric age categories per 2-year-intervals [14]. Also, Dffm can vary in children with different nutritional status (e.g. hydration status), physical activity level, ethnicity and disease status, but these variables were not included in the density models[14, 32]. All these factors could have added to the inter-method differences observed in present study. DXA is based on a 3-compartment-model and uses the attenuation of X-ray-energy passing different types of tissue[33, 34]. DXA-software differentiates bone, fat and other tissues. For pixels that contain mixed tissues, the software calculates the three parameters based on fixed algorithms using bone-edge-detection[33]. These tools are based on a constant hydration status of FFM but it is known that the hydration status of FFM in children decreases with age[35]. Furthermore, it has been reported that DXA-software encounters difficulties differentiating tissues in objects with a smaller body size[36]. DXA-software might, therefore, be less accurate in young children, despite pediatric software options.

Our revised Dffm-estimates are higher compared to those of Lohman *et al*[14], which are used as default in the BODPOD machine for age 3-5 years. Our estimates are in line with a study from Wells *et al*[16], who revised Dffm-estimates for children aged ≥5 years using a 4-compartment model. The Wells *et al* estimates were more accurate compared to Lohmans estimates in healthy 5.5-year old children, when validated against a 3-compartment model, including isotope dilution[17]. We have now added revised Dffm estimates for younger children, aged 3-5 years.

We observed that the inter-method differences were significantly larger in children born preterm compared to full-term children. Although using our revised Dffm-estimates improved ADP-results, considerable bias, fixed and proportional, remained present. This could have several explanations. First, very preterm-born children are prone to experience impaired growth resulting in smaller body size as compared to full-term born peers[37], as also seen in our cohort. The aforementioned limitations of DXA-software in subjects with small body size may, therefore, hamper accurate assessment of body composition in this group[36]. More importantly, children born very preterm show a different pattern of body composition and Dffm over childhood. While FM in preterm children was observed to be higher around term age, studies later in childhood reported lower FM and FFM as compared to full-term born children[5, 38]. Furthermore, recent studies showed that bone mineral content and density were also lower in preterm born children at the age of 5-9 years as compared to full-term born children[39, 40]. Besides, incorrect assumptions about thoracic gas volume could lead to incorrect body composition estimates by ADP[10].

Preterm born children, with or without bronchopulmonary dysplasia (BPD), more often have reduced lung volumes or impaired lung function in mid childhood[41]. All these variables may complicate accurate assessment of body composition by ADP in the preterm population, in which accurate information on body composition is important for long-term health. Given the observed proportional bias between ADP and DXA, a low FM% will lead to greater inter-method differences. Moreover, a recent study in over 900 subjects aged 4-22 years showed that leaner body types have lower FFM hydration and consequently higher Dffm[32]. Altogether, we suggest that caution is needed when interpreting ADP-results of this specific patient group. In fact, it warrants further research to compose separate Dffm-estimates for children with deviant body composition trajectories, such as preterm born children.

Strength of this study is the relatively large number of healthy full-term born children who underwent an ADP and DXA assessment within one hour. To our knowledge, this is the first study to provide revised Dffm-models for ADP in full-term born children aged 3-5 years. Furthermore, comparing results with a group of very preterm born children emphasizes the challenges of assessing body composition in children at risk for deviant growth patterns. We also acknowledge several limitations. We revised the Dffm-model using FM% prediction not from a 4-compartment but from a 3-compartment model as reference method. Yet, a 4-compartment model has not been investigated in children below the age of 5.5 years[34]. Although we observed improvement of ADP results using our revised Dffm-estimates, future studies should explore how the revised estimates hold in pediatric populations elsewhere. In particular, including sufficient numbers of children from different ethnical groups would increase external generalizability. Moreover, our findings suggest the need for specific Dffm-models for different patient groups with deviant body composition trajectories, such as preterm born children. Because the 3-compartment model was validated for healthy subjects, it proved not suitable as a reference for the very preterm group in our study. Further research, using a 4-compartment model including isotope dilution, in larger cohorts is needed to calculate and validate Dffm-estimates for particular patient groups (e.g. other growth disorders). Lastly, development of cheaper but reliable methods also applicable in lower-resource settings could improve body composition measurement in a broader sense.

Conclusion

Despite revised age and sex-specific Dffm-estimates for ADP, results of ADP and DXA remained not comparable and should not be used interchangeably in the longitudinal assessment of body composition in children aged 3-5 years, especially not in very preterm born children of that age.

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Author contributions: AHK designed the Sophia Pluto study, whereas MV and KJ designed the BOND study. IvB, VB, AB, MV and AHK were in charge of data collection. IvB performed the statistical analysis. Drafting the manuscript was primarily done by IvB and VB. All authors were involved in interpretation of the data, revising the manuscript critically for important intellectual content. All authors had final approval of the version to be published and agreed to be accountable for all aspects of the work.

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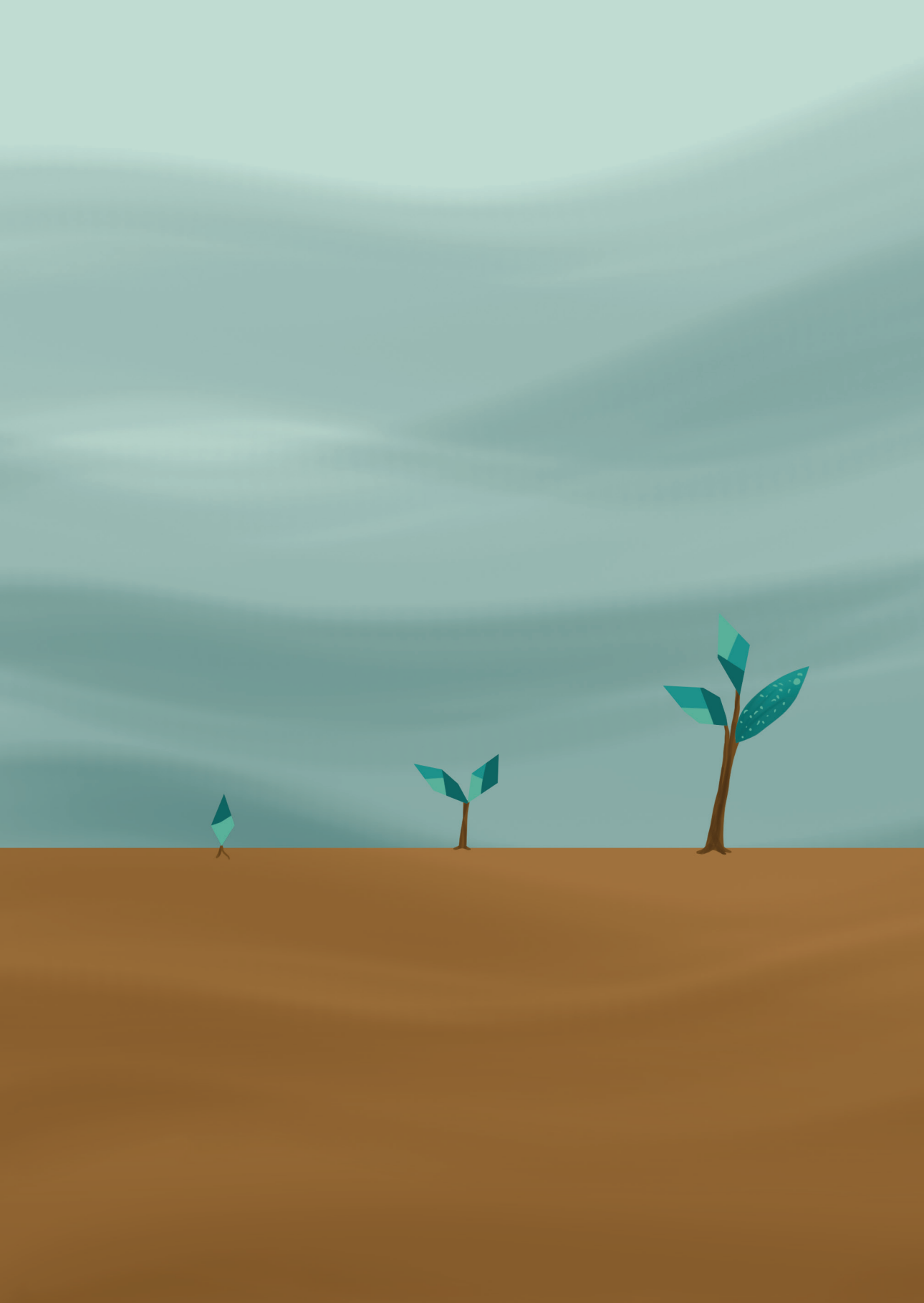
Supplement

Supplementary Table 1. Body composition parameters assessed by ADP and DXA between age 3-5 years

	Age 3 years			Age 4 years	Age 5 years		
	Full-term	Very preterm	*p*-value	Full-term	Full-term	Very preterm	*p*-value
FM (kg)							
DXA	4.86 (1.20)	3.92 (0.79)	**0.001**	5.10 (1.21)	5.35 (1.30)	4.70 (1.39)	**0.009**
ADP default	3.67 (1.47)	3.34 (1.04)	0.344	4.16 (1.39)	4.24 (1.46)	2.12 (1.31)	**<0.001**
ADP revised	4.04 (1.46)	3.71 (1.03)	0.341	4.59 (1.42)	4.64 (1.32)	2.60 (1.39)	**<0.001**
FM%							
DXA	30.30 (4.76)	26.06 (4.01)	**0.001**	28.70 (4.86)	26.98 (4.60)	23.52 (4.93)	**<0.001**
ADP default	23.06 (7.65)	22.30 (5.69)	0.677	23.50 (6.66)	21.52 (6.65)	10.58 (5.52)	**<0.001**
ADP revised	25.53 (8.37)	25.05 (6.86)	0.815	26.01 (7.41)	22.87 (7.78)	10.96 (5.93)	**<0.001**
FFM (kg)							
DXA	11.07 (1.15)	11.11 (1.48)	0.893	12.56 (1.51)	14.35 (1.68)	15.08 (1.19)	**0.028**
ADP default	12.00 (1.32)	11.52 (1.36)	0.169	13.35 (1.67)	15.31 (2.05)	17.36 (2.10)	**<0.001**
ADP revised	11.63 (1.27)	11.15 (1.34)	0.157	12.92 (1.61)	14.73 (1.99)	16.88 (2.02)	**<0.001**

Data are expressed as mean (SD). P-value term vs preterm is difference between mean difference in term and very preterm born children. Abbreviations: ADP= air displacement plethysmography; DXA=dual energy X-ray absorptiometry; FM= fat mass; FM% = fat mass percentage; FFM= fat free mass, LoA= limits of agreement (95% CI)



Chapter 4

Anthropometry-based prediction of body composition in early infancy compared to Air Displacement Plethysmography

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Abstract

Background: Anthropometry-based equations are commonly used to estimate infant body composition. However, existing equations were designed for newborns or adolescents. We aimed to (a) derive new prediction equations in infancy against air-displacement plethysmography (ADP PEA POD) as the criterion, (b) validate the newly developed equations in an independent infant cohort and (c) compare them with published equations (Slaughter-1988, Aris-2013, Catalano-1995).

Methods: Cambridge Baby Growth Study (CBGS), UK, had anthropometry data at 6 weeks (N = 55) and 3 months (N = 64), including skinfold thicknesses (SFT) at four sites (triceps, subscapular, quadriceps and flank) and ADP-derived total body fat mass (FM) and fat free mass (FFM). Prediction equations for FM and FFM were developed in CBGS using linear regression models and were validated in Sophia Pluto cohort, the Netherlands, (N = 571 and N = 447 aged 3 and 6 months, respectively) using Bland–Altman analyses to assess bias and 95% limits of agreement [LoA].

Results: CBGS equations consisted of sex, age, weight, length and SFT from three sites and explained 65% of the variance in FM and 79% in FFM. In Sophia Pluto, these equations showed smaller mean bias than the three published equations in estimating FM: mean bias [LoA] 0.008 [-0.489-0.505] kg at 3 months and 0.084 [-0.545-0.713] kg at 6 months. Mean bias in estimating FFM was 0.099 [-0.394-0.592] kg at 3 months and -0.021 [-0.663-0.621] kg at 6 months.

Conclusions: CBGS prediction equations for infant FM and FFM showed better validity in an independent cohort at ages 3 and 6 months than existing equations.

Introduction

Nutritional and growth patterns during early life have been associated with risks for obesity and cardiometabolic diseases later in life [1-4]. This association has been continuously reported even in the current studies and reviews [5-7]. Quantification of infant body composition enables accurate estimation of the effects of early-life nutrition on growth and the putative developmental mechanisms leading to later co-morbidities. Weight for length and body mass index (BMI) are widely used as early adiposity screening tools [8]; however, those parameters do not distinguish between fat mass (FM) and fat free mass (FFM), the relative proportions of which vary markedly during infancy [9]. Moreover, in pediatric population, BMI often produces imprecise estimate of adiposity, and it varies greatly with age and gender [10, 11].

Several methods are available to assess infant body composition. These include Dual energy X-ray Absorptiometry (DXA) [12], Quantitative nuclear Magnetic Resonance (QMR) [13], Bioelectrical Impedance Analysis (BIA) [14], Total Body Electrical Conductivity (TOBEC)[15] stable isotope dilution and air-displacement plethysmography (ADP). BIA and TOBEC are non-invasive, safe, portable, inexpensive and widely available, but their use in infants is limited by poor accuracy [14-18]. Prediction studies in infants using BIA as the criterion are also scarce [17, 18]. DXA and QMR provide more accurate estimates of infant body composition; however, they are often infeasible because they require infants to lie still, even with the use of sedative agents [12, 13]. In addition, DXA uses ionizing radiation, and results could vary depending on the type of scans and softwares used [19-22]. Accordingly, the use of DXA in infants is limited, and detailed body composition data in this population are not abundant [17, 18].

ADP PEA POD is a non-invasive whole-body densitometry device to estimate infant body composition (total body FM and FFM). It is accurate and reliable in young infants when assessed against DXA [23-25], although there was also a study reporting high correlation between those two instruments with significant difference [19]. Nevertheless, ADP PEA POD is limited to infants weighing between 1 and 8 kg, thus usually it cannot be used for infants older than 6 months. The equipment is relatively expensive, is not portable, and the process is often time-consuming, so is impractical to use in many large-scale population studies [26]. Furthermore, some parents report anxiety in leaving their young infants in the closed ADP PEA POD system for around 2 minutes [26]. Therefore, in research studies, estimates of infant body composition are often derived using anthropometry-based equations [8, 27]. However, many of those equations include uncommonly collected measures (e.g., calf circumference [28] and flank skinfolds [29]) that are not available in infant cohort studies.

In this study, we aimed to develop new anthropometry-based equations for the prediction of total body FM and FFM in infancy against ADP PEA POD as the criterion, in a UK cohort, the Cambridge Baby Growth Study (CBGS). We also aimed to determine the accuracy of these new equations and three existing childhood anthropometry-based equations (Slaughter *et al* [16], Aris *et al* [23] and Catalano *et al* [30], Table 1), in an independent birth cohort, Sophia Pluto study, The Netherlands, using ADP PEA POD as the reference method. While Aris *et al* [23] and Catalano *et al* [30] were derived among neonatal populations, Slaughter *et al* [16] involved individuals aged 8-29 years old. Although the age range used in those three published equations was different from ours, they are frequently used in studies involving infants and children and were built using relevant anthropometry measures and skinfold sites.

Table 1. Published anthropometry-based prediction equations for body composition in children

First author (year)	Prediction equation	SFT site(s)	Participants age	Reference method
Slaughter *et al* (1988)[16]	Boys: %BF = 1.21 * Sum SFT – 0.008 * (Sum SF)² – 1.7 Girls: %BF = 1.33 * Sum SFT – 0.013 * (Sum SFT)² – 2.5	Triceps and subscapular (mm)	8–29 years	Underwater weighing to measure body density and deuterium oxide dilution to measure body water
Aris *et al* (2013)[23]	FM (kg) = –0.022 + 0.307 * Weight (kg) – 0.077 * Sex ^ – 0.019 * GA (weeks) + 0.028 x SFT	Subscapular (mm)	1–3 days	ADP PEA POD
Catalano *et al* (1995)[30]	FM (kg) = 0.54657 + 0.39055 * Weight (kg) + 0.0453 * SFT – 0.03237 * Length (cm)	Flank (mm)	1–3 days	TOBEC

^Sex (1 = boy; 0 = girl). Abbreviations: FM=fat mass; FFM=fat free mass; GA=gestational age; SFT=skinfold thickness; TOBEC= total body electrical conductivity.

Subjects and methods

Derivation cohort

The new anthropometry-based prediction equations were derived in CBGS, a longitudinal birth cohort study set up in 2001 at a single maternity hospital in Cambridge, United Kingdom, to investigate the prenatal and postnatal determinants of infancy weight gain, body composition and adiposity [31]. To provide detailed growth measures in the first weeks of life, N = 150 mother–infant pairs born between 2015 and 2018 underwent a more intensive measurement protocol. All infants were singleton, vaginally delivered at term, of normal weight mothers with no significant pregnancy comorbidities and had normal birth weight. This analysis included a cohort subgroup of 77 infants with ADP PEA POD measurements. There were in total 119 measurements employed to derive the equations, N = 55 at 6 weeks and N = 64 at 3 months. There was no significant difference in 6 weeks and 3 months anthropometry between the subgroup and the whole cohort (data not shown). The study was approved by the Cambridge Local Research Ethics Committee, and all mothers gave written informed consent.

Validation cohort

The anthropometry-based equations developed in CBGS were validated in an independent birth cohort study, Sophia Pluto, a prospective study to collect longitudinal data on measured growth and body composition among large group of healthy infants born at term. Mothers were recruited between 2013 and 2018, from several maternity wards in and near Rotterdam, The Netherlands.

Infant anthropometry

Infant anthropometry data were collected by trained pediatric research nurses, following standard protocols. Weight was measured to the nearest 1 g using a Seca 757 electronic baby scale (Seca, Birmingham, UK). Length was measured to the nearest 0.1 cm using an Infantometer (Seca 416). Waist circumference was measured at the midpoint between the lowest rib margin and the iliac crest to the nearest 0.1 cm using a non-stretchable fiber-glass tape (Chasmors Ltd, London, United Kingdom) in CBGS and a measuring tape (Seca, Hamburg, Germany) in Sophia Pluto. Skinfold thickness (SFT) measures were taken in triplicate from the left side of the body at four sites, including triceps, subscapular, flank (suprailiac), biceps (Sophia Pluto only) and quadriceps (CBGS only) using a calibrated Holtain Tanner/Whitehouse Skinfold Caliper (Holtain, Crymych, United Kingdom) in CBGS and using a Skinfold caliper (Slimguide C-120, Creative Health) in Sophia Pluto. Infant body composition parameters (FM%, FM and FFM) were estimated using

ADP PEA POD (COSMED/Life Measurement Inc., Concord, California), which directly measures body volume and body weight to calculate body density. Infant FM% was calculated from body density assuming the density of fat to be 0.9007 kg/L. Age- and sex-specific densities of FFM were computed using the data of Fomon et al [32]. FM and FFM were calculated from body weight and FM%. ADP PEA POD was calibrated every day, according to the instructions of the manufacturer. In the CBGS, ADP PEA POD was conducted twice, at 6 weeks and 3 months old, while in Sophia Pluto, it was conducted twice at 3 and 6 months.

Statistical analysis

In CBGS, stepwise multivariable regression models were performed to derive the optimal prediction of ADP PEA POD derived FM and FFM, using sex, age, length, weight and skinfold thicknesses as independent variables. The equations involved three sites of skinfolds measurement which were commonly measured by both studies: triceps, subscapular and flank (suprailiac). Quadriceps skinfold was omitted due to its unavailability in the validation cohort.

In Sophia Pluto, FM and FFM values were predicted using newly developed equations and three other childhood prediction equations (Table 1). Agreement between predicted and ADP PEA POD measured FM and FFM values was assessed using one-sample paired Student's *t* test, bivariate correlation, linear regression analysis and Bland–Altman analyses. In each Bland–Altman plot, the *y*-axis represents the difference or bias between equation-predicted and ADP PEA POD measured values with limits of agreement [LoA] described as the 95% confidence range (mean bias ± 1.96 SDS), while the *x*-axis represents the mean values of the two methods being compared (FM or FFM predicted from each corresponding equation and their absolute measured values from ADP PEA POD). The possibility of predicted results being affected by the magnitude of the measured values was assessed by running a correlation analysis between the mean (of the values measured by ADP PEA POD as the reference and each alternative equation) and the difference of values between the reference and each equation. Moreover, proportional bias was also calculated using linear regression, with the difference between measured and predicted FM/FFM acting as the dependent variable while the average of measured and predicted FM/FFM acting as the independent variable.

Statistical analyses were performed using SPSS version 25.0 (IBM) and R version 1.0.136. A *p*-value less than 0.05 was considered statistically significant.

Results

Baseline characteristics of derivation and validation cohorts are summarized in Table 2. At birth, CBGS infants were heavier than Sophia Pluto's with comparable length. In contrast, both cohorts had similar weight average, while CBGS infants were shorter at 3 months of age. In addition, 92.4% CBGS subjects were of Caucasian, while Sophia Pluto included a more diverse population with 62.6% Caucasian (of which 93.8% were white Caucasian and 6.2% were Turkish/Moroccan), 27.1% of mixed ethnicities and the remaining 10.3% of other ethnicities (Asian, African, Latin American).

Table 2. Baseline cohorts' characteristics by sex

	Cohort					
	CBGS (N = 77)		Sophia Pluto (N = 571)		*p*-value*	
Descriptive	**Boys (55%)**	**Girls (45%)**	**Boys (54%)**	**Girls (46%)**	**Boys**	**Girls**
Birth						
GA (weeks)	40.05 ± 1.17	40.13 ± 1.15	39.65 ± 1.24	39.77 ± 1.24	**0.048**	**0.008**
Weight (kg)	3.55 ± 0.47	3.47 ± 0.4	3.45 ± 0.49[b]	3.31 ± 0.50	0.211	0.072
Length (cm)[a]	50.79 ± 1.89	50.21 ± 1.55	50.84 ± 2.18	49.91 ± 2.04	0.889	0.412
Age 3 months						
Weight (kg)	6.29 ± 0.73[b]	5.65 ± 0.54	6.26 ± 0.70[b]	5.70 ± 0.69	0.795	0.682
Length (cm)	60.87 ± 2.12[b]	59.58 ± 1.74	61.95 ± 2.07[b]	60.23 ± 2.18	**<0.010**	0.089
FM (kg)	1.42 ± 0.37	1.29 ± 0.39	1.42 ± 0.41[b]	1.32 ± 0.40	1.000	0.675
FFM (kg)	4.78 ± 0.42[b]	4.42 ± 0.47	4.84 ± 0.47[b]	4.37 ± 0.42	0.430	0.516
FMI (kg/m^2)	3.85 ± 0.98	3.62 ± 1.04	3.68 ± 1.01	3.62 ± 1.02	0.313	0.96
FFMI (kg/m^2)	12.95 ± 0.88[b]	12.41 ± 0.95	12.57 ± 0.91[b]	12.05 ± 0.86	**0.011**	**0.022**

*Values are mean ± SD. FMI= fat mass index, calculated by dividing FM (kg) by length squared (m^2).26 FFMI= fat free mass index, calculated by dividing FFM (kg) by length squared (m^2). Abbreviations: GA= gestational age; FFM= fat free mass; FM= fat mass. p-values are based on independent t test. Significant p-values (< 0.05) are indicated in bold. * p-value between CBGS and Sophia Pluto of the same sex (i.e., boys = CBGS boys vs Sophia Pluto boys, girls = CBGS girls vs Sophia Pluto girls). [a] Birth length available in Sophia Pluto cohort: boys n = 210, girls n = 152. [b] Significantly different between boys and girls (p < 0.05) in the same infant group (i.e., CBGS boys vs girls, Sophia Pluto boys vs girls).*

Table 3. CBGS-derived equations to predict ADP PEA POD measured infant FM and FFM combining 6 weeks and 3 months measurements

Model			Weight (kg)	Length (cm)	Sex	Age (days)
Dependent variable: FM (kg)						
1	Wt + L + Sex + Age	B	0.624	−0.088	−0.070	0.001
		± SE	± 0.06*	± 0.02*	± 0.05	± 0.002
2	*Model 1* + SFT	B	0.512	−0.074	−0.037	0.002
		± SE	± 0.08*	± 0.02*	± 0.05	± 0.002
Dependent variable: FFM (kg)						
1	Wt + L + Sex + Age	B	0.407 ± 0.06*	0.067	0.034	−0.002
		± SE		± 0.02*	± 0.05	± 0.002
2	*Model 1* + SFT	B	0.528	0.052	−0.001	−0.002
		± SE	± 0.08*	± 0.02*	± 0.06	± 0.002

*Based on 119 infant measurements at ages 5-16 weeks. Abbreviations: B=unstandardized beta; FFM=fat free mass; FM=fat mass; RMSE=root mean squared error; SE=standard error; sex (1 = male, 0 = female); SFT-f=flank (suprailiac) skinfold thickness; SFT-s=subscapular skinfold thickness; SFT-t=triceps skinfold thickness. * $p < 0.05$ for statistically significant B.*

Derivation of anthropometry-based prediction equations

Infant weight and length appeared as significant predictors of infant body composition, while infant sex, gestational age (GA) and postnatal age at visit were not. However, since the equations were derived using the stepwise method with pragmatic approach, infant sex and age at visit were still included in the models. The proportion of variance explained by the derived prediction equations was greater in FFM than FM models. Infant weight, length, sex and visit age explained 63% and 77% variance of the FM and FFM models, respectively (Table 3). The addition of SFTs only added a further 2% of variance proportion explained in both FM and FFM models. Furthermore, among the three SFT sites included, only flank SFT appeared as a significant predictor of infant FFM.

We also developed a number of other prediction equations for FM and FFM in CBGS using subsets of the available infant anthropometry parameters. While their predictive abilities are somewhat weaker than the above equations, these will allow a wider application in infant cohort studies that have collected limited anthropometric measurements (Table S1). The final equations to be validated in the Sophia Pluto (Table 3) were chosen by taking R^2 and root mean squared error (RMSE) into consideration.

	SFT-t (mm)	SFT-s (mm)	SFT-f (mm)	Constant	R^2	RMSE
				2.870 ± 0.79	0.63	0.262
	0.041 ± 0.02	0.008 ± 0.03	0.011 ± 0.02	2.167 ± 0.86	0.65	0.258
				-1.703 ± 0.83	0.77	0.276
	−0.005 ± 0.03	−0.014 ± 0.02	−0.046 ± 0.02*	-0.954 ± 0.90	0.79	0.271

Independent validation in Sophia Pluto

The performance of CBGS equations was assessed against ADP PEA POD measured FM values as the criterion in Sophia Pluto infants and compared to three existing childhood equations. The CBGS equation was the only method that had the least significant difference (Table 4). Predicted values from CBGS equations did not differ with the absolute values from ADP PEA POD for FM at 3 months (p = 0.402) and for FFM at 6 months (p = 0.171). Accordingly, while all five equations predicted FM with strong positive correlations with ADP PEA POD measured FM values at both 3 and 6 months (Pearson coefficients >0.7), mean bias was lowest for CBGS-derived FM values (0.008 kg; LoA [-0.489-0.505] (Figure 1 and Table 5). All of the correlation analyses between the mean (of the values measured by ADP PEA POD as the reference and each alternative equation) and the difference of values between the reference and each equation resulted in significant negative correlations, with CBGS equations had the least negative Pearson correlation coefficients (Table 5). Negative proportional bias was also detected for predicted FM values derived from all four equations, but again the extent of this bias was smallest when using the CBGS equations (Table 5 and Figure S1).

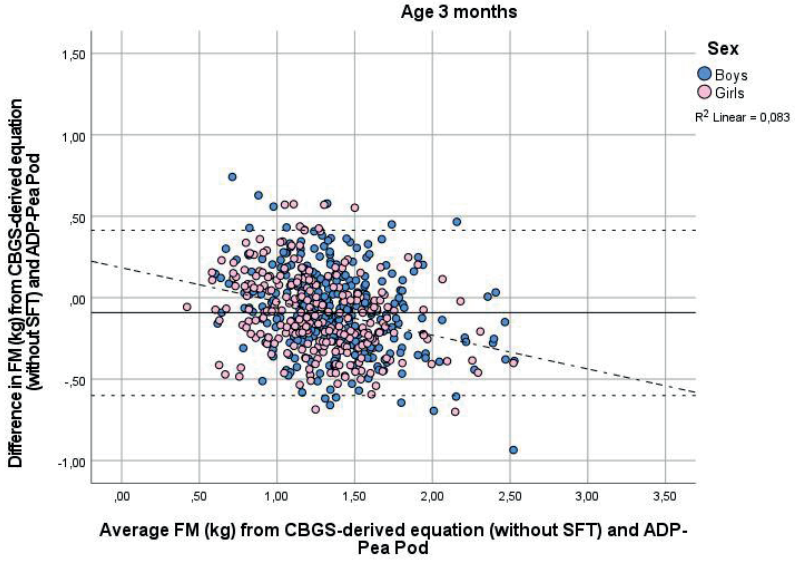
Table 4. FM and FFM values predicted by anthropometry-based equations vs measured by ADP PEA POD among Sophia Pluto infants

		Slaughter *et al*	Aris *et al*	Catalano *et al*	CBGS (no SFT)	CBGS (with SFT)	ADP PEA POD
Age 3 months							
N = 571 (264 girls), mean age = 92.3 days							
FM (kg)	All		1.23 ± 0.24	1.25 ± 0.30	1.29 ± 0.34	1.39 ± 0.35[a]	1.37 ± 0.41
	Boys	1.00 ± 0.27					1.42 ± 0.41
	Girls	0.88 ± 0.23					1.32 ± 0.40
FFM (kg)	All				4.68 ± 0.43	4.74 ± 0.45	4.62 ± 0.50
	Boys						4.84 ± 0.47
	Girls						4.37 ± 0.42
Age 6 months							
N = 447 (211 girls), mean age = 183.4 days							
FM (kg)	All		1.73 ± 0.29	1.69 ± 0.36	1.84 ± 0.43	1.96 ± 0.42	1.86 ± 0.51
	Boys	1.28±0.32					1.85 ± 0.51
	Girls	1.18±0.31					1.86 ± 0.51
FFM (kg)	All				5.62 ± 0.49	5.77 ± 0.52[b]	5.76 ± 0.58
	Boys						6.02 ± 0.53
	Girls						5.46 ± 0.49

Values are mean ± SD. Paired t test (compared to ADP PEA POD), all, $p < 0.05$, except [a] p-value = 0.402 and [b] p-value = 0.171. Abbreviations: FFM= fat free mass; FM= fat mass.

FFM was predicted only using CBGS equations, and these values were strongly correlated with FFM measured by ADP PEA POD at both time points (Pearson coefficients >0.8). Similarly, FFM predicted by CBGS equations showed small mean bias compared to ADP PEA POD measured FFM (0.099 kg; LoA [-0.394 - 0.592]).

A



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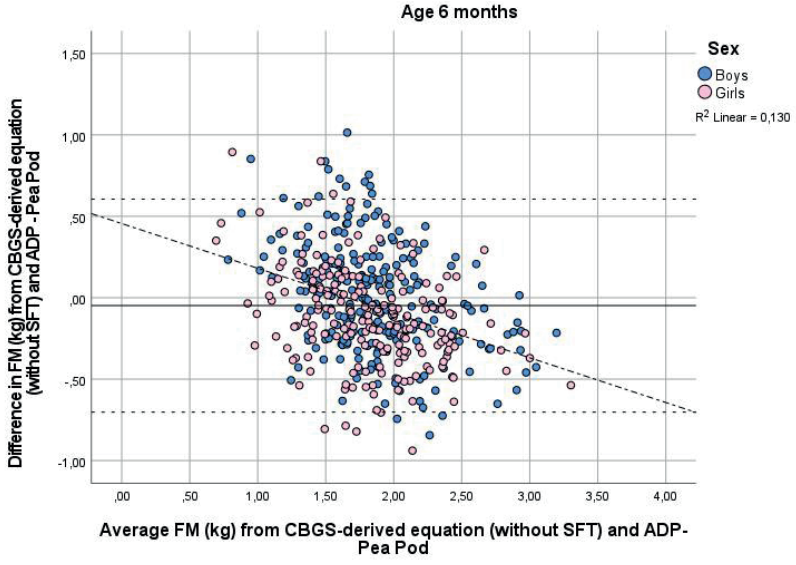

Figure 1. Bland–Altman plots *showing mean bias (solid line) and limits of agreement (LoA, represented by 95% CI; dotted horizontal line) between FM values estimated by the CBGS equation (without skinfolds) vs values measured by ADP PEA POD as the criterion method among Sophia Pluto infants at age 3 (A), and 6 months (B).*

Table 5. Bland and Altman and regression analyses of body composition values estimated by prediction equations against ADP PEA POD measurements

	Correlation		Bland-Altman	
	Pearson R	*p*-value	Mean bias (kg)	LoA (95% CI)
Age 3 months				
FM				
Slaughter *et al* (boys)	0.736	<0.001	-0.422	-0.987 - 0.143
Slaughter *et al* (girls)	0.730	<0.001	-0.440	-1.000 - 0.123
Aris *et al*	0.798	<0.001	-0.151	-0.675 - 0.373
Catalano *et al*	0.800	<0.001	-0.131	-0.628 - 0.366
CBGS no SFT	0.785	<0.001	-0.093	-0.600 - 0.414
CBGS with SFT	0.794	<0.001	0.008	-0.489 - 0.505
FFM				
CBGS no SFT	0.867	<0.001	0.049	-0.453 - 0.551
CBGS with SFT	0.872	<0.001	0.099	-0.394 - 0.592
Age 6 months				
FM				
Slaughter *et al* (boys)	0.722	<0.001	-0.589	-1.317 - 0.139
Slaughter *et al* (girls)	0.733	<0.001	-0.677	-1.382 - 0.029
Aris *et al*	0.755	<0.001	-0.144	-0.860 - 0.572
Catalano *et al*	0.774	<0.001	-0.192	-0.852 - 0.468
CBGS no SFT	0.767	<0.001	-0.048	-0.702 - 0.606
CBGS with SFT	0.789	<0.001	0.084	-0.545 - 0.713
FFM				
CBGS no SFT	0.821	<0.001	-0.171	-0.833 - 0.491
CBGS with SFT	0.832	<0.001	-0.021	-0.663 - 0.621

Abbreviations: B= unstandardized beta; CI= confidence interval; FFM= fat free mass; FM=fat mass; LoA= limit of agreement; SE=standard error of B; SFT: skinfold thicknesses

[a] Correlation between the mean (of the reference/ADP PEA POD and each alternative equation) and the difference between methods.

	Proportional bias		Correlation between mean and difference [a]	
	B ± SE	*p*-value	Pearson R	*p*-value
	-0.470 ± 0.043	<0.001	-0.535	<0.001
	-0.613 ± 0.045	<0.001	-0.645	<0.001
	-0.570 ± 0.026	<0.001	-0.676	<0.001
	-0.348 ± 0.027	<0.001	-0.472	<0.001
	-0.207 ± 0.029	<0.001	-0.288	<0.001
	-0.189 ± 0.028	<0.001	-0.271	<0.001
	-0.178 ± 0.022	<0.001	-0.318	<0.001
	-0.125 ± 0.022	<0.001	-0.234	<0.001
	-0.600 ± 0.049	<0.001	-0.630	<0.001
	-0.592 ± 0.050	<0.001	-0.637	<0.001
	-0.718 ± 0.032	<0.001	-0.736	<0.001
	-0.469 ± 0.032	<0.001	-0.568	<0.001
	-0.275 ± 0.034	<0.001	-0.361	<0.001
	-0.300 ± 0.032	<0.001	-0.410	<0.001
	-0.261 ± 0.029	<0.001	-0.390	<0.001
	-0.188 ± 0.029	<0.001	-0.299	<0.001

Discussion

In this study, we derived new anthropometry-based prediction equations for FM and FFM in UK infants aged 5-16 weeks using ADP PEA POD as the criterion method. In the CBGS, infant weight and length appeared as significant predictors of infant body composition, whereas infant sex, gestational age (GA) and postnatal age at visit were not. Using stepwise method with pragmatic approach to derive the prediction equations, infant sex and age at visit were still included in the models. Many studies have reported that there are sex differences in body composition [33, 34]. These equations were then validated among Dutch infants aged 3 and 6 months in an independent cohort, Sophia Pluto.

In the Sophia Pluto cohort, the CBGS-derived equations produced more accurate predictions of infant FM compared to the other existing FM prediction equations published by Slaughter *et al*, Aris *et al* and Catalano *et al*. Based on paired *t t*est, predicted values from CBGS equations were the most accurate to ADP PEA POD results compared to the other published equations, in both FM and FFM.

Of note, although the participants involved in Slaughter's equations were much older than our infant population (Table 2), comparing our equation to theirs is still considered relevant. This is because Slaughter's equations are frequently used in studies involving pediatric population, including those of younger groups [12, 23], especially when data harmonization is needed across cohorts [35].

All equations produced significant negative proportional biases (Table 5), suggesting negative correlations between the mean and the difference of the predicted vs the actual FM/FFM values. This means that the performance of each equation depends on the magnitude of the actual values of FM/FFM, and all equations tend to over- and underestimate FM/FFM in those with lower and higher measured values (by the ADP PEA POD), respectively. Compared to the other equations, CBGS equations had the smallest proportional biases.

To our knowledge, this is one of the few studies testing the combinations of anthropometric parameters to build body composition prediction equations with the use of ADP PEA POD as the criterion method. We aimed to predict absolute FM and FFM, rather than relative or FM%, since previous studies have reported better correlations between those absolute values with anthropometry [33]. The correlation coefficients of predicted and measured values of FFM were slightly higher than FM values, but the mean differences were similar.

We observed that weight and length were the main contributors in predicting infant FM and FFM. Infant weight has been consistently reported in previous studies to be the most essential predictor of infant FM [23, 27, 33]. Apart from weight, Deierlein *et al* reported that other predictors included infant SFT (triceps, subscapular and quadriceps), sex, age at measurement and ethnicity [27]. Infant weight and sex were also described predictors of infant FM in a Singapore cohort (Aris *et al*), together with GA [23]. However, we did not find infant sex or GA to be significant contributors to our prediction equations. We postulate that this is due to the limited heterogeneity in ethnicity among CBGS infants, and the difference in age range covered by CBGS (5-16 weeks) compared to those other studies (1-3 days post-delivery) [23, 36]. Nonetheless, although they were not statistically significant, we still included infant sex and age at measurement in the prediction equations as biologically plausible contributors.

We found that SFTs contributed only modestly to the prediction of both FM and FFM. Lingwood *et al* also found that SFT did not improve their predictions equations beyond weight, length and sex [33]. Nonetheless, SFT were still included in the equations (Table 3) since they increased the R^2, decreased the RMSE and therefore increased the precision of the equations, although not by much.

Furthermore, of all three SFT sites included in the equations, only flank SFT appeared to be a significant independent predictor of infant FFM (Table 3). Since flank skinfold reflects central adiposity, this result could be speculatively interpreted as central fatness contributing more to the FFM estimation. However, if all SFT sites in CBGS cohort were considered, additional analyses showed that both flank and quadriceps SFTs were the most significant contributors to the prediction of FFM (Supplementary materials). Interestingly, flank SFT was also determined as the most significant FM predictor in Catalano's equation [30]. Since Sophia Pluto did not measure quadriceps SFT, this parameter could not be included in the equations taken forward for validation.

The proportional biases in the CBGS equations were smaller than those of the other equations, but they were all significant when compared to the criteria method (ADP PEA POD, Table 5 and Figure S1). Therefore, accurate body composition measurement during infancy should be pursued by ADP PEA POD or DXA, whilst equations can be employed as proxies to estimate fat/fat free mass where body composition instrument is not available.

While the derivation sample included a wide distribution of FM% (6.5-38.6%) and a relatively wide age range (38-112 days), we acknowledge some limitations. Firstly, the skinfold thickness measurements in the derivation and validation cohorts were conducted using different tools. However, despite the use of different calipers, the CBGS equations still produced smaller proportional biases compared to the other established equations. Second, since all CBGS infants were vaginally delivered with normal birth weight and born of healthy mothers with normal pre-pregnancy BMI, the equations might not be applicable in population with a high rate of Caesarean section and high variance of maternal pre-pregnancy BMI or infant's birth weight. Third, both prediction and validation cohorts included only healthy and term infants, thus our findings may not relevant for preterm infants. However, our validation cohort also included severe small-for-gestational-age (SGA) infants with birthweight/length less than −2.5 z-score. Regarding ethnicity, although our derivation cohort was predominantly white Europeans, Sophia Pluto as the independent validation cohort included more diverse ethnicities with at least 37% of them were non-Caucasian. Although Aris *et al* did not find ethnicity to be significant in their FM equation derived in Asian infants[23], a recent systematic review reported differences in infant body composition between ethnicities[37]. Therefore, the applicability of our equations to other ethnic populations remains in question.

Conclusions

We derived and validated new anthropometry-based equations for infant FM and FFM using simple parameters often measured in infant studies. These new equations appeared to be more robust in predicting infant FM and FFM when compared to other published childhood equations despite the presence of proportional bias. These equations are fit for use in longitudinal infant cohorts or trials, when reference methods, such as ADP PEA POD, are not feasible.

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Conflict of interest: The authors declare no conflict of interest.

Authors contributions: LO, IvB, EDLR and AHK had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. DD, KO and IH were involved in the conduction of the CBGS and AHK for Sophia Pluto. LO and IvB were responsible for infant recruitment and clinic visit in the CBGS and Sophia Pluto, respectively. LO and IvB performed statistical analyses. LO and EDLR drafted the manuscript. IvB, AHK, KK, DD and IH helped to improve the manuscript. All authors contributed to interpretation of data, critically revised the article for important intellectual content and approved the final version.

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4

Supplement

Supplementary Table 1. Results of other anthropometric parameter combinations to predict FFM

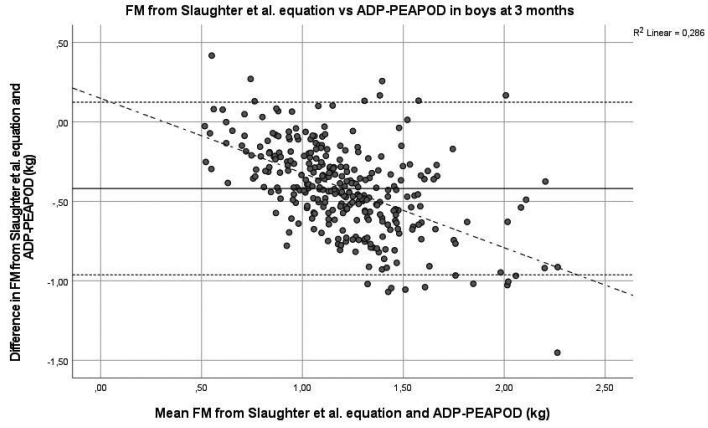
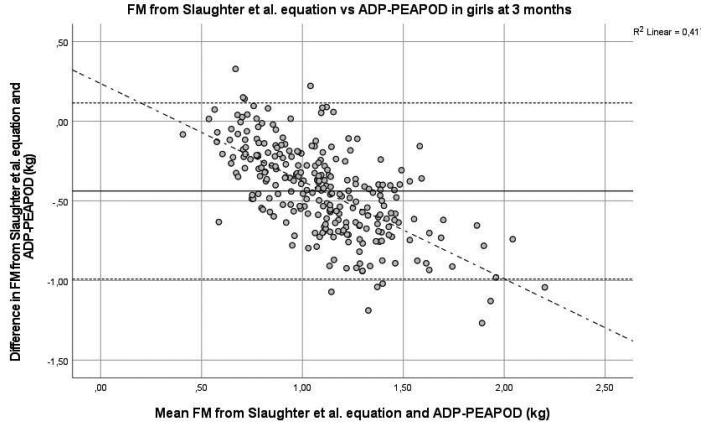
No			W	H	S	A
1	**W+H**	B±SE	0.406* ±0.06	0.059* ±0.02		
2	**W+H+S+A**	B±SE	0.407* ±0.06	0.067* ±0.02	0.034 ±0.05	-0.002 ±0.002
3	**W+H+S+A+WC**	B±SE	0.39* ±0.09	0.068* ±0.02	0.034 ±0.05	-0.002 ±0.002
4	**W+H+S+A+SSF**	B±SE	0.595* ±0.08	0.042* ±0.02	-0.011 ±0.05	-0.002 ±0.002
5	**W+H+S+A+WC+SSF**	B±SE	0.558* ±0.1	0.044* ±0.02	-0.013 ±0.05	-0.002 ±0.002
6	**W+H+S+A+ CSF**	B±SE	0.497* ±0.08	0.054* ±0.02	0.015 ±0.06	-0.002 ±0.002
7	**W+H+S+A+ PSF**	B±SE	0.584* ±0.08	0.045* ±0.02	-0.011 ±0.05	-0.001 ±0.002
8	**W+H+S+A+ all individual SF**	B±SE	0.587* ±0.09	0.045* ±0.02	-0.009 ±0.06	-0.001 ±0.002
9	**W+H+S+A+ WC + individual SF**	B±SE	0.555* ±1.0	0.047* ±0.02	-0.011 ±0.06	-0.001 ±0.002
10	**W+H+S+A+ SFs**	B±SE	0.475* ±0.08	0.057* ±0.02	0.015* ±0.06	-0.002 ±0.002
11	**W+H+S+A+ SFf**	B±SE	0.471* ±0.07	0.058* ±0.02	0.023 ±0.05	-0.002 ±0.002
12	**W+H+S+A+ SFt**	B±SE	0.494* ±0.07	0.057* ±0.02	0.004 ±0.05	-0.002 ±0.002
13	**W+H+S+A+ SFq**	B±SE	0.57* ±0.08	0.047* ±0.02	-0.001 ±0.05	-0.001 ±0.002
14	**W+H+S+A+ SFs+SFf+SFt**	B±SE	0.528* ±0.08	0.052* ±0.02	-0.001 ±0.06	-0.002 ±0.002
15	**W+H+S+A+WC+ SFs+SFf+SFt**	B±SE	0.507* ±0.1	0.053* ±0.02	-0.002 ±0.06	-0.002 ±0.002

W = weight (kg), H = height (cm), S = sex (1=male, 0=female), WC = waist circumference (cm), A = age at visit (days), SFs = skinfold subscapular (mm), SFf = skinfold flank (suprailiac), SFt = skinfold triceps, SFq = skinfold quadriceps, SSF = sum skinfolds (mm): tricep+subscapular+quadriceps+flank (suprailiac), CSF= sum of central skinfolds (mm): subscapular+flank,

WC	SFs	SFf	SFt	SFq	Constant	R^2	SEE
					-1.352 ±0.41	0.77	0.276
					-1.703 ±0.83	0.774	0.276
0.005±0.02					-1.867 ±1.01	0.774	0.277
				-0.018*±0.006	-0.586 ±0.874	0.793	0.265
0.012±0.02				-0.019*±0.006	-0.969 ±1.01	0.794	0.266
		-0.019±0.01			-1.123 ±0.89	0.78	0.273
				-0.033*±0.009	-0.731 ±0.84	0.797	0.263
	-0.026 ±0.02	0.006 ±0.03	-0.006 ±0.02	-0.035* ±0.02	-0.74 ±0.89	0.798	0.266
0.011±0.02	-0.024 ±0.02	0.003 ±0.03	-0.007 ±0.02	-0.036 ±0.02	-1.073 ±1.02	0.799	0.267
	-0.031 ±0.02				-1.222 ±0.91	0.778	0.275
		-0.025 ±0.02			-1.323 ±0.86	0.779	0.274
			-0.053* ±0.02		-1.166± 0.84	0.786	0.27
				-0.043* ±0.01	-0.858 ±0.83	0.795	0.264
	-0.005 ±0.03	-0.014 ±0.02	-0.046* ±0.02		-0.954 ±0.9	0.788	0.271
0.007 ±0.02	-0.006 ±0.03	-0.014 ±0.02	-0.045 ±0.02		-1.168 ±1.04	0.788	0.272

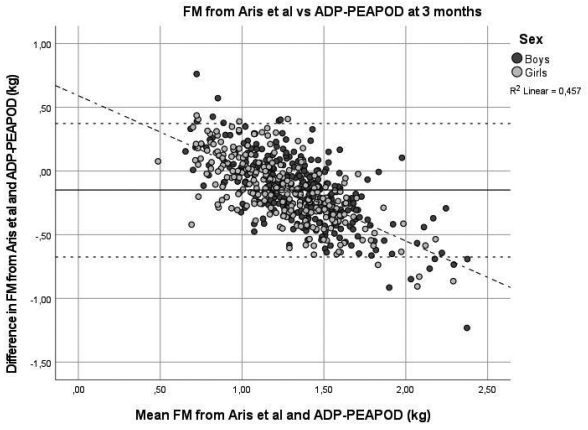
*PSF= sum of peripheral skinfolds (mm): triceps+quadriceps B = unstandardized beta±standard error, β = standardized beta, SEE = standard error of the estimate, calculated as the root mean squared error *p<0.05 for statistically significant BHighlighted row: the FFM equation validated in the independent cohort (Sophia Pluto)*

Supplementary Figure 1. Bland-Altman plots demonstrating the agreement between predicted and ADP PEA POD measured FM (kg) or FFM (kg) among Sophia-Pluto infants

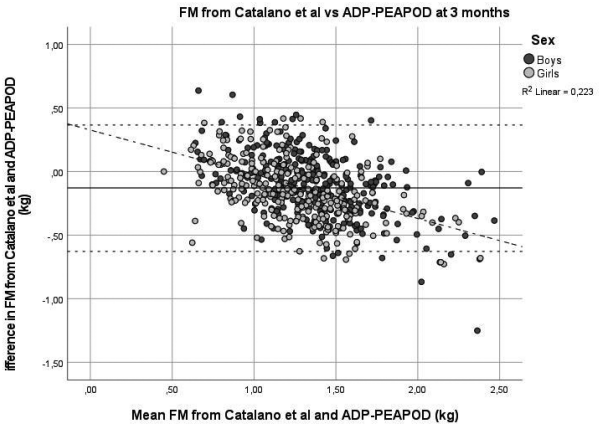
Age 3 months	
Equation	**Bland-Altman plot**
FM estimation	
Slaughter et al (boys)	
Slaughter et al (girls)	

Supplementary Figure 1. Continued

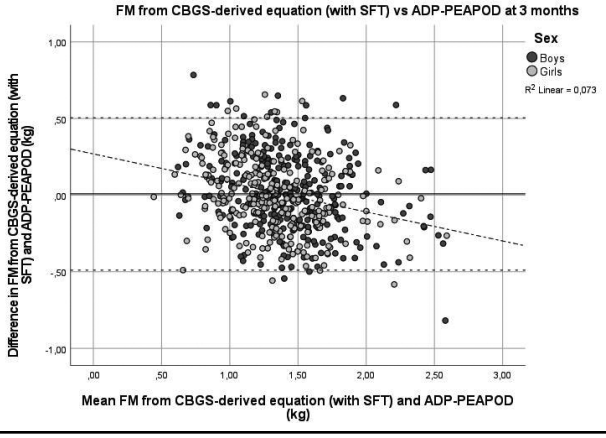
Aris et al



Catalano et al



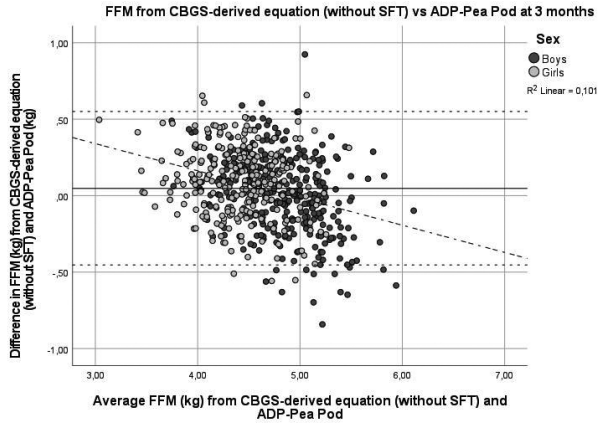
CBGS-with SFT



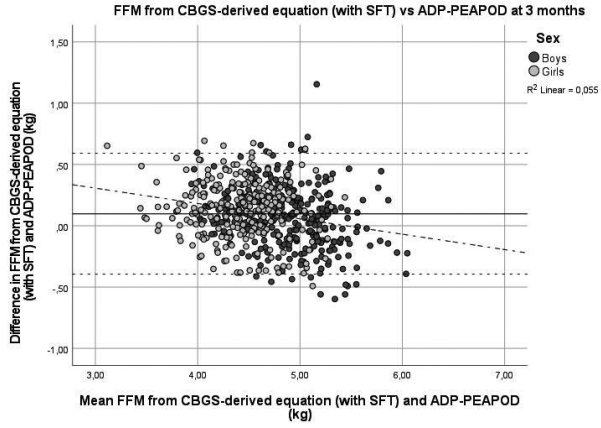
Supplementary Figure 1. Continued

FFM estimation

CBGS- without SFT

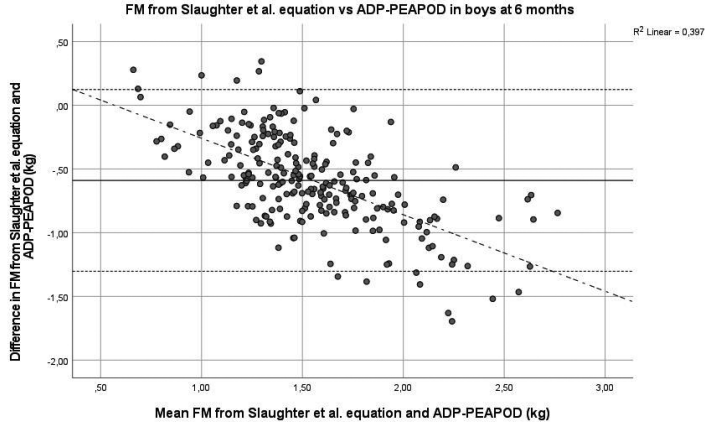


CBGS-with SFT

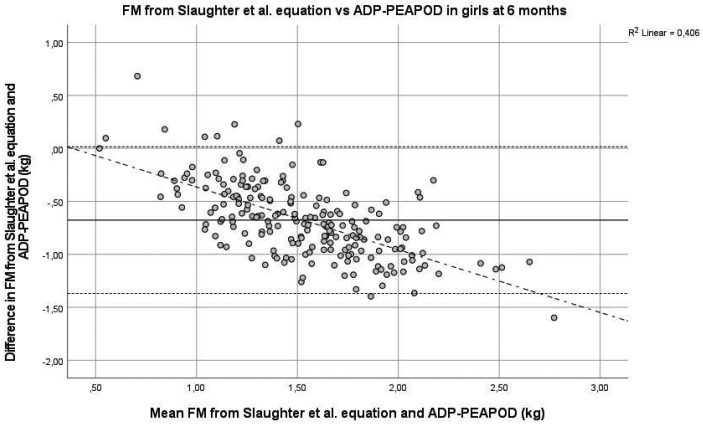
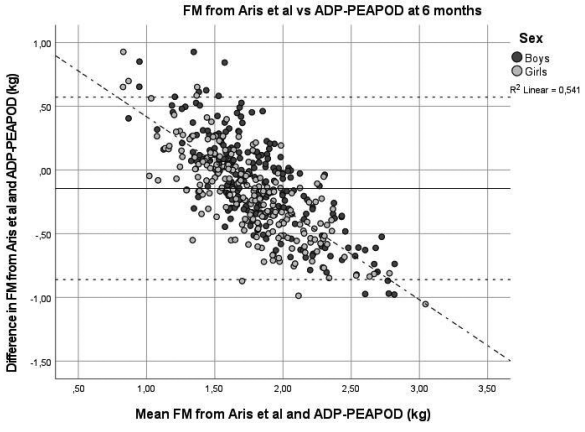


FM estimation

Slaughter et al (boys)

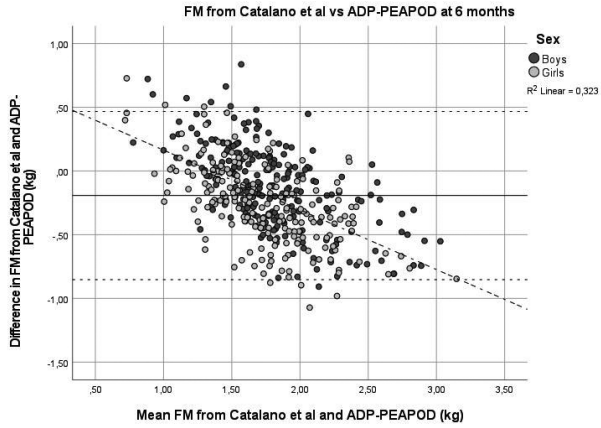


Supplementary Figure 1. Continued

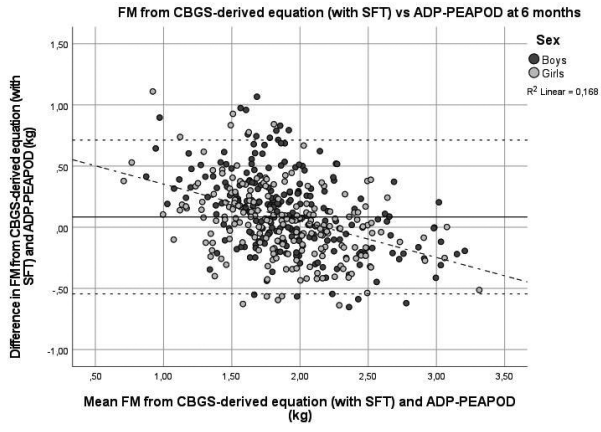
Age 6 months	
Equation	**Bland-Altman plot**
Slaughter et al (girls)	
Aris et al	

Supplementary Figure 1. Continued

Catalano et al

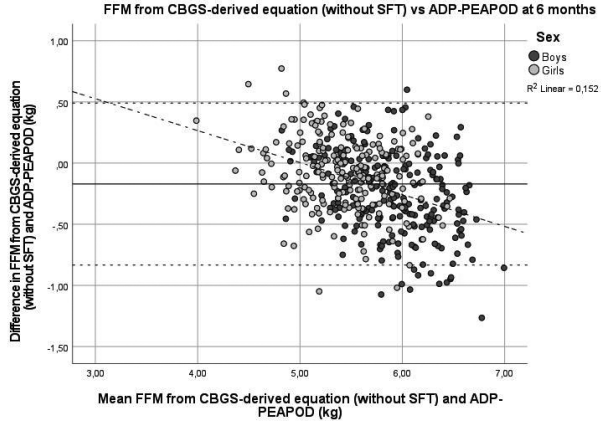


CBGS-with SFT



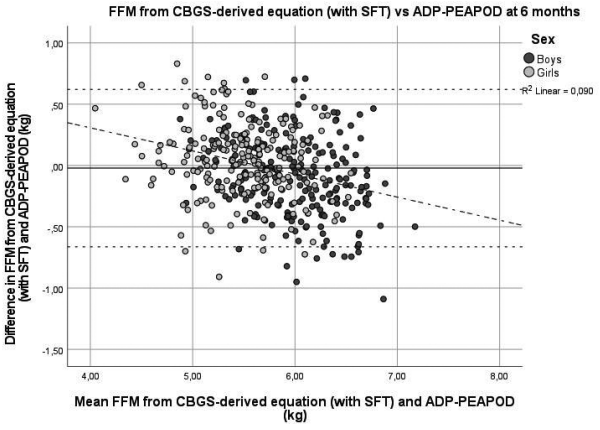
FFM estimation

CBGS-without SFT



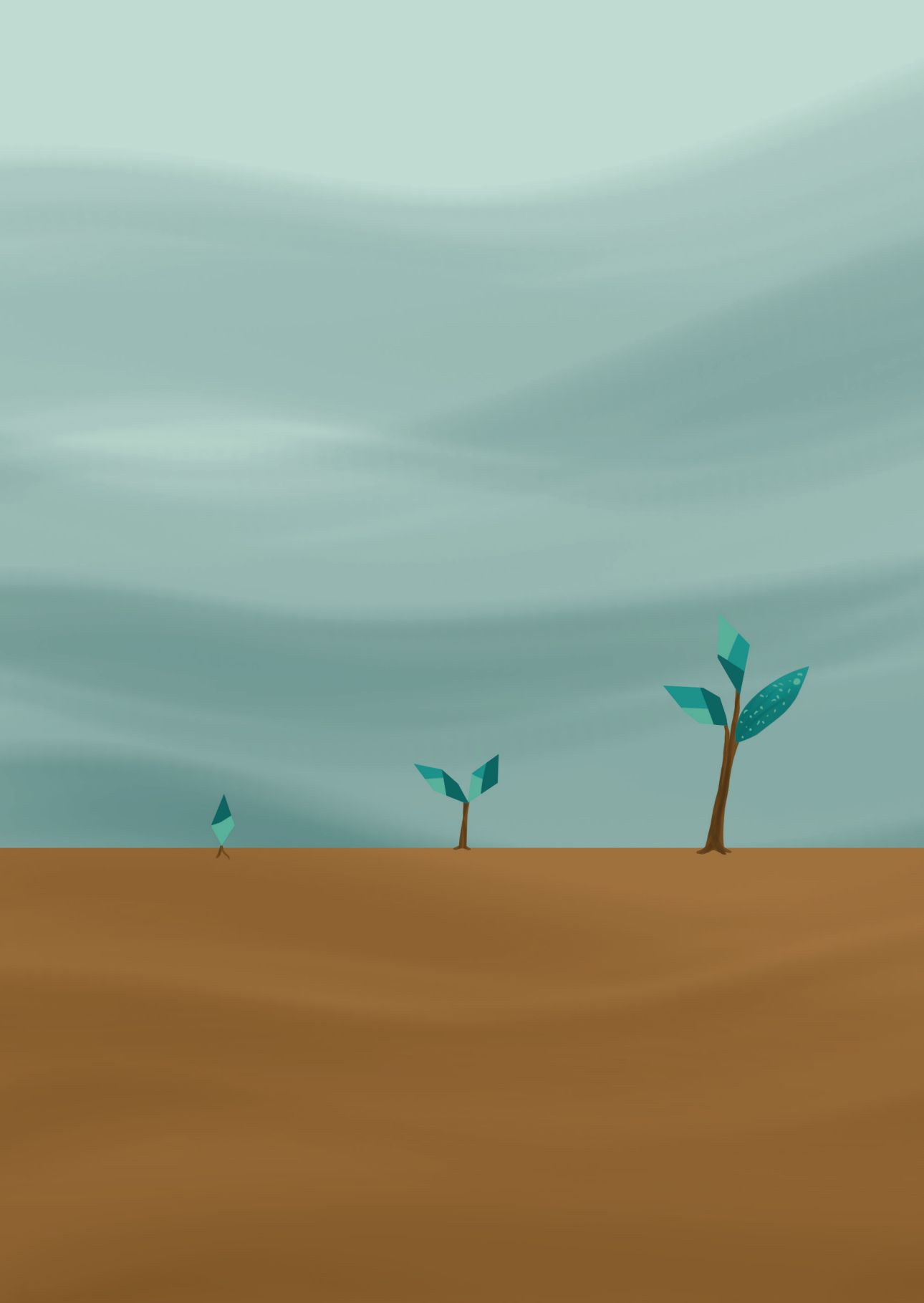
Supplementary Figure 1. Continued

CBGS-with SFT



Part II

Determinants of adiposity programming in early life



Chapter 5

Fat mass and fat free mass track from infancy to childhood: new insights in body composition programming in early life

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Abstract

Background: Early life is a critical window for adiposity programming. This study investigated whether fat mass percentage (FM%), fat mass index (FMI), abdominal fat, and fat free mass (FFM) in early life track into childhood and whether there are sex differences and differences between infant feeding types.

Methods: Detailed body composition was longitudinally measured by Air Displacement Plethysmography, Dual energy X-ray Absorptiometry, and abdominal ultrasound in 224 healthy, term-born children. Measurements were divided into tertiles. Odds ratios (OR) of remaining in the highest tertile of FM%, FMI, abdominal subcutaneous and visceral fat, and FFM-index (FFMI) were calculated from early life to age 4 years.

Results: High FM% and FMI tracked from age 3 and 6 months to age 4 years (OR = 4.34 [$p = 0.002$] and OR = 6.54 [$p < 0.001$]). High subcutaneous abdominal fat tracked from age 6 months to age 4 years (OR = 2.30 [$p = 0.012$]). High FFMI tracked from age 1, 3, and 6 months to age 4 years (OR = 4.16 [$p = 0.005$], 3.71 [$p = 0.004$], and 3.36 [$p = 0.019$]). In non-exclusively breastfed infants, high FM% tracked from early life to age 4 years, whereas this was not the case for exclusively breastfed infants. There was no tracking in visceral fat or sex differences.

Results: Infants with high FM%, FMI, subcutaneous abdominal fat, and FFMI in early life are likely to remain in the highest tertile at age 4 years. Exclusive breastfeeding for 3 months is potentially protective against having high FM% at age 4 years.

Introduction

Childhood obesity is a global public health threat, with an alarming rising incidence. Globally, 38 million children under the age of 5 years were overweight or obese in 2019 [1]. Obesity at a young age has direct adverse consequences as well as long-term morbidity, such as cardiovascular disease, insulin resistance and type 2 diabetes mellitus. Without intervention, children with overweight or obesity have a high risk of having overweight or obesity as adults [1]. It is, therefore, important to unravel which period during childhood has a great influence on adiposity programming.

It has been reported that weight-to-height and BMI standard deviation score (SDS) track from childhood into adulthood [2]. A high BMI at age 5 years and fast increase in BMI SDS in early childhood are predictive for overweight and obesity in adolescence [3]. Our research group found that rapid weight gain in the first months of life is an important risk factor for having overweight or obesity and having an unfavorable metabolic profile in early adulthood [4]. This indicates that especially the first months of life are a critical window for adiposity programming and development of non-communicable diseases later in life [4, 5]. However, it has been demonstrated that infants and young children with a comparable weight or BMI have a highly variable body composition and fat distribution [6-9]. Excessive fat mass and visceral fat accumulation are important contributors to the development of an unfavorable metabolic outcome [10, 11]. We found that infants with a rapid rise in fat mass in the first 6 months of life have higher fat mass trajectories until 2 years of age [12]. Despite the development of accurate methods for body composition measurement in infants and young children, such as Air Displacement Plethysmography (ADP) [13, 14], data about tracking of high fat mass (FM) and fat free mass (FFM) from infancy to childhood are not yet available.

Therefore, we evaluated the tracking of high fat mass percentage (FM%) and abdominal fat distribution, measured as high abdominal subcutaneous and visceral fat, in our prospective cohort from age 1, 3 and 6 months until the age of 4 years. We also determined whether high fat free mass index (FFMI) would track from infancy to childhood. We hypothesized that infants with high FM%, abdominal subcutaneous or visceral fat in early infancy would continue to have high body fat measures at 4 years of age. In addition, we investigated whether this tracking would be different between boys and girls or between types of infant feeding.

Methods

Subjects

This current study was embedded in the Sophia Pluto study, a birth cohort study of healthy infants, aiming to provide detailed data on body composition from early life to childhood [6, 12]. Infants were recruited from several maternity wards in Rotterdam, the second largest city in The Netherlands, between 2013 and 2017. All participants met the following inclusion criteria: born term (≥37 weeks' gestation) and singleton, with an uncomplicated neonatal period and without severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation and at least 4 years of age with complete follow-up.

Exclusion criteria were maternal disease or medication that could interfere with fetal growth, including maternal corticosteroids and diabetes mellitus, known congenital or postnatal disease or intrauterine infection that could interfere with growth. The Medical Ethics Committee of Erasmus Medical Center approved the study and written informed consent was given by all parents or caregivers with parental authority.

Data collection and measurements

Outpatient clinic visits were scheduled at 1, 3 and 6 months and at 4 years. Birth data were taken from hospital and midwife records. Maternal and paternal characteristics and feeding type and habits were obtained by interviews at the clinic visits and completed questionnaires from both parents. Infant feeding mode was categorized as exclusive breastfeeding (EBF) if infants had EBF for at least the first 3 months of life. Exclusive formula feeding (EFF) was defined as starting EFF before 1 month of age. Mixed feeding was defined as starting a combination of breastfeeding and formula feeding between 1 and 3 months of age.

Anthropometrics

Weight was measured to the nearest 5 grams by an electronic infant scale (Seca 717) at 1, 3 and 6 months and by a flat scale (Seca 876) at 4 years. Length was measured twice in supine position to the nearest 0.1 cm by an Infantometer (Seca 416) at 1, 3 and 6 months and in upright position by a stadiometer (Seca 213) at 4 years. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Body composition

Body composition was measured with ADP by PEA POD (Infant Body Composition System (COSMED)) during the visits at 1, 3 and 6 months of age, as described in detail elsewhere [6]. ADP was conducted using the same machine. It was used and calibrated daily according to the user's manual [15]. ADP was validated earlier against a reference four-compartment model, and reliability was determined with a percent coefficient of variance of 7.9% for FM% [14].

When the infant exceeded the weight limit of 8 kg at 6 months, body composition was measured by Dual energy X-ray Absorptiometry (DXA) scan (GE Prodigy Advance R000279 and encore software version 14.1). A vacuum cushion was used to avoid movement artifacts, which we have reported to have similar results at age 6 months compared with ADP [16]. At age 4 years, body composition was determined using the same DXA machine, without vacuum cushion. Children wore only underwear and they were swaddled in a cotton blanket. During the study, the same DXA machine was used and calibrated daily, according to the protocol recommended by the supplier [17]. Percent coefficient of variance for FM% was determined earlier to be between 0.39% and 4.49% [18].

FM% was calculated as fat mass in kilograms divided by total body weight in kilograms times 100%. Fat mass index (FMI) was calculated as fat mass in kilograms divided by length in meters squared (kg/m^2). FFMI was calculated as FFM in kilograms divided by length in meters squared (kg/m^2).

Abdominal fat

Abdominal subcutaneous and visceral fat were determined as single plane depth in centimeters (cm), at every visit starting from 3 months, using ultrasound (Prosound 2 ultrasound with a UST-9137 convex transducer; Hitachi Aloka Medical, Ltd.) [19]. Both were measured in supine position, placing the transducer on the intercept of the xiphoid line and the waist circumference measurement plane. Visceral fat was measured in the longitudinal plane from the peritoneal boundary to the corpus of the lumbar vertebra with a probe depth of 9 cm and subcutaneous fat in the transvers plane from the cutaneous boundary to the linea alba with a probe fat of 4 cm. Minimal pressure was applied. Validity and reproducibility of measurements were confirmed previously [19]. The relative interobserver technical error of measurement was 3.2% for visceral fat and 3.6% for subcutaneous fat.

Statistical analysis

SDS for birth length and birth weight was calculated and corrected for gestational age and sex. SDS for length and weight was calculated at every visit, based on Dutch references, using Growth Analyser software (http://www.growthanalyser.org) [20]. Using World Health Organization (WHO) classification, overweight at age 4 years was defined as a weight-for-length >2 SDS and obesity was defined as a weight-for-length >3 SDS [1]. Underweight was defined as an weight-for-age <-2 SDS [21].

Baseline characteristics and body composition measurements are expressed as mean (SD). Not-normally distributed values are expressed as median (interquartile range). Independent Student *t* test was used to determine differences in the baseline characteristics and body composition measurements between boys and girls. Because of the lack of longitudinal reference values for FM%, FMI, FFMI, and abdominal subcutaneous and visceral fat measured by ultrasound from infancy until 4 years of age, we used categorical outcomes. Boys and girls were divided at each time point into sex-specific tertiles for FM%, FFMI, abdominal subcutaneous and visceral fat, and BMI SDS, and these were subsequently merged into group tertiles for "high," "moderate," and "low". Logistic regression models were used to calculate the odds ratio (OR) for having high FM%, FMI, FFMI, abdominal subcutaneous and visceral fat, or BMI SDS at 4 years of age, based on being in the high group at 1, 3, and 6 months. OR was calculated using the low group at 4 years of age as the reference category. An OR above 1 for remaining in the high group over time was considered as significant tracking. All logistic regression models were corrected for infant feeding mode until age 3 months. Additional adjustment for sex did not change the results and thus was not included in the final models. If infant feeding type was a significant factor in one of the models, logistic regression models were conducted for EBF and non-EBF infants separately. In order to determine differences between sex, logistic regression models were performed for boys and girls separately.

Statistical tests were performed with SPSS Statistics version 25.0 (IBM Corp.). Results were regarded as statistically significant if $p < 0.05$.

Results

Clinical characteristics are presented in Table 1. The total group consisted of 224 infants, and 53.6% was male. Weight and length were different between boys and girls at 1, 3, and 6 months of age. Based on the WHO criteria, most children at 4 years of age had normal weight, and only eight (3.6%) children had overweight or obesity. FM%, FFMI, abdominal subcutaneous and visceral fat, and BMI SDS were divided into "high", "moderate," and "low" groups. Distribution of these groups is presented in Table 2.

Table 1. Child characteristics

	Boys n = 120 (53.6 %)	**Girls n= 104 (46.4 %)**	***p* value**
Birth			
Gestational age (weeks)	39.58 (1.26)	39.86 (1.08)	0.080
Weight SDS	0.29 (1.01)	0.14 (1.05)	0.270
Length SDS*	0.70 (1.14)	0.63 (1.15)	0.724
Ethnicity (%)			0.064
Caucasian	69.2%	65.4%	
Black	1.7%	10.6%	
Asian	0.8%	1.0%	
Latin	0.8%	0	
Other	22.5%	19.2%	
Missing	5.0%	3.8%	
Delivery mode (%)			0.080
Vaginal	60.8%	74.0%	
Caesarian section	39.2%	26.0%	
Age 1 month			
Weight (kg)	4.36 (0.55)	4.09 (0.51)	**<0.001**
Weight-for-length SDS	-0.05 (0.86)	-0.03 (0.88)	0.896
Length (cm)	54.93 (2.12)	53.85 (2.21)	**<0.001**
Length SDS	0.11 (0.91)	-0.02 (0.90)	0.284
Age 3 months			
Feeding mode until 3 months			0.783
EBF (%)	40.8%	43.3%	
EFF (%)	29.2%	25.0%	
Mix (%)	30.0%	31.7%	

Table 1. Continued

	Boys n = 120 (53.6 %)	Girls n= 104 (46.4 %)	*p* value
Weight (kg)	6.19 (0.67)	5.73 (0.58)	**<0.001**
Weight-for-length SDS	0.26 (0.90)	0.20 (0.93)	0.630
Length (cm)	61.95 (2.10)	60.41 (1.97)	**<0.001**
Length SDS	0.42 (0.85)	0.26 (0.80)	0.146
Age 6 months			
Weight (kg)	7.92 (0.82)	7.34 (0.75)	**<0.001**
Weight-for-length SDS	0.10 (0.94)	0.07 (0.96)	0.813
Length (cm)	68.67 (2.17)	66.70 (2.09)	**<0.001**
Length SDS	0.24 (0.87)	0.10 (0.82)	0.244
Age 4 years			
Weight (kg)	17.52 (2.16)	17.18 (2.12)	0.221
Weight-for-length SDS	0.18 (1.18)	0.22 (0.92)	0.745
Underweight	8 (6.7%)	2 (1.9%)	0.188
Normal weight	106 (88.3%)	100 (96.2%)	
Overweight	4 (3.3%)	1 (1.0%)	
Obese	2 (1.7%)	1 (1.0%)	
Length (cm)	104.98 (3.91)	104.31 (4.28)	0.233
Length SDS	-0.22 (0.91)	-0.24 (0.97)	0.889

*Data expressed as mean (SD). Significant data are bold. *Birth length was available in 71 boys and 60 girls. Abbreviations: EBF= exclusive breastfeeding, EFF= exclusive formula feeding, mix= mixed feeding, SDS = standard deviation score*

Tracking of FM% and FMI

High FM% tracked from age 3 and 6 months to 4 years, with OR = 4.34 (p = 0.002) and OR = 6.54 (p < 0.001), respectively (Table 3). High FMI also tracked from age 3 and 6 months to 4 years, with OR = 2.62 (p = 0.027) and OR = 5.68 (p = 0.001), respectively. There was no tracking from age 1 month to 4 years, and there was no difference in tracking of high FM% or FMI between boys and girls (data not shown).

Tracking of FFMI

High FFMI tracked from age 1, 3, and 6 months to 4 years, with OR = 4.16 (p = 0.005), OR = 3.71 (p = 0.004), and OR = 3.36 (p = 0.019), respectively (Table 3). We found no difference in tracking of high FFMI between sexes.

Table 2. "High" and "low" groups of FM%, FMI, FFMI, abdominal subcutaneous and visceral fat and BMI SDS

		FM%	FMI (kg/m²)	FFMI (kg/m²)	Abdominal subcutaneous fat (cm)	Visceral fat (cm)	BMI SDS (kg/m²)
Age 1 month							
High	♂	>18.03	>2.57	>12.41	*NA*	*NA*	>0.77
	♀	>18.80	>2.60	>11.91	*NA*	*NA*	>0.74
Low	♂	<14.27	<2.09	<11.65	*NA*	*NA*	<-0.19
	♀	<14.50	<2.01	<11.35	*NA*	*NA*	<-0.02
Age 3 months							
High	♂	>24.23	>3.91	>12.77	>0.45	>2.59	>0.68
	♀	>25.73	>4.18	>12.31	>0.43	>2.55	>0.60
Low	♂	<21.20	<3.29	<12.11	<0.36	<2.07	<-0.10
	♀	<20.43	<3.10	< 11.65	<0.34	<1.99	<-0.17
Age 6 months							
High	♂	>26.00	>4.33	>13.13	>0.47	>2.44	>0.55
	♀	>28.03	>4.69	>12.47	>0.47	>2.39	>0.52
Low	♂	<21.40	<3.42	<12.36	<0.37	<2.00	<-0.18
	♀	<23.50	<3.74	<12.00	<0.37	<1.84	<-0.17
Age 4 years							
High	♂	>28.63	>4.64	>11.95	>0.41	>2.31	>0.62
	♀	>31.80	>5.18	>11.41	>0.42	>2.48	>0.74
Low	♂	<25.23	<3.90	<11.36	<0.32	<1.83	<-0.28
	♀	<27.63	<4.25	<10.64	<0.31	<1.89	<-0.12

Sex-specific "high" and "low" groups for FM%, FMI, FFMI, abdominal subcutaneous fat, visceral fat and BMI SDS. Abbreviations: BMI SDS = Body Mass Index Standard Deviation Score, FM%= Fat Mass percentage, FMI =Fat Mass Index, FFMI = Fat Free Mass Index, ♂ = boys, ♀= girls, NA= not applicable

Tracking of abdominal fat

Median visceral and subcutaneous abdominal fat decreased with age. High abdominal subcutaneous fat tracked from age 6 months to 4 years, with OR = 2.30 (p = 0.035) (Table 3). No tracking of visceral fat was found. There was no sex difference in tracking of high abdominal fat.

Tracking of BMI

For the sake of comparison with literature data, we also evaluated the tracking of BMI SDS (Table 3). High BMI SDS tracked from 1, 3, and 6 months to 4 years, with OR = 3.15 (p = 0.012), OR = 6.50 (p < 0.001), and OR = 7.74 (p < 0.001), respectively. No sex differences in tracking of high BMI SDS were found.

Table 3. Odds ratio for body composition tracking from age 1, 3 and 6 months to 4 years

	At age 4 years					
	High FM%	High FMI	High FFMI	High subcutaneous fat	High visceral fat	High BMI SDS
Age 1 month						
High	1.70 (0.72-3.98) p=0.225	1.48 (0.63-3.48) p=0.369	**4.16 (1.52-11.36) p=0.005**	*NA*	*NA*	**3.15 (1.29-7.66) p=0.012**
Age 3 months						
High	**4.34 (1.72-10.92) p=0.002**	**2.62 (1.12-6.16) p=0.027**	**3.701 (1.50-9.15) p=0.004**	1.76 (0.79-3.95) p=0.168	1.03 (0.50-2.14) p=0.932	**6.50 (2.45-17.28) p<0.001**
Age 6 months						
High	**6.54 (2.37-18.07) p<0.001**	**5.68 (1.96-16.45) p=0.001**	**3.36 (1.22-9.23) p = 0.019**	**2.30 (1.06-4.99) p=0.035**	1.63 (0.74-3.62) p=0.228	**7.74 (2.67-22.45) p<0.001**

Values are odds ratios (95% confidence interval) and p-value for having high body composition outcome at 4 years of age, adjusted for feeding mode until age 3 months, estimated by logistic regression with the "low"-group as reference category. Significant data are boldfaced. Abbreviations: FM%= Fat Mass percentage, FMI = Fat Mass Index, FFMI = Fat Free Mass Index, BMI SDS = Body Mass Index Standard Deviation Score, NA=not applicable

Influence of infant feeding type

Tracking of high FM% was influenced by infant feeding type. We analyzed tracking of FM% separately for EBF infants and non-EBF infants (children with EFF and mixed feeding; Table 4). In the EBF infants, there was no significant tracking of high FM% from early life to 4 years of age. However, in non-EBF infants, we found tracking from age 3 and 6 months to 4 years, with OR = 4.00 (p = 0.006) and OR = 7.33 (p = 0.001), respectively

There was no influence of infant feeding type in tracking of high FFMI, abdominal subcutaneous and visceral fat, and BMI SDS to 4 years of age.

Table 4. Odds ratio for fat mass percentage tracking from age 1, 3 and 6 months to 4 years in EBF and non-EBF-infants

	At age 4 years	
	EBF	Non-EBF
	FM% High	FM% High
Age 1 month		
FM% High	1.00 (0.42 – 2.40) p=1.00	1.33 (0.56 – 3.16) p=0.514
Age 3 months		
FM% High	1.43 (0.54-3.75) p=0.469	**4.00 (1.50-10.66) p=0.006**
Age 6 months		
FM% High	2.20 (0.76-6.33) p=0.144	**7.33 (2.20-24.50) p=0.001**

Values are odds ratios (95% confidence interval) and p-value for FM% at 4 years of age in EBF and in non-EBF infants estimated by logistic regression with the "low"-group as reference category. Significant data are boldfaced. Abbreviations: FM%= Fat Mass percentage, EBF= exclusive breastfeeding until 3 months of age, non-EBF = exclusive formula feeding or mixed feeding until 3 months of age

Discussion

In the present study, we show that infants in the highest tertile of FM%, FMI, abdominal subcutaneous fat, and FFMI in early life, had high odds to remain in the highest tertile at 4 years of age. In contrast to EBF infants, non-EBF infants tracked in the highest FM% tertile from early life to 4 years of age, indicating a difference in tracking between infant feeding types.

High FM% and FMI tracked from age 3 and 6 months to 4 years, indicating that infants with a high FM% and FMI in the first 6 months of life are more likely to still have high FM% and FMI at 4 years of age. Previously, others found a moderate association between FM% and FMI at 3-4 months and 4 years of age [22, 23]. Our research group showed that accelerated gain in weight-for-length during the first 6 months of life was associated with increased FM% at 21 years [4]. We found previously, in the Sophia Pluto study, that a rapid rise in FM% in the first 6 months of life and not thereafter was associated with higher FM% trajectories until the age of 2 years [12]. Altogether, these findings support the presence of a critical window for adiposity programming in the first 6 months of life.

High FFMI tracked from age 1, 3 and 6 months to 4 years. Our results are in line with those of an Ethiopian birth cohort study showing a positive association between FFM at birth with FFMI at 4 years of age [24] and FFM at the age of 5 years measured by ADP [25]. However, tracking of high FFM from infancy to childhood has never been described. As high FFMI tracked from early life to childhood, this could indicate that the first months of life are not only a critical window for adiposity programming, but also for FFM programming. In contrast to FM%, we found tracking of high FFMI from age 1 month to 4 years. This might suggest that FFMI tracks from an earlier age than FM%. A systematic review reported consistent associations between birth weight and lean body mass in term-born children, adolescents and adults, while this consistent association was not found for fat mass [26]. A study in preterm infants reported a similar FM% at 52 weeks postmenstrual age as in term-born infants, while the lower FFM persisted [27]. This could indicate that the last trimester of pregnancy is potentially also an important period for the programming of FFM, particularly as we showed that FFMI already tracks from 1 month of age.

We found tracking of high abdominal subcutaneous fat from age 6 months to 4 years, but there was no tracking of abdominal visceral fat to 4 years. In older children and adults, especially truncal and visceral fat largely contribute to an unfavorable metabolic health profile [28, 29]. Some research groups found abdominal subcutaneous

fat to be associated with an unfavorable metabolic profile in children [11, 30]. However, these findings are contradictive, as others showed that abdominal subcutaneous fat might have a protective effect against the adverse effects of visceral fat [31]. We found tracking of abdominal subcutaneous fat from infancy to childhood, which might suggest that subcutaneous fat is also programmed during the first months of life. In contrast, we did not find tracking of visceral fat from early life to 4 years of age. Other research groups measured visceral fat by ultrasound or computed tomography in 6- to 8-year-old children or reported tracking of abdominal visceral fat from age 2 to 6 years [32-34]. Altogether, this suggests that the visceral fat depot develops at an older age than the abdominal subcutaneous depot, which would potentially explain why we did not find tracking of visceral fat from early life to 4 years of age.

Our results show that infant feeding influences tracking of FM%. EBF infants had lower odds of having high FM% at 4 years, compared to infants with EFF or mixed feeding. We also found tracking to be different between EBF and non-EBF infants. EBF infants had no significant tracking of FM% from early life, whereas non-EBF infants had a high OR to track from 3 and 6 months to 4 years of age. An explanation could be that adiposity programming is different in EBF and non-EBF infants. Our research group found earlier that appetite-regulating hormones were different between EBF and EFF infants at age 3 months [35]. Also, differences in gut microbiota and serum metabolic profile have been reported between EBF and EFF infants in early life, which all could potentially influence adiposity programming [36, 37]. It has been described that EBF is protective for the development of childhood obesity [38]. Our findings support that EBF in early life has a potentially protective effect on adiposity programming later in life.

Tracking of FM%, FMI and FFMI was not different between girls and boys. It is known that girls have a higher FM% and lower FFM from early age onwards [6, 39], but present findings show, that tracking of body composition until age 4 years does not have sex differences.

For the sake of comparison with literature data, we also investigated the tracking of BMI SDS. High BMI SDS tracked from age 1, 3 and 6 months to 4 years, which is in line with previous data [2, 3]. However, it is known that children with a comparable weight or BMI might have different body fat or fat distribution [6-8]. Therefore, tracking in BMI is not predictive for later body fat and fat distribution.

This is one of the first studies, to our knowledge, reporting tracking of FM%, FMI, FFMI and abdominal fat distribution from early life to 4 years of age. The strengths of this study are the longitudinal and detailed measurements of body composition in a large group of 224 healthy, term-born children for a period of 4 years. Although, ADP by PEA POD (COSMED) and DXA had a very small difference in FM% of 0.9% at age 6 months, these measurements were considered similar as there was no proportional bias [16]. Therefore, it is very unlikely tracking results were influenced by the measuring methods used. A limitation is the lack of longitudinal reference values for fat mass percentage (FM%), fat mass index (FMI), fat free mass index (FFMI) and abdominal subcutaneous and visceral fat measured by ultrasound from infancy until 4 years of age. Therefore, we used categorical outcomes to investigate tracking from infancy to young childhood. We did not investigate tracking of abdominal subcutaneous and visceral fat from 1 month of age, because performing ultrasound measurements at 1 month of age has proven to be very exhausting for the infants. Since we did not found tracking of abdominal subcutaneous and visceral fat from age 3 months to 4 years, we do not expect to find tracking of abdominal fat from 1 month to 4 years of age.

Conclusion

FM% and FMI in the highest tertiles tracked from age 3 and 6 months to 4 years, high abdominal subcutaneous fat tracked from 6 months to 4 years of age and high FFMI tracked from age 1, 3 and 6 months to 4 years. High FM% tracked from 3 and 6 months to 4 years in non-EBF infants, whereas tracking was not significant in infants with EBF. Our longitudinal data support the presence of a critical window of adiposity and FFM programming in the first 6 months of life and a protective effect of EBF on adiposity programming.

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Author Contributions The study concept was developed by AHK and LB. Research was conducted by IvB, KdF and LB. Data analysis and drafting the manuscript was primarily done by IvB and AHK. All authors were involved in writing the manuscript and had final approval of the submitted version.

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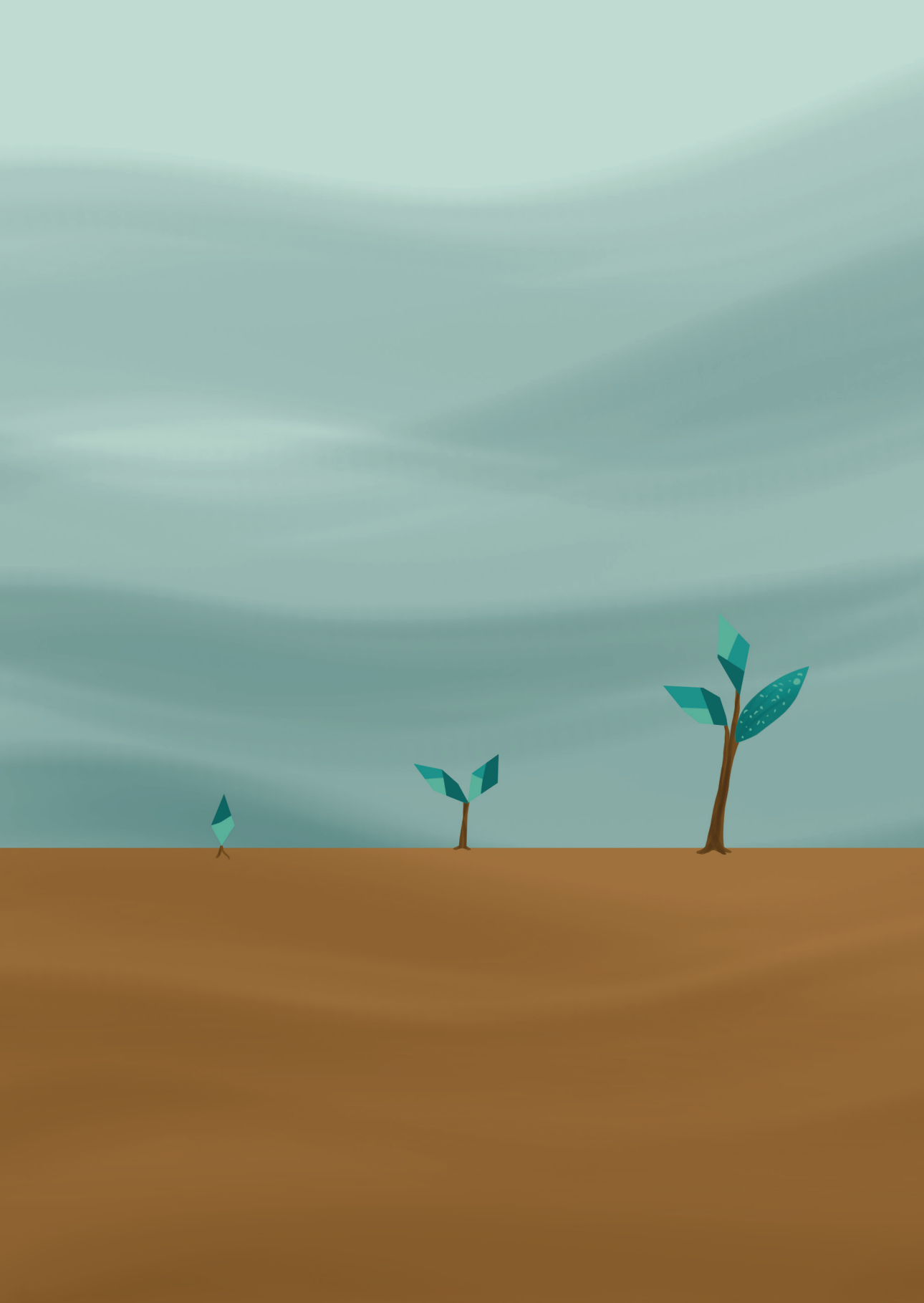

Chapter 6

Metabolomics in early life and the association with body composition at age 2 years

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Abstract

Background: Early life is a critical window for adiposity programming. Metabolic profile in early life may reflect this programming and correlate with later life adiposity. We investigated if metabolic profile at 3 months of age is predictive for body composition at 2 years and if there are differences between boys and girls and between infant feeding types.

Methods: In 318 healthy term-born infants, we determined body composition with skinfold measurements and abdominal ultrasound at 3 months and 2 years of age. High throughput metabolic profiling was performed on 3 month blood samples. Using random forest machine learning models, we studied if the metabolic-profile at 3 months can predict body composition outcomes at 2 years of age.

Results: Plasma metabolite profile at 3 months was found to predict body composition at 2 years, based on truncal: peripheral fat skinfold-ratio (T:P-ratio), with a predictive value of 75.8%, sensitivity of 100% and specificity of 50%. Predictive value was higher in boys (Q^2 = 0.322) than girls (Q^2 = 0.117). Of the 15 metabolite variables most strongly associated with T:P-ratio, 11 were also associated with visceral fat at 2 years of age.

Conclusions: Several plasma metabolites (LysoPS(22:2), dimethylarginine and others) at 3 months associate with body composition outcome at 2 years. These results highlight the importance of the first months of life for adiposity programming.

Introduction

Childhood obesity is an increasing and worldwide problem. In 1990, 32 million young children had overweight or obesity and this number increased to 41 million in 2016 [1]. Obesity at young age does not only cause short-term morbidity, but also increases the risk of developing non-communicable diseases (NCD) in later life, such as insulin resistance, type 2 diabetes and cardiovascular disease [1, 2].

The first months of life are a critical window for metabolic programming affecting adult outcome and body composition [3, 4]. It has been reported that a high weight-to-height SD-score and a high BMI in childhood are predictive for overweight and obesity in adolescence and adulthood [5, 6]. It is also known however, that a similar body weight or BMI may be accompanied by a different body composition or fat mass percentage, especially in infants and young children [7, 8]. We have previously reported that rapid weight gain in the first 6 months of life is an important risk factor for a higher fat mass in early adulthood [3] and that infants with a rapid rise in fat mass during the first 6 months of life have higher fat mass percentage trajectories during the first 2 years of life [9]. Fat mass and its distribution play an important role in the development of unfavorable metabolic outcomes [10, 11]. Especially excessive truncal and visceral fat accumulation compared to peripheral fat is associated with an unfavorable metabolic profile [12]. The ability to identify infants at risk of obesity at an early stage, will provide the opportunity to develop more targeted preventative strategies.

Also feeding type during the first few months of life influences body composition, with infants receiving exclusive breastfeeding exhibiting different weight trajectories with more subcutaneous fat accumulation and different serum concentrations of appetite regulating hormones compared to infants receiving exclusive formula feeding [13, 14].

An unfavorable body composition with excessive body fat and more visceral fat is associated with an adverse lipoprotein profile in children and adults, especially with high LDL cholesterol levels, which increases the risk of cardiovascular disease [15, 16]. However, not only the standard lipoproteins, but also several hundred lipid species were found in infant plasma [17]. These could potentially be early biomarkers for unfavorable metabolic outcomes.

Koulman *et al* found that the metabolic and lipid profile of exclusively breastfed infants is different from exclusively formula fed infants. In breastfed infants, total phosphatidylcholine levels are higher and linoleic acid is less incorporated in

palmitate into the phospholipid fraction as compared to that of formula-fed infants [18, 19]. In formula-fed infants, also the amount of formula feeding did influence the metabolic profile. In addition, in infants aged 3 months, phosphatidylcholine (PC) (18:1/16:0) and PC plasmalogen (34:1) were associated with accelerated weight gain, while phosphatidylcholine (20:4/18:0), PC plasmalogen (36:4), Sphingomyelin (d18:1/16:0) had an association with poor weight gain [18, 20].

These differences in metabolic profile and phospholipid composition could change the endogenous lipid metabolism and, thus, have consequences for adiposity programming and vice versa. We, therefore, hypothesized that specific metabolites in early life associate with body composition parameters at 2 years. The primary objective was to investigate if metabolites at 3 months of age are associated with, and even may predict specific body composition outcomes at age 2 years in a cohort of healthy infants. Second, we aimed to investigate if any metabolites, predictive of 2 years body composition, already associated with body composition parameters at 3 months. Lastly, we investigated if the metabolite profile at 3 months was different between boys and girls and between infants with exclusive breastfeeding and those with exclusive formula feeding.

Methods

Subjects

The cohort consisted of infants participating in the Sophia Pluto study, an ongoing birth cohort study of healthy infants, aimed to provide detailed data on early growth- and body composition trajectories in infancy and childhood. Infants were recruited between January 2013 and November 2017, from several maternity wards in Rotterdam, the second largest city in The Netherlands. All participants met the following inclusion criteria: born term (≥37 weeks of gestation), an uncomplicated neonatal period, without severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation.

Exclusion criteria were maternal disease or medication that could interfere with fetal growth, including maternal corticosteroids, insulin-dependent (gestational) diabetes mellitus, known congenital or postnatal disease or intrauterine infection that could interfere with infant growth. The Medical Ethics Committee of Erasmus Medical Centre approved the study. We obtained written informed consent of all parents/caregivers with parental authority.

Data collection and measurements

Trained staff carried out the measurements according to standard procedures at 3 months and at 2 years. Birth data were taken from medical records. Parental characteristics and feeding type were obtained by standardized interviews at the clinic visits and by questionnaires. Details about frequency and amount of infant feeding and dates of changes in feeding mode were recorded. Exclusive breastfeeding (EBF) was defined as receiving only mother's milk until at least the age of 3 months. Exclusive formula feeding (EFF) was defined as receiving only infant formula starting before the age of 1 month. Mixed feeding (mix) was defined as starting with formula feeding between 1 and 3 months of age.

Anthropometrics

Weight was measured to the nearest 5 grams by an electronic infant scale (Seca 717, Hamburg, Germany). Length was measured twice in supine position to the nearest 0.1 cm by an infantometer (Seca 416). BMI was calculated as weight (kg) / length^2 (m^2). Head, waist and hip circumference were measured to the nearest 0.1 cm by a circumference measuring tape (Seca 201). Skinfolds were measured to the nearest mm with a skinfold caliper (Slimguide C-120, Creative Health) at every visit on 4 sites on the left side of the body: biceps, triceps, subscapular and suprailliac. The intra-observer intra class correlation coefficient (ICC) and inter-observer ICC

were determined earlier; 0.88 and 0.76, resp. [21]. Peripheral skinfolds were calculated as triceps + biceps. Truncal skinfolds were calculated as subscapular + suprailliac [21]. The truncal:peripheral skinfold ratio (T:P-ratio) was calculated as truncal skinfolds divided by peripheral skinfolds.

SD-scores of weight, length and weight-for-length were calculated using Growth Analyser software (http://www.growthanalyser.org) [22].

Abdominal fat

Abdominal visceral and subcutaneous fat were determined at 3 months and 2 years, using ultrasound (Prosound 2 ultrasound with a UST-9137 convex transducer [both Hitachi Aloka Medical, Zug, Switzerland]). Fat depths were measured in supine position, with the transducer on the intercept of the xiphoid line and the waist circumference measurement plane. Visceral fat was measured in the longitudinal plan from the peritoneal boundary to the corpus of the lumbar vertebra with a probe depth of 9 cm and abdominal subcutaneous fat in the transvers plan from the cutaneous boundary to the linea alba with a probe depth of 4 cm. Minimal pressure was applied. Validity and reproducibility of measurements were confirmed in the Cambridge Baby Growth Study (CBGS), the relative interobserver technical error of measurement was 3.2% for visceral fat and 3.6% for subcutaneous fat [23]. If the vertebra were not visualized, measurements were considered unsuccessful and were excluded from analyses. The abdominal subcutaneous:visceral fat ratio (S:V-ratio) was calculated as abdominal subcutaneous fat divided by visceral fat.

Sample collection

Blood samples were collected at 3 months and 2 years by capillary toe or finger prick sampling after the infants had fasted for a minimum of 2 hours. Blood was collected in heparin microtubes (BD Microtainer®, 200-400 µL) and centrifuged to prepare plasma. The samples were stored at – 80°C until analysis. Plasma samples were transported on dry ice to the University of Cambridge (UK) for metabolic profiling.

Metabolic profiling

Metabolic profiling was performed with high throughput platform in the Metabolic Research Laboratories of the Institute of Metabolic Science in Cambridge. The samples were analysed using liquid chromatography mass spectrometry method [24] ultimately yielding results of the absolute and relative concentration of 349 individual metabolites and lipids. The protein precipitation liquid extraction protocol was used as described previously [24]. Briefly, 50 µL of plasma was transferred into a

2 mL screw cap Eppendorf plastic tube (Eppendorf, Stevenage, UK). Immediately, 650 µL of chloroform was added to each sample, followed by thorough mixing. Then, 100 µL of the internal standards (5 µM in methanol), 100 µL of the carnitine internal standards (5 µM in methanol) and 150 µL of methanol was added to each sample, followed by thorough mixing, after which 400 µL of acetone was added to each sample. The samples were vortexed and centrifuged for 10 minutes at ~20 000 g to pellet any insoluble material. The supernatant was pipetted into separate 2 mL screw cap amber-glass auto-sampler vials (Agilent Technologies, Cheadle, United Kingdom). The organic extracts were evaporated to dryness using a Concentrator Plus system (Eppendorf, Stevenage, UK) run for 60 minutes at 60˚C . The samples were reconstituted in 100 µL of a 2: 1: 1 mixture of propan-2-ol, acetonitrile and water, and then thoroughly vortexed. The reconstituted sample was transferred into a 250 µL low-volume vial insert inside a 2 mL amber glass auto-sample vial ready for liquid chromatography with mass spectrometry detection (LC-MS) analysis.

Chromatographic separation was achieved using Shimadzu HPLC System (Shimadzu UK Limited, Milton Keynes, UK) with the injection of 10 µl onto a Waters Acquity UPLC® CSH C18 column (Waters, Hertfordshire, UK); 1.7 µm, I.D. 2.1 × 50 mm^2, maintained at 55˚C. Mobile phase A was 6:4 acetonitrile and a 10 mM ammonium formate solution in water. Mobile phase B was 9:1 propan-2-ol and a 10 mM ammonium formate solution in acetonitrile. The flow was maintained at 500 µl/min through the following gradient: 0.00 min_40% mobile phase B; 0.40 min_43% mobile phase B; 0.45 min_50% mobile phase B; 2.40 min_54% mobile phase B; 2.45 min_70% mobile phase B; 7.00 min_99% mobile phase B; 8.00 min_99% mobile phase B; 8.3 min_40% mobile phase B; and 10 min_40% mobile phase B. The sample injection needle was washed using 9:1, propan-2-ol and acetonitrile. The mass spectrometer used was the Thermo Scientific Exactive Orbitrap with a heated electrospray ionization source (Thermo Fisher Scientific, Hemel Hempstead, UK). The mass spectrometer was calibrated immediately before sample analysis using positive and negative ionization calibration solution (recommended by instrument manufacturer). Additionally, the mass spectrometer scan rate was set at 4 Hz, giving a resolution of 25 000 (at 200 m/z) with a full-scan range of m/z 100–1800 with continuous switching between positive and negative mode.

Data processing

All .RAW files were converted to .mzXML files using msConvert (ProteoWizard) [25]. Converted files were subsequently processed in R (v3.3.1) using the CAMERA package [26] with peak picking performed using the centWave method as this enables for the deconvolution of closely eluting and slightly overlapping peaks.

Metabolite variables included within the final dataset were defined as peaks that had an intensity at least 3 times higher in analytical samples relative to the extraction blanks and that was present in at least 90% of the analyzed samples. If possible, metabolite variables were putatively annotated by matching measured accurate masses to entities in the Human Metabolome database (www.hmdb.ca).

Statistical analysis

Clinical characteristics are expressed as mean and standard deviation (SD) or as median and interquartile range [IQR] when not normally distributed. Differences in clinical characteristics were determined by independent Student's *t* test or Mann-Whitney *U*-test for non-parametric parameters. Pearson's correlation coefficient was used to determine bivariate correlations. Exact power calculations for this type of experiments were not readily available at the design of the project. Previous analyses of the lipid profiles in the Cambridge Baby Growth Study showed significant associations with catch-up growth[18] using around 215 samples. By using the Sophia Pluto cohort, that is almost double in sample size, sufficient power was considered to find metabolites that are associated with fat distribution.

Using WHO classification, overweight was defined as a weight-for-length > 2 SDS and obesity was defined as a weight-for-length > 3 SDS [1]. Underweight was defined as an weight-for-age < -2 SDS [27] .

To analyze the association between metabolite profile and six measures of body composition, peripheral and truncal fat, subcutaneous and visceral fat and the ratio of truncal : peripheral fat and the ratio of abdominal subcutaneous : visceral fat, individuals were stratified into tertiles of each body composition measure ('high', 'middle' and 'low'). Multivariate analysis was performed using principal component analysis (PCA). Partial least squares – discriminant analyses (PLS-DA), performed in SIMCA v13.0 (Umetrics, Umeå, Sweden), were used to identify associations between the metabolite profiles generated from samples collected at 3 months of age and body composition at 2 years of age, with all data logarithmically transformed (base10) and scaled to unit variance (UV) in all models. The performance of the generated models was based on cumulative correlation coefficients R^2X[cum] and R^2Y[cum] (PLS-DA only) to assess what percentage of the variation in the X and Y variables was explained by the model. The predictive performance of these models was based on the 7-fold cross validation Q^2[cum] and the significance of the model was determined using ANOVA of the cross validation residuals (CV-ANOVA).

To estimate if it is possible to determine body composition at 2 years of age using metabolite profile data from 3 months of age, random forest machine learning models were performed in R (V3.3.1). Each of the body compositions measures were split into a training (70%) and testing set (30%). The performance of these models was assessed by looking at overall classification accuracy of predicating 'high' or 'low' body composition measures, as well as the sensitivity and specificity of the predictions made in the testing set. Univariate analysis of metabolites of interest was performed using generalized linear models (GLM) calculated in R (v3.3.1). We corrected for possible confounders: sex, birth weight and feeding type. Additional corrections for BMI SDS at age 3 months, weight-for-length SDS and total skinfolds at age 2 years did not change the results. To determine differences between boys and girls and between the different types of feeding type, models were performed for boys and girls separately and for EBF, EFF and mixed feeding separately.

Controlling for the false discovery rate (FDR) was done by calculating a Bonferroni corrected p-threshold based on all 600 metabolite variables ($p = 8.33 \times 10^{-5}$).

Results

The study group consisted of 318 healthy infants of the Sophia Pluto cohort with complete body composition data and blood samples. One hundred forty-three (45%) were girls and 66.4% of the infants was Caucasian. Clinical characteristics are presented in Table 1. Of all infants, 38.7% received exclusive breastfeeding (EBF) until the age of 3 months and 25.5% of the infants were exclusive formula fed (EFF). This did not differ between boys and girls. Body composition parameters were not different between boys and girls, except for visceral fat at 3 months, which is higher in boys than in girls (p = 0.017). Abdominal subcutaneous and visceral fat and truncal : peripheral fat ratio (T:P-ratio) ratio decreased over time from age 3 months to 2 years. Based on the WHO criteria for weight-for-length SDS, 93.1% of the infants had normal weight, 5.7% was underweight and 1.3% had overweight at 2 years of age. None of the infants classified had obesity. This was not different between boys and girls. Infants were divided in tertiles based on T:P-ratio (Table 2).

Table 1. Clinical characteristics

	All	**Boys**	**Girls**	***p*-value**
Birth	***N*= 318**	***N*=175**	***N*=143**	
Gestational age (weeks)	39.74 (1.21)	39.66 (1.28)	39.83 (1.13)	0.224
Sex (%)		55.0	45.0	0.479
Birth weight SDS	0.28 (1.15)	0.42 (1.08)	0.11 (1.20)	**0.017**
Birth length SDS *	0.68 (1.20)	0.83 (1.19)	0.49 (1.19)	0.058
Ethnicity (%)				0.060
Caucasian	211 (66.4%)	122 (69.7%)	89 (62.2%)	
Black	21 (6.6%)	5 (2.9%)	16 (11.2%)	
Asian	3 (0.9%)	1 (0.6%)	2 (1.4%)	
Latin	1 (0.3%)	1 (0.6%)	0	
Other	64 (20.1%)	35 (20.0%)	29 (20.3%)	
Missing	18 (5.7%)	11 (6.2%)	7 (4.9%)	
Mode of delivery				0.479
Vaginal	219 (68.9%)	115	94	
Caesarian section	98 (30.8%)	59	39	
Missing	1 (0.3%)	1	0	

Table 1. Continued

	All	Boys	Girls	*p*-value
Age 3 months	***N*= 318**	***N*=175**	***N*=143**	
Age (months)	2.99 [2.92 – 3.09]	2.99 [2.92-3.09]	2.99 [2.92-3.06]	0.615
Feeding mode				0.597
Exclusive breastfeeding	123 (38.7%)	64	59	
Exclusive formula feeding	81 (25.5%)	48	33	
Mix feeding	113 (35.5%)	62	51	
Weight-for-length SDS	0.22 (1.01)	0.27 (0.95)	0.15 (1.08)	0.306
Length SDS	0.35 (0.87)	0.49 (0.81)	0.17 (0.92)	**0.001**
Peripheral skinfolds (mm)	15 [13-16]	15 [13-17]	14 [13-16]	0.139
Truncal skinfolds (mm)	13 [11.5-16]	13 [11-16]	14 [12-17]	0.633
T:P-ratio	0.93 [0.81 – 1.09]	0.92 [0.81 – 1.07]	1.00 [0.81-1.11]	0.112
Abdominal subcutaneous fat (cm)	0.41 [0.32 -0.49]	0.41 [0.34 – 0.49]	0.40 [0.32 - 0.49]	0.787
Visceral fat (cm)	2.36 (0.57)	2.42 (0.58)	2.29 (0.54)	**0.048**
S:V-ratio	0.17 [0.13-0.22]	0.16 [0.13-0.21]	0.18 [0.14-0.23]	0.111
Age 2 years	***N*=318**	***N*=175**	***N*= 143**	
Age (months)	24.02 [23.92 – 24.25]	24.02 [23.92 – 24.21]	24.08 [23.92-24.35]	0.119
Weight-for-length SDS	-0.41 (1.06)	-0.41 (1.13)	-0.41 (0.97)	0.945
Underweight	18 (5.7%)	10	8	0.720
Normal	296 (93.1%)	162	134	
Overweight	4 (1.3%)	3	1	
Obese	0	0	0	
Length SDS	0.22 (1.01)	0.28 (1.04)	0.15 (0.96)	0.250
Peripheral skinfolds (mm)	16 [14-19]	16 [14-19]	16 [14 – 18]	0.741
Truncal skinfolds (mm)	13 [11 – 15]	13 [11-15]	13 [11 – 15]	0.994
T:P-ratio	0.79 [0.67 – 0.93]	0.79 [0.68 – 0.94]	0.78 [0.65 – 0.92]	0.686
Abdominal subcutaneous fat (cm)	0.32 [0.26 -0.40]	0.32 [0.26 – 0.40]	0.34 [0.26-0.39]	0.787
Visceral fat (cm)	2.12 [1.82 -2.51]	2.11 [1.77 - 2.49]	2.14 [1.84 – 2.56]	0.387
S:V-ratio	0.16 [0.13-0.22]	0.16 [0.12-0.21]	0.15 [0.12-0.20]	0.280

*Data expressed as mean (SD) or median [IQR]. Significant p-values are boldfaced. SDS = Standard Deviation Score, BMI = Body Mass Index, T:P-ratio =Truncal:peripheral skinfold ratio, S:V-ratio= Subcutaneous : visceral fat ratio. * Birth weight SDS N=175*

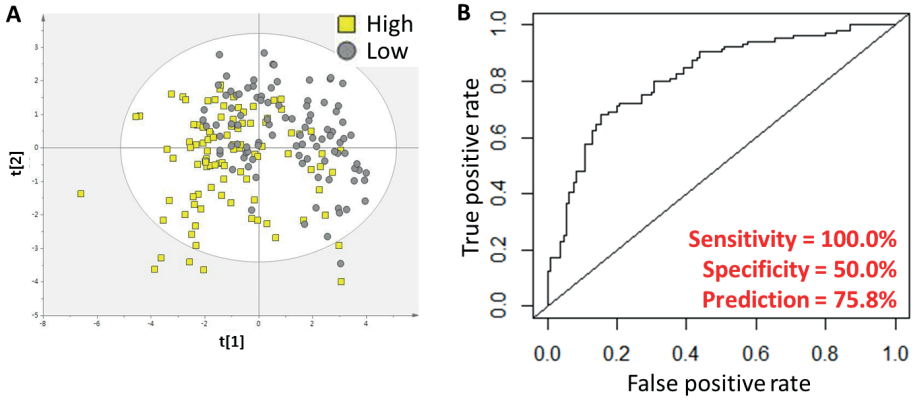
Table 2. Clinical characteristics and body composition of infants with high, middle and low trunk:peripheral fat ratio

	Low	Middle	High
T:P-ratio (range)	<0.72	0.72 – 0.88	>0.88
T:P-ratio (mean ± SD)	0.62 ± 0.07	0.79 ± 0.05	1.03 ± 0.12
Sex (F/M)	51/55	39/49	43/60
Feeding type (EBF/EFF/Mix)	39/30/37	36/23/29	39/25/39
Age 3 months			
Peripheral skinfolds (mm)	14.48 ± 2.54	14.95 ± 2.78	14.96 ± 2.94
Trunk skinfolds (mm)	12.97 ± 3.04*	14.47 ± 3.15*	14.76 ± 3.87*
Total skinfolds (mm)	27.45 ± 5.21*	29.42 ± 5.70*	29.72 ± 9.93*
Abdominal subcutaneous fat (cm)	0.41 ± 0.11	0.40 ± 0.11	0.43 ± 0.12
Visceral fat (cm)	2.41 ± 0.56	2.35 ± 0.57	2.34 ± 0.57
Age 2 years			
Peripheral skinfolds (mm)	18.13 ± 6.25*	16.98 ± 5.23*	14.77 ± 4.77*
Trunk skinfolds (mm)	11.18 ± 6.22*	13.36 ± 6.85*	15.12 ± 6.69*
Total skinfolds (mm)	29.31 ± 5.25	30.34 ± 5.23	29.89 ± 4.77
Abdominal subcutaneous fat (cm)	0.32 ± 0.09	0.33 ± 0.09	0.36 ± 0.10
Visceral fat (cm)	2.24 ± 0.64	2.27 ± 0.57	2.08 ± 0.51*

** Indicates a significant ($p<0.05$) difference between groups. All p-values were calculated using generalized linear models applied simultaneously to all three groups. Abbreviations: EBF= exclusively breastfed, EFF= exclusively formula fed, F= female, M=male, Mix= Mixed fed, SD= standard deviation, T:P-ratio = truncal:peripheral skinfold ratio*

Association between metabolite variables at 3 months and body composition at 2 years

There was a modest association between the plasma metabolite profile at 3 months of age and body composition at 2 years of age, measured as truncal : peripheral ratio (T:P-ratio) (R^2X = 0.224, R^2Y = 0.351, Q^2 = 0.185, CV-ANOVA = $5.71x10^{-8}$) (Figure 1). Using random forest, modest predictions for infants with high and low T:P-ratio at 2 years were achieved using 3 month plasma metabolite profiles with an predictive performance of 75.8%, a sensitivity of 100% and a specificity of 50.0%. Meaning that 100% of the infants with a high T:P-ratio at 2 years of age was predicted to have a high T:P-ratio based on their metabolite profile at 3 months, while 50.0% of the infants with a low T:P-ratio prediction based on their plasma metabolite profile did have a low T:P-ratio measured at 2 years of age.



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Predicted	Actual: Low	Actual: High
Low	17	0
High	17	30

Figure 1. Evaluation of the ability for 3 month metabolite profile to predict truncal:peripheral fat ratio at 2 years. *A) Scores plot of PLS-DA model calculated on individuals with 'high' and 'low' truncal:peripheral fat ratio after the dataset had been stratified into three groups of 'high', 'middle' and 'low' truncal:peripheral fat ratio B) Receiver operating characteristic curve showing diagnostic ability of the model to identify individuals with high and low truncal:peripheral fat ratio C) prediction matrix showing rates of correct classification.*

Of the 15 most strongly associated metabolite variables with T:P-ratio at 2 years of age, two passed 'false discover rate' (FDR) correction based on a Bonferroni corrected *p*-threshold ($p = 8.33 \times 10^{-5}$) and eight passed Benjamini-Hochberg based on all 600 variables (Table 3). Of these, nine metabolites were annotated: Lysophosphatidylserine 22:2 (LysoPS (22:2)) had a fold change of 1.48 ($p = 2.32 \times 10^{-5}$) in infants with a high T:P-ratio compared to infants with low T:P-ratio. Meaning that the relative abundance of LysoPS (22:2) was 48% higher in infants with a high T:P-ratio at 2 years, compared to infants with low a T:P-ratio at 2 years of age. For dimethylarginine, esterone glucoronide ($C_{24}H_{30}O_8$), the C13 isotope of hydroxypentaoxolanostenoic acid ($C_{30}H_{40}O_8$), hydroxyprogesterone glucoronide ($C_{27}H_{38}O_9$), lysophosphatidylethanolamine (20:1) (LysoPE(20:1)) the fold changes were 1.85 ($p = 0.0002$), 1.65 ($p = 0.0003$), 1.31 ($p = 0.0003$), 1.31 ($p = 0.0007$) and 1.09 ($p = 0.0005$), resp. Other annotated metabolites were lysophosphatidylglycerol (16:0) (LysoPG(16:0)), $C_{30}H_{40}O_8$ and lysophosphosphatidic acid (22:1) (LysoPA (22:1)). All had a fold change above 1.

Table 3. Panel of 15 plasma metabolite variables at 3 months most strongly associated with truncal:peripheral fat ratio at 2 years

			All Individuals		
Metabolite variables	*m/z*	*Rt (min)*	***p*-value**	**Corrected *p*-value**	**Fold change**
LysoPS(22:2)	578.3481	0.49	$5.99x10^{-06\ a,b}$	$2.16x10^{-05\ a,b}$	1.48
Unkown	1015.6761	0.39	$2.28x10^{-05\ a,b}$	$3.79x10^{-05\ a,b}$	1.57
Dimethylarginine	203.0533	0.33	0.0001 [b]	0.0002 [b]	1.85
$C_{24}H_{30}O_8$	447.2815	0.47	0.0002 [b]	0.0003 [b]	1.65
C^{13} isotope of $C_{30}H_{40}O_8$	530.3357	0.52	0.0003 [b]	0.0003 [b]	1.31
$C_{27}H_{38}O_9$	507.3104	0.50	0.0004 [b]	0.0007 [b]	1.31
Unknown	431.2617	0.39	0.0005 [b]	0.0007 [b]	1.38
LysoPE(20:1)	508.3468	0.66	0.0008 [b]	0.0005 [b]	1.09
Unknown	590.3393	0.47	0.0018	0.0017	1.48
LysoPG(16:0)	486.2760	0.54	0.0022	0.0026	1.14
$C_{30}H_{40}O_8$	529.3246	0.47	0.0034	0.0034	1.60
LysoPA(22:1)	495.3132	0.86	0.0040	0.0051	1.41
Unknown	200.8896	0.319	0.0043	0.0031	0.91
Unknown	162.9649	0.367	0.0046	0.0075	2.03
Unknown	222.8750	0.38	0.0050	0.0022	0.87

'p-value' are the unadjusted p-values. 'Corrected p-values' have been adjusted for sex, birth weight and feeding type. All p-values were calculated using generalized linear models comparing high and low tertiles of trunk:peripheral ratio. Fold change is calculated relative to the 'low' group. [a] denotes passing FDR based on Bonferroni [b] denotes passing FDR based on Benjamini-Hochberg.

Having identified candidate biomarkers at age 3 months for the T:P-ratio at 2 years of age, we wanted to determine the inter-relation between the biomarkers, body composition and potentially confounding factors. After correcting for sex, birth weight and feeding type (Table 3) all biomarkers remained significant ($p < 0.05$) with two passing a Bonferroni corrected *p*-threshold and six passing Benjamini–Hochberg correction. Additional corrections for BMI SDS at age 3 months, weight-for-length SDS and total skinfolds at age 2 years did not change the results (data not shown). Of the 15 metabolite variables, four were already associated with T:P ratio at 3 months of age ($p < 0.05$) (Table S1).

Boys		Girls	
***p*-value**	**Fold change**	***p*-value**	**Fold change**
$4.95x10^{-05}$	1.58	0.021	1.37
0.0002	1.64	0.033	1.49
0.0001	2.20	0.074	1.50
0.0009	1.80	0.032	1.49
0.0069	1.26	0.016	1.36
0.0015	1.36	0.072	1.26
0.0003	1.50	0.104	1.26
0.0053	1.08	0.059	1.09
0.0063	1.49	0.052	1.47
0.0240	1.11	0.054	1.17
0.0075	1.73	0.114	1.46
0.0122	1.42	0.101	1.39
0.0066	0.91	0.069	0.90
0.0021	2.76	0.418	1.29
0.1124	0.95	0.006	0.79

Putative annotations: $C_{24}H_{30}O_8$ – Esterone Glucoronide, $C_{27}H_{38}O_9$ –Hydroxyprogesterone Glucoronide, $C_{30}H_{40}O_8$ – Hydroxypentaoxolanostenoic acid. Abbreviations: LysoPA = lysophosphophatidic acid, LysoPE = lysophosphatidylethanolamine, LysoPG = lysophosphatidylglycerol, LysoPS = lysophosphatidylserine, m/z = mass-to-charge ratio, Rt = Retention time in minutes.

Eleven of the 15 metabolite variables, identified at age 3 months, were significantly associated with visceral fat at 2 years of age ($p < 0.05$), though none passed FDR based on either Bonferroni or Benjamini-Hochberg (Table 4). All had a fold change below 1, meaning that the relative abundance of these metabolite variables was lower at 3 months in infants with higher visceral fat at 2 years. T:P-ratio and visceral fat were not correlated, R = −0.073 (p = 0.220). We also found that some of the 15 metabolite variables were associated with peripheral and trunk fat levels although none of these associations passed FDR (data not shown).

Table 4. Panel of 15 plasma metabolite variables at 3 months associated with visceral fat at 2 years

	All Individuals		
	***p*-value**	**Corrected *p*-value**	**Fold change**
LysoPS(22:2)	0.003	0.004	0.75
Unkown	0.007	0.008	0.72
Dimethylarginine	0.002	0.003	0.62
$C_{24}H_{30}O_8$	0.009	0.010	0.71
C^{13} isotope of $C_{30}H_{40}O_8$	0.005	0.008	0.79
$C_{27}H_{38}O_9$	0.014	0.016	0.81
Unknown	0.209	0.207	0.88
LysoPE(20:1)	0.081	0.096	0.95
Unknown	0.023	0.028	0.74
LysoPG(16:0)	0.046	0.036	0.89
$C_{30}H_{40}O_8$	0.002	0.002	0.60
LysoPA(22:1)	0.042	0.051	0.77
Unknown	0.435	0.459	1.03
Unknown	0.001	0.003	0.49
Unknown	0.715	0.627	0.98

'p-value' are the unadjusted p-values. 'Corrected p-values' have been adjusted for sex, birthweight and feeding type. All p-values were calculated using generalized linear models comparing high and low tertiles of visceral fat at 2 years of age. Fold change is calculated relative to the 'low' group.

Difference between boys and girls

We found differences in the association between the 15 metabolite variables and the T:P-ratio between boys and girls (Figure S1). In boys, the model had a predictive performance of 32.2% between individuals in the highest and lowest tertile of T:P-ratio at 2 years (R^2X = 0.204, R^2Y = 0.595, Q^2 = 0.322, CV-ANOVA = 3.05×10^{-8}). In girls, the predictive performance of the model was 11.7% (R^2X = 0.197, R^2Y = 0.539, Q^2 = 0.117, CV-ANOVA = 0.038). In boys, all metabolic variables except for one unknown metabolite variable showed a significant ($p < 0.05$) association with T:P-ratio at 2 years. Of these 14 metabolic variables, 13 had a fold change greater than 1, which means that the relative abundance of the metabolic variables were higher in boys with high T:P-ratio compared to boys with low T:P-ratio. In girls, 5 out of 15 metabolic variables were significantly associated with T:P-ratio (Table 3). Four of these had a fold change

Boys		Girls	
***p*-value**	**Fold change**	***p*-value**	**Fold change**
0.084	0.80	0.010	0.68
0.109	0.78	0.026	0.64
0.032	0.62	0.025	0.61
0.105	0.74	0.028	0.66
0.108	0.83	0.015	0.73
0.191	0.86	0.025	0.74
0.209	0.85	0.523	0.91
0.124	0.95	0.299	0.95
0.142	0.77	0.071	0.69
0.332	0.93	0.077	0.83
0.046	0.64	0.010	0.55
0.236	0.82	0.079	0.71
0.518	1.02	0.595	1.04
0.007	0.44	0.090	0.55
0.203	0.93	0.511	1.06

Putative annotations: $C_{24}H_{30}O_8$ – Esterone Glucoronide, $C_{27}H_{38}O_9$ –Hydroxyprogesterone Glucoronide, $C_{30}H_{40}O_8$ – Hydroxypentaoxolanostenoic acid. Abbreviations: EBF = exclusively breastfed, EFF = exclusively formula fed, LysoPA = lysophosphophatidic acid, LysoPE = lysophosphatidylethanolamine, LysoPG = lysophosphatidylglycerol, LysoPS = lysophosphatidylserine, Mix = Mixed fed

greater than 1. An unknown metabolite variable had an fold change of 0.79 (p = 0.006) in girls, meaning that this metabolite variable had a lower relative abundance in girls with a high T:P-ratio, compared to girls with a low T:P-ratio. It was the only metabolic variable that was stronger associated with T:P-ratio in girls compared to boys.

Effect of feeding type

T:P-ratio at 2 years of age was not different between infants with exclusive breastfeeding (EBF), exclusive formula feeding (EFF) and mixed feeding. Of the 15 metabolite variables, lysophosphatidylethanolamine (20:1) (LysoPE(20:1)) and the isotope of Lysophosphoglycerol (16:0) (LysoPG(16:0)) were significantly associated with feeding type (Table 5). LysoPE(20:1) had a higher relative abundance in EBF-infants and LysoPG(16:0) had an higher relative abundance in EFF-infants.

Table 5. Relative abundance of 15 plasma metabolite variables in different feeding types

	Relative Abundance			Difference
	EBF	**Mix**	**EFF**	***p*-value**
LysoPS(22:2)	1.57	1.57	1.68	0.505
Unknown	0.88	0.94	0.99	0.357
Dimethylarginine	3.92	3.98	4.59	0.338
$C_{24}H_{30}O_8$	6.83	7.31	8.19	0.202
C^{13} isotope of $C_{30}H_{40}O_8$	8.30	7.52	7.62	0.351
$C_{27}H_{38}O_9$	35.97	38.51	38.76	0.442
Unknown	2.83	2.91	3.30	0.159
LysoPE(20:1)	48.04	44.90	43.70	**0.002**
Unknown	27.92	27.69	31.88	0.345
LysoPG(16:0)	6.10	7.23	7.40	**0.001**
$C_{30}H_{40}O_8$	13.20	12.92	14.14	0.684
LysoPA(22:1)	8.84	8.67	9.77	0.452
Unknown	1.88	1.91	1.92	0.568
Unknown	4.09	3.92	3.77	0.702
Unknown	1.07	1.18	1.15	0.193

All p-values were calculated using generalized liner models comparing relative abundance in exclusive breast fed, mixed and exclusive formula fed infants. Significant p-values are boldfaced. Relative abundance is shown in arbitrary units. Putative annotations: $C_{24}H_{30}O_8$ – Esterone Glucoronide , $C_{27}H_{38}O_9$ –Hydroxyprogesterone Glucoronide, $C_{30}H_{40}O_8$ – Hydroxypentaoxolanostenoic acid. Abbreviations: LysoPA = lysophosphophatidic acid, LysoPE = lysophosphatidylethanolamine, LysoPG = lysophosphatidylglycerol, LysoPS = lysophosphatidylserine

Discussion

Our data show that the plasma metabolite profile at 3 months of age can modestly predict body composition at 2 years of age, based on truncal : peripheral fat ratio (T:P-ratio), with a predictive value of 75.8%, sensitivity of 100% and a specificity of 50%. The predictive value was better in boys than in girls. Of the 15 metabolite variables at 3 months of age that were most strongly associated with the T:P-ratio at 2 years, 11 were also associated with visceral fat at 2 years of age.

We are the first to describe potential biomarkers at 3 months of age which are associated with body composition outcome at 2 years of age. It has been reported that metabolic biomarkers are different in lean children and children with obesity, with especially branched chain amino acids (BCAAs) concentrations being higher in individuals with obesity [28]. Second, metabolic biomarkers have been reported to be potentially predictive for unfavorable metabolic outcome in adults, adolescents and school children with overweight and obesity [29, 30]. However, it has never been described before that plasma metabolite profile at 3 months of age is associated with future visceral fat and proxy of body composition, such as skinfold measurements [4, 31], in a large cohort of healthy infants.

Our results support the hypothesis of a critical window of adiposity programming in early life [3, 4]. It has been reported that growth and body composition, especially in the first 6 months of life, are important for the development of body composition later in life and for the adult metabolic profile [3, 9]. We now add that the plasma metabolic profile in early life is potentially involved in the adiposity programming and contributes to adiposity at 2 years of age. Our results show that only four of the associated plasma metabolites associated with T:P-ratio at 2 years were also associated with T:P-ratio at 3 months of life. This means the plasma metabolites we found are independent of the biological progress of body composition.

Of the 15 most strongly associated metabolite variables, 11 were also significantly associated with abdominal visceral fat at 2 years of age, with a fold change below 1. Meaning, infants with high visceral fat at 2 years of age, had lower relative abundance of these metabolite variables at 3 months of age. This is remarkable, since we found the metabolite variables to be associated with T:P-ratio with a fold change above 1. We found visceral fat and T:P-ratio not to be correlated at 2 years of age. It has been described that skinfold measurements are correlated with total body fat [31]. However, this is mostly based on the amount of subcutaneous fat instead of visceral fat [32]. This could possibly explain, why we found opposite association

between T:P-ratio and visceral fat. Second, it has been described that especially excessive visceral fat is associated with an unfavorable metabolic outcome, with more insulin resistance and a higher risk of diabetes mellitus type 2 [12, 33]. Since we found an association between metabolite variables at 3 months of age with visceral fat at 2 years, our findings could suggest that metabolite profile in early life is also important for programming the metabolic outcome later in life.

Our results showed that plasma dimethylarginine and lysophosphatidylserine 22:2 (LysoPS 22:2) at 3 months of age are highly correlated with body composition outcomes at 2 years. In adults, higher dimethylarginine levels have been associated with dyslipidemia and accelerated atherosclerosis and found to be predictive for cardiovascular events [34, 35]. One research group described dimethylarginine levels to be higher in teenagers with obesity compared to their lean peers [36]. LysoPS 22:2 has been mainly studied in rodent and *in vitro* studies. These studies show that LysoPS is involved in glucose uptake in muscle and adipose tissue [37, 38]. In contrast, it has been reported that LysoPS levels are lower in hepatic tissue from adults with obesity, compared to lean peers [39]. Since we found LysoPS to be associated with visceral fat at 2 years of age with a fold change below 1, the mechanism for LysoPS involved with intra-abdominal fat could possibly differ from subcutaneous fat. We also found plasma lysophosphatidic acid(22:1) (LysoPA (22:1)) at 3 months of age to be associated with body composition outcome at 2 years. LysoPA has been described to interact with mTOR signalling, affecting body composition due to changes in lean body mass [40].

Dimethylarginine, LysoPS and LysoPA have been associated with inflammatory processes [41-43]. It was reported that LysoPS is an emerging class of signaling compounds [44] and could be interacting with Toll-like receptor dimers (TLR2/6) [45]. LysoPA has been reported to activate peroxisome proliferator-activated receptor gamma (PPAR-γ) [46]. Both Toll-like receptors and PPAR-γ are known to be involved in the development of inflammation, obesity and metabolic syndrome [47, 48]. Second, LysoPS, LysoPA, lysophosphatidylethanolamine (LysoPE) and lysophosphatidylglycerol (LysoPG) are all deacylated products of phospholipids and are the result of phospholipase A activity [46]. Phospholipase A2 is important in lipid metabolism and it has been described that serum levels of phospholipase A2 are increased in patients with obesity and inflammation, due to activation of pro-inflammatory pathways in preadipocytes [49, 50]. Our findings could, therefore, suggest that the identified metabolites are involved in adiposity development and systematic low grade inflammatory processes from early age onwards.

Plasma metabolite variables at 3 months of age had a higher predictive value in boys than in girls. We found no differences between girls and boys in skinfold measurements and abdominal fat distribution at 2 years of age. However, it has been described that girls have a higher fat mass percentage and lower lean body mass, measured with air displacement plethysmography, compared to boys [7]. One research group also found differences in metabolic biomarkers associated with insulin resistance between female and male adolescents with obesity [51]. Our findings of metabolite differences already present at a very young age suggest that these metabolite variables may have different mechanism of action in the adiposity programming of boys and girls.

Two of the 15 metabolite variables showed a different relative abundance in infants with different feeding types. This is in line with our previous findings in other cohorts, where we identified differences in lipid profile between infants receiving exclusive breastfeeding compared to infants receiving formula feeding [18, 20, 52]. Since only 2 of the 15 metabolite variables were different across different infant feeding types, the associations we found between metabolite variables at 3 months of age and body composition outcome at 2 years of age seemed independent of infant feeding type.

This is the first study reporting plasma metabolite profile at 3 months that are associated with body composition outcome at 2 years. The strengths of our study are the longitudinal body composition measurements and collected blood samples in a large group of healthy infants combined with a validated technique to obtain a detailed metabolic profile. We acknowledge some limitations. Although we did not annotated all identified metabolite variables, with the level of detail we provide, others can replicate our work and future annotation is possible. We identified metabolic variables in a single cohort containing a very low number of infants developing overweight and no infants developed obesity at 2 years of age. It was, therefore, not possible to predict obesity based on metabolic profile. The metabolite variables we found, were associated with childhood body composition trajectories. They will, therefore, have to be validated in infants who develop overweight or obesity and in an independent external cohort to validate generalizability in healthy infants.

Conclusion

In conclusion, we found that the plasma metabolite profile at 3 months of age can modestly predict body composition at 2 years of age, measured as T:P-ratio. The predictive value was higher in boys than in girls. Of the 15 highest correlated plasma metabolite variables, 11 were also associated with visceral fat at 2 years. These findings contribute to our insight into adiposity programming in the first months of life.

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Author contributions: AK, AHK, SB, KO and DD were in charge of designing the study. IvB, KdF and AHK were in charge of the cohort, design, and collecting of the data and samples. SS and AK performed the metabolomics and bioinformatic analysis. Drafting the manuscript was primarily done by IvB and SS under supervision of AHK and AK. All authors were involved in writing the manuscript and had final approval of the submitted version.

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Supplement

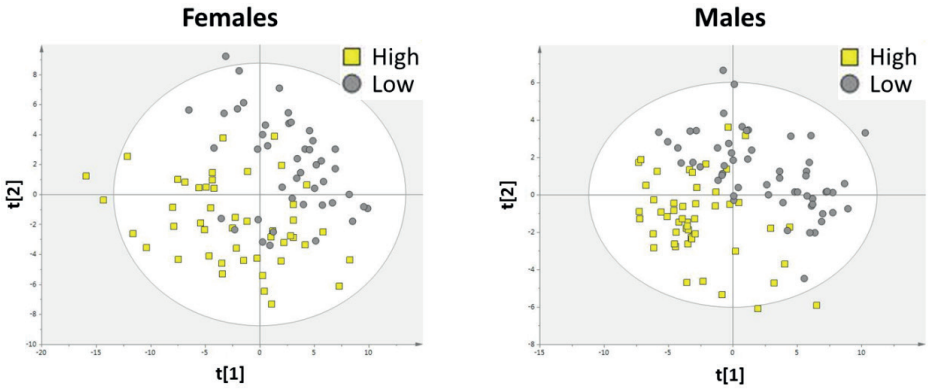
Supplement Table 1. Panel of 15 plasma metabolite variables at 3 months associated with truncal : peripheral fat ratio at 3 months

	All Individuals		
Metabolite variables	**p-value**	**Corrected p-value**	**Fold change**
LysoPS(22:2)	0.150	0.107	1.15
Unknown	0.074	0.058	1.25
Dimethylarginine	0.042	0.029	1.39
$C_{24}H_{30}O_8$	0.070	0.051	1.28
C^{13} isotope of $C_{30}H_{40}O_8$	0.026	0.014	1.20
$C_{27}H_{38}O_9$	0.051	0.038	1.18
Unknown	0.454	0.330	1.07
LysoPE(20:1)	0.147	0.061	1.04
Unknown	0.067	0.049	1.27
LysoPG(16:0)	0.034	0.041	1.12
$C_{30}H_{40}O_8$	0.013	0.009	1.53
LysoPA(22:1)	0.167	0.126	1.19
Unknown	0.465	0.318	0.98
Unknown	0.088	0.078	1.47
Unknown	0.097	0.164	1.08

'p-value' are the unadjusted p-values. 'Corrected p-values' have been adjusted for sex, birthweight and feeding type. All p-values were calculated using generalized linear models comparing high and low tertiles of trunk:peripheral ratio at 3 months of age. Fold change is calculated relative to the 'low' group.

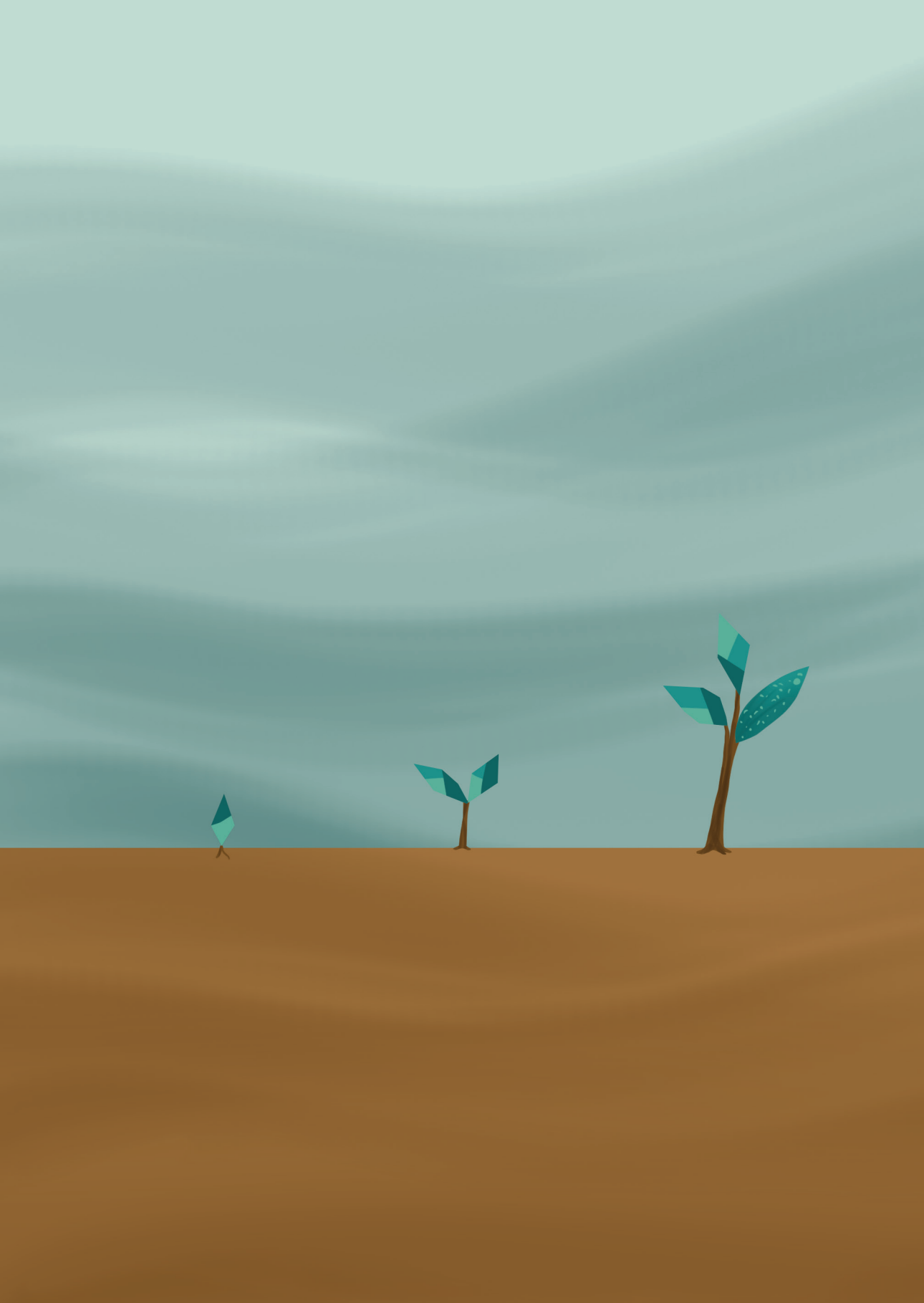
Boys		Girls	
p-value	Fold change	p-value	Fold change
0.241	1.17	0.353	1.13
0.114	1.31	0.310	1.21
0.124	1.42	0.171	1.38
0.074	1.41	0.361	1.20
0.009	1.33	0.542	1.07
0.118	1.20	0.180	1.18
0.292	1.15	0.811	1.03
0.269	1.04	0.279	1.05
0.050	1.41	0.399	1.18
0.041	1.15	0.270	1.10
0.013	1.80	0.237	1.34
0.186	1.23	0.447	1.16
0.390	0.97	0.643	0.98
0.095	1.66	0.498	1.25
0.272	1.06	0.327	1.08

Putative annotations: $C_{24}H_{30}O_8$ – Esterone Glucoronide, $C_{27}H_{38}O_9$ – Hydroxyprogesterone Glucoronide, $C_{30}H_{40}O_8$ – Hydroxypentaoxolanostenoic acid. Abbreviations: LysoPA = lysophosphophatidic acid, LysoPE = lysophosphatidylethanolamine, LysoPG = lysophosphatidylglycerol, LysoPS = lysophosphatidylserine



Supplement Figure 1. partial least squares – discriminant analysis modeling of the relationship between metabolite profile and high and low truncal:peripheral fat ratio in males and females separately.

Female (R^2X = 0.197, R^2Y = 0.539, Q^2 = 0.117, CV-ANOVA = 0.038) Male (R^2X = 0.204, R^2Y = 0.595, Q^2 = 0.322, CV-ANOVA = 3.05×10^{-8})



Chapter 7

Distinct infant feeding type-specific plasma metabolites at age 3 months associate with body composition at 2 years

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Abstract

Background: Early life is a critical window for adiposity programming and metabolic profile may affect this programming. We investigated if plasma metabolites at age 3 months were associated with fat mass, fat free mass and abdominal subcutaneous and visceral fat outcomes at age 2 years in a cohort of healthy infants and if these associations were different between infants receiving exclusive breastfeeding (EBF) and those with exclusive formula feeding (EFF).

Methods: In 318 healthy term-born infants, we determined body composition by Dual Energy X-ray absorptiometry (DXA) and visceral fat by abdominal ultrasound at 2 age years. High-throughput metabolic profiling was performed on blood samples collected at age 3 months. Tertiles were generated for each body composition outcome and differences in plasma metabolite levels at age 3 months between infants with high and low body composition outcomes at age 2 years were evaluated in general, as well as separately in EBF- and EFF-infants.

Results: Distinct plasma metabolite variables identified at age 3 months were associated with body composition at 2 years. These metabolites included several classes of lyso-phospholipids. Associations between the metabolites at age 3 months and fat mass index, fat mass percentage, fat free mass index and visceral fat at 2 years were predominantly found in EBF-infants.

Conclusions: Associations between plasma metabolite levels at age 3 months and high body fat mass at 2 years depend on infant feeding type. These findings contribute to our insight into the importance of infant feeding on adiposity programming in early life.

Introduction

Childhood obesity is a global public health threat [1]. Excess adiposity at young age has not only short-term, but particularly long-term morbidity, such as insulin resistance, type 2 diabetes mellitus and cardiovascular disease [1, 2]. The first months of life are a critical window for adiposity programming, affecting adult metabolic health and body composition [3]. Multifactorial risk factors influence this course, but the exact mechanisms have not yet been elucidated. Our research group found that especially rapid weight gain in the first 6 months of life is an important risk factor for a higher fat mass and an unfavorable metabolic profile in young adults [3]. In addition, infants with a rapid rise in fat mass in the first 6 months of life have higher fat mass percentage trajectories up until at least age 2 years [4]. Recently, we have added that fat mass and fat free mass track from early life to at least age 4 years [5]. Infant feeding is also reported to influence body composition during the first 6 months of life. Infants with exclusive breastfeeding have different growth trajectories with more subcutaneous fat accumulation, different serum concentrations of appetite regulating hormones and less tracking of fat mass percentage compared with formula fed infants [5-7]. It remains unclear how dietary exposure in the first months of life affects later life body composition and we seem to be lacking the tools to study this. Unravelling the risk factors involved in adiposity programming will increase the ability to identify infants at risk of excess adiposity at an early age and may thus provide the opportunity to develop more targeted preventative strategies.

Excessive body and visceral fat in children and adults are associated with adverse plasma lipid profiles [8, 9]. Especially branched chain amino acids (BCAAs) concentrations were reported to be higher in children with obesity [10]. In infants, the metabolic profile is also associated with growth patterns [11, 12]. At age 3 months, plasma levels of phosphatidylcholine (PC) (20:4/18:0), PC plasmalogen (36:4) and sphingomyelin (d18:1/16:0) have been associated with poor weight gain, while PC (18:1/16:0) and PC plasmalogen (34:1) were associated with accelerated weight gain [11, 12]. Recently, we have reported that LysoPC(22:2), dimethylarginine and several other plasma metabolites at age 3 months modestly predict the truncal-to-peripheral skinfold ratio (T:P-ratio) at age 2 years [13]. The metabolic profile in the first months of life could, therefore, potentially be involved in adiposity programming. However, the association between early life plasma metabolites and measured body composition including visceral fat in later in life has never been described.

The metabolic profile in early life is strongly influenced by infant feeding, since differences in metabolic profiles have been described between children with exclusive breastfeeding and exclusive formula feeding [11-14]. In breastfed infants, total PC levels in plasma are higher and linoleic acid is less incorporated than palmitate into the phospholipid fraction as compared to that of formula fed infants [12, 15]. However, after the cessation of breastfeeding and the introduction of complementary food, these differences in metabolic profile dissolve [12].

The metabolic profile in early life, whether or not influenced by infant feeding type, could have consequences for adiposity programming. The primary objective of the current study was to investigate if plasma metabolites at age 3 months are associated with fat mass, fat free mass, abdominal subcutaneous fat (ASF) and visceral fat outcomes at age 2 years in a cohort of healthy infants. Secondly, we investigated if these associations were different between infants with exclusive breastfeeding and those with exclusive formula feeding at age 3 months. We hypothesized that specific metabolites in early life are associated with body composition at age 2 years and that these associations depend on infant feeding type.

Methods

Subjects

The study population consisted of 318 healthy infants participating in the Sophia Pluto study, a birth cohort aiming to provide detailed data on early growth- and body composition trajectories in infancy and childhood. Infants were recruited from several maternity wards in Rotterdam, The Netherlands. All participants met the following inclusion criteria: born term (≥37 weeks of gestation) and singleton. Exclusion criteria were severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation or any maternal disease or medication use that could interfere with fetal growth, including maternal corticosteroids, diabetes mellitus or intrauterine infection, and known congenital or postnatal disease that could interfere with infant growth. The Medical Ethics Committee of Erasmus Medical Centre approved the study. We obtained written informed consent of all parents or caregivers with parental authority.

Data collection and measurements

Outpatient clinic visits were scheduled at age 3 months and 2 years. The same trained staff carried out the measurements according to local standard procedures. Birth data were taken from hospital and midwife records. Parental characteristics and feeding type were obtained by interviews at the clinic visits and by questionnaires. Exclusive breast feeding (EBF) was defined as receiving only mother's milk at the 3 months study visit. Exclusive formula feeding (EFF) was defined as receiving only infant formula at the 3 months study visit. Mixed feeding (mix) was defined as receiving both mother's milk and infant formula at the 3 months study visit.

Anthropometrics

Weight was measured to the nearest 5 grams by an electronic infant scale (Seca 717, Hamburg, Germany). Length was measured in supine position to the nearest 0.1 cm by an infantometer (Seca 416). SD-scores of weight, length and weight-for-length were calculated using Dutch references [16] by Growth Analyser software (www.growthanalyser.org).

Body composition

Body composition was measured with air displacement plethysmography (ADP) by PEA POD (Infant Body Composition System (COSMED)) during the visits at age 3 months, as described in detail elsewhere [17]. ADP was conducted using the

same machine. It was used and calibrated daily according to the user's manual [18]. ADP was validated earlier against a reference 4-compartment model and reliability was determined with a CV of 7.9% for fat mass percentage (FM%) [19].

At age 2 years, body composition was measured by DXA scan (GE Prodigy Advance R000279 and encore v14.1 software). Children wore only underwear and were swaddled in a cotton blanket. A vacuum cushion (465 75100, Schmidt, Germany) was used to avoid movement artifacts, which has similar results at age 6 months compared to ADP [20]. During the study, the same DXA machine was used and calibrated daily, according to the protocol recommended by the supplier [21]. CV for FM% was 3.2% [22]. Fat mass index (FMI) was calculated as fat mass divided by length in meters squared (kg/m^2) and fat free mass index (FFMI) as fat free mass divided by length in meters squared (kg/m^2).

Abdominal fat

Visceral and subcutaneous fat depth were determined at age 3 months and 2 years, using ultrasound (Prosound 2 ultrasound with a UST-9137 convex transducer (both Hitachi Aloka Medical, Zug, Switzerland). Assessments were made in supine position, with the transducer on the intercept of the xiphoid line and the waist circumference measurement plane. Visceral fat was measured in the longitudinal plan from the peritoneal boundary to the corpus of the lumbar vertebra with a probe depth of 9 cm and subcutaneous fat in the transverse plan from the cutaneous boundary to the linea alba with a probe depth of 4 cm. Minimal pressure was applied. Validity and reproducibility of measurements were confirmed in the Cambridge Baby Growth Study (CBGS), the relative interobserver technical error of measurement was 3.2% for visceral depth and 3.6% for subcutaneous depth [23]. If the vertebrae were not visualized, measurement was considered unsuccessful and was excluded from analyses.

Sample collection

Blood samples were collected at age 3 months and 2 years by capillary toe or finger prick sampling after the infants had fasted for ≥2 hours. Blood was collected in heparin microtubes (BD Microtainer®) and centrifuged to prepare plasma. The samples were stored at -80˚C until transportation on dry ice to the University of Cambridge (United Kingdom (UK)) for metabolomics analysis.

Metabolic profiling

Metabolic profiling was performed with the high throughput platform at the Metabolic Research Laboratories of the Institute of Metabolic Science, University of Cambridge, UK. The samples were analysed using a liquid chromatography mass

spectrometry method [24] ultimately yielding results of the absolute and relative concentration of 349 individual metabolites and lipids. The protein precipitation liquid extraction protocol was performed as described previously [24]. Briefly, 50 µL of plasma was transferred into a 2 mL screw cap Eppendorf plastic tube (Eppendorf, Stevenage, UK). Immediately, 650 µL of chloroform was added to each sample, followed by thorough mixing. Then, 100 µL of the internal standards (5 µM in methanol), 100 µL of the carnitine internal standards (5 µM in methanol) and 150 µL of methanol was added to each sample, followed by thorough mixing, after which 400 µL of acetone was added to each sample. The samples were vortexed and centrifuged for 10 minutes at ~20,000*g* to pellet any insoluble material. The supernatant was pipetted into separate 2 mL screw cap amber-glass auto-sampler vials (Agilent Technologies, Cheadle, UK). The organic extracts were evaporated to dryness using a Concentrator Plus system (Eppendorf, Stevenage, UK) run for 60 minutes at 60˚C. The samples were reconstituted in 100 µL of a 2: 1: 1 mixture of propan-2-ol, acetonitrile and water, and then thoroughly vortexed. The reconstituted sample was transferred into a 250 µL low-volume vial insert inside a 2 mL amber glass auto-sample vial ready for liquid chromatography with mass spectrometry detection (LC-MS) analysis.

Chromatographic separation was achieved using Shimadzu HPLC System (Shimadzu UK Limited, Milton Keynes, UK) with the injection of 10 µL onto a Waters Acquity UPLC® CSH C18 column (Waters, Hertfordshire, UK); 1.7 µm, I.D. 2.1 mm X 50 mm, maintained at 55˚C. Mobile phase A was 6:4 acetonitrile and a 10 mM ammonium formate solution in water. Mobile phase B was 9:1 propan-2-ol and a 10 mM ammonium formate solution in acetonitrile. The flow was maintained at 500 µL per minute through the following gradient: 0.00 minutes_40% mobile phase B; 0.40 minutes_43% mobile phase B; 0.45 minutes_50% mobile phase B; 2.40 minutes_54% mobile phase B; 2.45 minutes_70% mobile phase B; 7.00 minutes_99% mobile phase B; 8.00 minutes_99% mobile phase B; 8.3 minutes_40% mobile phase B; 10 minutes_40% mobile phase B. The sample injection needle was washed using 9:1, propan-2-ol and acetonitrile. The mass spectrometer used was the Thermo Scientific Exactive Orbitrap with a heated electrospray ionisation source (Thermo Fisher Scientific, Hemel Hempstead, UK). The mass spectrometer was calibrated immediately before sample analysis using positive and negative ionisation calibration solution (recommended by instrument manufacturer). Additionally, the mass spectrometer scan rate was set at 4 Hz, giving a resolution of 25,000 (at 200 m/z) with a full-scan range of m/z 100 to 1,800 with continuous switching between positive and negative mode.

Data processing

All .RAW files were converted to .mzXML files using msConvert (ProteoWizard) [25]. Converted files were subsequently processed in R (v3.3.1) using the CAMERA package [26] with peak picking performed using the centWave method as this enables for the deconvolution of closely eluting and slightly overlapping peaks. Metabolite variables included within the final dataset were defined as peaks that had an intensity at least 3 times higher in analytical samples relative to the extraction blanks and that was present in at least 90% of the analyzed samples. For each sample all signals are expressed relative to the total signal strength. If possible, metabolite variables were putatively annotated by matching measured accurate masses to entities in the Human Metabolome database (www.hmdb.ca) and the metabolomics workbench (www.metabolomicsworkbench.org). Resulting identities were cross-checked manually based on retention time and isotopic pattern and additional ion adducts (Supplementary Table 1).

Statistical analysis

Because body composition outcomes have sex-specific differences from an early age onwards, tertiles for FM%, FMI, FFMI, ASF and visceral fat were generated for boys and girls separately. These were subsequently merged into group tertiles for 'high', 'moderate' and 'low' (Supplementary Table 2) [5]. Comparison was done using Mann-Whitney *U*-test for non-parametric parameters, as most metabolite signals were not normally distributed based on manual checks of histograms. Differences between metabolic biomarkers, in 'high' versus 'low' tertiles, were calculated using non-parametric Wilcoxon rank-sum tests, for the total group (EBF, EFF and mix fed infants combined) and for EBF- and EFF-infants separately. To correct the false discovery rate, we used a lenient p-value cut-off $p < 0.005$, based on half the square root of the signals and the high degree of co-dependency between the signals as described previously [11].

Results

Clinical characteristics are presented in Table 1. Of the total study population, 55.0% was male, 71.8% was Caucasian. One hundred twenty one infants were exclusively breastfed (EBF) and 130 exclusively formula fed (EFF) at age 3 months. FMI and abdominal subcutaneous fat (ASF) were highest in EBF-infants at age 3 months. At age 2 years, there were no differences in clinical characteristics between EBF and EFF.

Plasma metabolites at age 3 months and body composition at 2 years

At age 3 months, two plasma metabolites were associated with FMI and FM% at age 2 years (Table 2). At age 3 months, dimethylarginine plasma level was higher in EBF-infants with high FMI at age 2 years compared with those with low FMI. Oxidised triglyceride (TG 55:10; O) levels at age 3 months were higher in the total group with a high FMI at 2 years and higher at age 3 months in the total group and in EBF-infants with high FM% at 2 years. For EFF-infants, these metabolites at 3 months did not show a relationship with FMI or FM% at 2 years.

Three other metabolites at age 3 months were associated with FFMI at age 2 years. LPE 18:2 levels at age 3 months were higher in EBF-infants with high FFMI at age 2 years, compared to EBF-infants with low FFMI. Two, not further identified, phospholipids species showed higher levels at age 3 months in the total group with high FFMI at age 2 years compared to those with low FFMI. Again, none of these 3 metabolites showed a relationship with FFMI at 2 years in EFF-infants.

Plasma metabolites at age 3 months and abdominal fat at 2 years

Three metabolites at age 3 months associated with abdominal subcutaneous fat (ASF) at age 2 years (Table 3). Oxycholesterol levels at age 3 months were lower in infants with high ASF at age 2 years in all infants. PC 42:8 at age 3 months was higher in all and in EBF-infants with high ASF at age 2 years, whereas PC 38:3 level at age 3 months was only higher in EFF-infants with high ASF at age 2 years.

Table 1. Clinical characteristics

	Exclusive breast feeding at age 3 months	Exclusive formula feeding at age 3 months	*p*-value
N	121	130	
Boys (%)	62 (51.2%)	76 (58.5%)	0.250
Gestational age (weeks)	39.91 (1.15)	39.45 (1.22)	**0.002**
Etnicity			0.054
Caucasian	76 (62.8%)	95 (73.1%)	
Black	8 (6.6%)	9 (6.9%)	
Asian	1 (0.8%)	1 (0.8%)	
Latin-American	1 (0.8%)	0	
Mix	32 (26.4%)	15 (11.5%)	
Missing	3 (2.5%)	10 (7.7%)	
Birth weight SDS	0.29 (1.12)	0.36 (1.16)	0.630
Birth length SDS*	0.89 (1.03)	0.40 (1.34)	**0.020**
Age 3 months			
Weight SDS	0.58 (1.14)	0.46 (1.06)	0.371
Length SDS	0.43 (0.80)	0.34 (0.90)	0.390
FM%	23.46 (4.57)	22.05 (4.84)	**0.021**
FMI (kg/m²)	3.79 (0.99)	3.54 (10.96)	0.051
FFMI (kg/m²)	12.20 (0.87)	12.37 (0.94)	0.153
ASF (cm)	0.43 (0.12)	0.39 (0.11)	**0.005**
Visceral fat (cm)	2.29 (0.56)	2.41 (0.60)	0.121
Age 2 years			
Weight SDS	-0.26 (1.06)	-0.22 (1.03)	0.746
Length SDS	0.20 (0.97)	0.30 (0.96)	0.393
FM%	17.56 (3.75)	18.02 (3.72)	0.391
FMI (kg/m²)	2.82 (0.77)	2.89 (0.75)	0.550
FFMI (kg/m²)	13.09 (0.88)	13.00 (0.85)	0.439
ASF (cm)	0.35 (0.09)	0.33 (0.10)	0.292
Visceral fat (cm)	2.15 (0.58)	2.22 (0.60)	0.361

*Data expressed as mean (SD) or N(%). Significant p-values are boldfaced. *birth length available in 75 infants with exclusive breastfeeding, 64 infants with exclusive formula feeding. Abbreviations: ASF= abdominal subcutaneous fat, FM%= fat mass percentage, FMI =Fat Mass Index, FFMI = Fat Free Mass Index, SDS = Standard Deviation Score.*

Table 2. Relative metabolite levels at age 3 months associated with body composition at age 2 years

Metabolite	Feeding type	Relative plasma metabolite levels at age 3 months (mol ratio; mean ± SD)		*p*-value
FMI (kg/m²) at age 2 years		**Low tertile**	**High tertile**	
Dimethylarginine	All	2.92E-05 (±3.27E-05)	3.83E-05 (±4.22E-05)	0.1331
	EBF	1.89E-05 (±2.04E-05)	5.43E-05 (±4.91E-05)	**0.0007**
	EFF	4.5E-05 (±4.04E-05)	2.85E-05 (±3.26E-05)	0.0950
TG 55:10; O ($M+Na^+$)	**All**	4.30E-05 (±1.11E-05)	4.78E-05 (±0.87E-05)	**0.0030**
	EBF	4.24E-05 (±1.09E-05)	4.82E-05 (±0.85E-05)	0.0207
	EFF	4.26E-05 (±1.09E-05)	4.76E-05 (±0.84E-05)	0.0571
FM% at age 2 years		**Low tertile**	**High tertile**	
TG 55:10; O ($M+Na^+$)	**All**	4.24E-05 (±1.13E-05)	4.78E-05 (±0.80E-05)	**0.0007**
	EBF	4.17E-05 (±1.10E-05)	4.88E-05 (±0.68E-05)	**0.0030**
	EFF	4.22E-05 (±1.09E-05)	4.73E-05 (±0.82E-05)	0.0476
FFMI (kg/m²) at age 2 years		**Low tertile**	**High tertile**	
LysoPE 18:2 (M+1)	All	0.000230 (±0.00012)	0.000280 (±0.00016)	0.0275
	EBF	0.000199 (±0.00006)	0.000282 (±0.00013)	**0.0010**
	EFF	0.000260 (±0.00016)	0.000260 (±0.00017)	0.9918
$C_{48}H_{94}N_2O_8P$	**All**	0.001587 (±0.00051)	0.001842 (±0.00036)	**0.0004**
	EBF	0.001606 (±0.00062)	0.002005 (±0.00030)	**0.0025**
	EFF	0.001556 (±0.00044)	0.001712 (±0.00035)	0.1218
$C_{48}H_{96}N_2O_8P$	**All**	0.000776 (±0.00029)	0.000912 (±0.00025)	**0.0020**
	EBF	0.000834 (±0.00023)	0.000964 (±0.00025)	0.0329
	EFF	0.000779 (±0.00029)	0.000859 (±0.00024)	0.2343

Metabolite variables (protonated ions ($M+H^+$) or as given, further details in suppl info table 1) associated with FMI, FM% and FFMI. Metabolite levels are provided as molar ratio. Normalised to total signal strength data for each metabolite signal. Tertiles were merged from sex-specific tertiles (further details in suppl info table 2). p-value is difference in plasma level between high and low tertile. Significant p-value <0.005 (corrected for FDR) are boldfaced. Abbreviations: EBF: exclusive breastfed infants, EFF: exclusive formula fed infants, FFMI = fat free mass index, FMI = fat mass index, FM%= Fat mass percentage, LysoPE= lysophosphatidylethanolamine, TG..O= oxidised triglyceride

Twelve other metabolites at age 3 months were associated with visceral fat at 2 years (Table 3). These were mainly lyso-phosopholipids (LysoPC 14:0, LysoPC 16:3, LysoPC 16:1, LysoPC 16:0, LysoPS 21:1, LysoPS 25:6 and LysoPA 23:1), as well as dimethylarginine, diacylglycerol (DG 40:10) and sphingomyelin (SM 35:2;O2).

All plasma levels of LysoPCs and the DG were higher at age 3 months in infants with high visceral fat at age 2 years, while all three LysoPSs, LysoPA, SM and dimethylarginine levels were higher at age 3 months in infants with low visceral fat at 2 years. These results were mostly driven by EBF-infants. Only for LysoPA 23:1, the difference between the tertiles was more prominent in EFF-infants.

Table 3. Relative metabolite levels at age 3 months associated with abdominal subcutaneous and visceral fat at 2 years.

Metabolite	**Feeding type (n)**	**Relative plasma metabolite levels at age 3 months (mol ratio; mean ± SD)**		**p-value**
Subcutaneous fat at age 2 years		**Low tertile**	**High tertile**	
Oxid Chol ($M-2H_2O$)	**All**	3.17E-06 (±1.59E-06)	2.55E-06 (±1.51E-06)	**0.0032**
	EBF	2.63E-06 (±1.31E-06)	2.57E-06 (±1.64E-06)	0.8617
	EFF	3.19E-06 (±1.63E-06)	2.45E-06 (±1.42E-06)	0.0344
PC 42:8	**All**	0.000734 (±0.00030)	0.000892 (±0.00028)	**0.0002**
	EBF	0.000687 (±0.00037)	0.000951 (±0.00023)	**0.0013**
	EFF	0.000762 (±0.00023)	0.000823 (±0.00031)	0.3177
PC 38:3	All	0.012896 (±0.00355)	0.014161 (±0.00318)	0.0104
	EBF	0.014895 (±0.00423)	0.015504 (±0.00356)	0.5220
	EFF	0.011468 (±0.00218)	0.012929 (±0.00150)	**0.0009**
Visceral fat at age 2 years		**Low tertile**	**High tertile**	
Dimethylarginine	**All**	4.79E-05 (±4.84E-05)	2.93E-05 (±3.45E-05)	**0.0025**
	EBF	5.16E-05 (±4.58E-05)	2.66E-05 (±3.28E-05)	0.0122
	EFF	5.06E-05 (±5.20E-05)	2.98E-05 (±3.53E-05)	0.0498
LysoPC 14:0 (M+1)	**All**	2.06E-05 (±0.94E-05)	2.62E-05 (±1.25E-05)	**0.0005**
	EBF	2.0E-05 (±0.99E-05)	2.49E-05 (±1.19E-05)	0.0760
	EFF	2.1E-05 (±0.91E-05)	2.57E-05 (±1.32E-05)	0.0691
LysoPC 16:3	**All**	5.02E-05 (±2.04E-05)	6.15E-05 (±2.24E-05)	**0.0003**
	EBF	4.67E-05 (±1.62E-05)	5.86E-05 (±2.16E-05)	0.0150
	EFF	5.43E-05 (±2.28E-05)	5.94E-05 (±2.17E-05)	0.3270
LysoPC 16:1	**All**	0.000106 (±5.01E-05)	0.000125 (±4.14E-05)	**0.0046**
	EBF	0.000109 (±4.08E-05)	0.000139 (±3.72E-05)	**0.0020**
	EFF	8.99E-05 (±3.87E-05)	0.000104 (±3.35E-05)	0.0971
LysoPC 16:0	**All**	0.006089 (±0.0016)	0.006783 (±0.0014)	**0.0020**
	EBF	0.005690 (±0.0014)	0.006414 (±0.0015)	0.0459
	EFF	0.006257 (±0.0017)	0.007016 (±0.0014)	0.0430

Table 3. Continued.

Metabolite	Feeding type (n)	Relative plasma metabolite levels at age 3 months (mol ratio; mean ± SD)		p-value
Subcutaneous fat at age 2 years		**Low tertile**	**High tertile**	
LysoPC 16:0 (M+1)	**All**	0.001566 (±0.00064)	0.001831 (±0.00049)	**0.0016**
	EBF	0.001434 (±0.00055)	0.001608 (±0.00062)	0.2352
	EFF	0.001688 (±0.00064)	0.001986 (±0.00037)	0.0181
LysoPA 23:1	**All**	0.000155 (±0.00016)	9.10E-05 (±0.00012)	**0.0069**
	EBF	0.000179 (±0.00016)	8.25E-05 (±0.00012)	0.0665
	EFF	0.000159 (±0.00016)	9.6E-05 (±0.00012)	**0.0016**
LysoPS 21:1 (M+1)	**All**	0.000375 (±0.00018)	0.000283 (±0.00015)	**0.0002**
	EBF	0.000408 (±0.00016)	0.000278 (±0.00013)	**0.0006**
	EFF	0.000371 (±0.00019)	0.000301 (±0.00017)	0.0900
LysoPS 22:2	**All**	1.78E-05 (±1.17E-05)	1.35E-05(±0.88E-05)	**0.0044**
	EBF	1.94E-05 (±1.20E-05)	1.26E-05 (±0.85E-05)	**0.0096**
	EFF	1.75E-05 (±1.15E-05)	1.36E-05 (±0.85E-05)	0.1034
LysoPS 25:6	**All**	0.000164 (±8.41E-05)	0.000124 (±7.04E-05)	**0.0005**
	EBF	0.000169 (±8.48E-05)	0.000112 (±6.24E-05)	**0.0023**
	EFF	0.000169 (±8.86E-05)	0.000138 (±7.52E-05)	0.1088
DG 40:10 ($M+NH_4^+$)	**All**	0.000286 (±0.00017)	0.000359 (±0.00018)	**0.0033**
	EBF	0.000244 (±0.00011)	0.000286 (±0.00012)	0.1490
	EFF	0.000374 (±0.00023)	0.000401 (±0.00022)	0.6005
SM 35:2; O_2	**All**	0.000956 (±0.00018)	0.000881 (±0.00018)	**0.0040**
	EBF	0.001029 (±0.00013)	0.000955 (±0.00016)	0.0432
	EFF	0.000909 (±0.00019)	0.000815 (±0.00016)	0.0232

Metabolite variables (protonated ions ($M+H^+$) or as given, and further details in suppl info table 1) associated with FMI, FM% and FFMI. Metabolite levels are provided as molar ratio. Normalised to total signal strength data for each metabolite signals. Tertiles were merged from sex-specific tertiles (further details in suppl info table 2). p-value is difference in plasma level between high and low tertile. Significant p-value <0.005 (corrected for FDR) are boldfaced. Abbreviations: DG= diacylglycerol, EBF: exclusive breastfed infants, EFF: exclusive formula fed infants, LysoPA= lysophosphatidic acid, LysoPC=lysophosphatidylcholine, LysoPS= lysophosphatidylserine, Oxid Chol= Oxycholesterol, PC= phosphatidylcholine, SM= sphingomyelin

Discussion

Our data show that several plasma metabolites at age 3 months are associated with progression towards a high FMI, FM%, FFMI and visceral fat at age 2 years. The associated metabolites are mainly different classes of lyso-phospholipids. Most of the associations were strongly dependent of infant feeding type, and we found most differences in plasma metabolite levels at age 3 months, between low and high tertile body fat mass infants at age 2 years to be apparent in EBF-infants, while only a few associations were seen for EFF-infants.

Lyso-phospholipids are phospholipids with only one fatty acid attached to the glycerol backbone and are mostly the result of the hydrolysis of phospholipids by lipases [27] and through the autotaxin-LysoPA-lipid phosphate phosphatase 3 axis [28]. Several of the identified metabolites (dimethylarginine, LysoPA, LysoPS, LysoPE and LysoPG) are known to be associated with pro-inflammatory pathways in preadipocytes [29, 30] and with inflammatory processes [31-33], mainly via interaction with Toll-like receptor dimers [34] and peroxisome proliferator-activated receptor gamma (PPAR-γ) [35], and/or the development of cardiovascular disease via G protein-coupled receptors (GPCRs)[36]. Our findings could, therefore, suggest that the identified metabolites at age 3 months are involved in adiposity development and systemic low-grade inflammatory processes, seemingly starting from early age onwards.

Our findings also show that the association between metabolite profile at age 3 months and body composition at age 2 years is dependent of infant feeding type. From our previous work, we know that feeding type and milk composition has a major impact on metabolic profiles of infants [11-14]. We now add that these differences in metabolic profile in early life, can potentially be involved in differences in adiposity programming between EBF- and EFF-infants.

Differences between plasma metabolite levels at age 3 months and 'high' or 'low' FM%, FMI, FFMI and visceral fat at 2 years were predominantly seen in EBF-infants. In EBF-infants, early life plasma metabolites which associated with later body composition were predominately LysoPC, LysoPE and sphingomyelin (SM). These polyunsaturated phospholipids were previously reported to be related to weight-to-age and weight-to-length trajectories until age 2 years [37]. Also, LysoPC and SM plasma levels are reported to be different between infants with EBF and EFF [38, 39]. This could potentially be linked to the milk fat globule membrane (MFGM), which coats fat droplets in human milk, but is absent in formula feeding [38, 39]. This

phospholipid layer consists mostly of PC and PE in combination with sphingolipids, predominantly in SM form [39]. The MFGM is associated with neurodevelopmental outcomes and microbiome development [39]. Our findings suggest that the MFGM could potentially also be involved in adiposity programming.

In EFF-infants, LysoPA was associated with visceral fat outcomes. Like LysoPC and LysoPE, this metabolite is a lyso-phospholipid, but from a different class. Lyso-phospholipids levels are a marker for phospholipid turnover and the product of lipases [40],while LysoPA in EFF-infants is generally not a major phospholipid in the circulation, but involved in biosynthesis of the more abundant classes of phospholipids like the PC lipids [41]. Together, these findings suggest that phospholipids are important in body composition development and fat distribution and that the supply and incorporation of phospholipids is different between EBF- and EFF-infants.

With the rapid increase in childhood obesity, it is imperative that we understand what factors predispose infants to unfavourable body composition, so that we can develop approaches to identify infants at risk for excess adiposity and adequate preventive tools. Our work shows that already at age 3 months, metabolic processes occur that could be involved in differences in future body composition. Moreover, it shows that caution is needed to combine the results for EBF-infants with those from EFF-infants. Our findings could contribute to more targeted and individualized preventive strategies, as well as an opportunity for early treatment options by improving infants diet, either directly for formula, or indirectly through the mother for breastmilk. Further work in this area is, therefore, crucial.

Our work has several strengths and weaknesses. It is important to note the exceptional quality of the body composition data that was crucial to show how both breast- and formula feeding have very different long-term effects on body composition, through the infant's metabolism. Another strength was the use of tertiles which were balanced for sex, which helped to assess the relationship between plasma metabolite variables and body composition outcomes. However, from these observational data it is difficult to infer causality. The differences between the plasma metabolites precede the body composition differences, but can be either the cause or the result of mechanisms and processes in metabolism that influence later body composition. The impact of feeding type, although expected, was much clearer than anticipated. As none of the metabolite associations with body composition outcomes were independent from feeding type, analysis of the whole cohort diluted any signal, whereas testing of the EBF and EFF sub-groups

reduced the statistical power. Another limitation was its scoping nature in relation to the specific metabolites involved. However, there was no clear evidence in the literature to enable us to focus the metabolite profiling. Our findings show that especially lyso-phospholipid analysis is important, hence future projects can use more targeted and quantitative methods to validate and further understand these relationships.

Conclusion

In conclusion, distinct plasma metabolites at age 3 months are associated with body composition at 2 years, measured as FMI, FM%, FFMI and abdominal fat distribution. Associations between plasma levels at age 3 months and high or low body composition outcomes were infant feeding type specific and were predominantly found in EBF-infants. These findings contribute key insights into the importance of the type of infant feeding on adiposity programming in early life.

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Supplement

Supplementary Table 1. Metabolite specifics

Metabolite	m/z	Rt (min)
$C_{48}H_{94}N_2O_8P$	857.6781	4.61
$C_{48}H_{96}N_2O_8P$	859.6862	4.83
Dimethylarginine	203.0533	0.34
DG 40:10 ($M+NH_4^+$)	678.5259	2.45
LysoPA 23:1	529.3246	0.47
LysoPC 14:0 (M+1)	469.3134	0.46
LysoPC 16:0	496.3389	0.53
LysoPC 16:0 (M+1)	497.3403	0.52
LysoPC 16:1	494.3186	0.47
LysoPC 16:3	490.2937	0.45
LysoPE 18:2 (M+1)	479.3198	0.52
LysoPS 21:1 (M+1)	567.336	0.50
LysoPS 22:2	578.3481	0.49
LysoPS 25:6	612.3477	0.52
PC 38:3	812.6204	4.16
PC 42:8	858.671	4.61
SM 35:2; O_2	715.5723	3.84
TG 55:10; O ($M+Na^+$)	927.6729	4.87

Metabolite specifics, presented as mass-to-charge raio and retention time. Abbreviations: m/z= mass-to-charge ratio, Rt = retention time in minutes

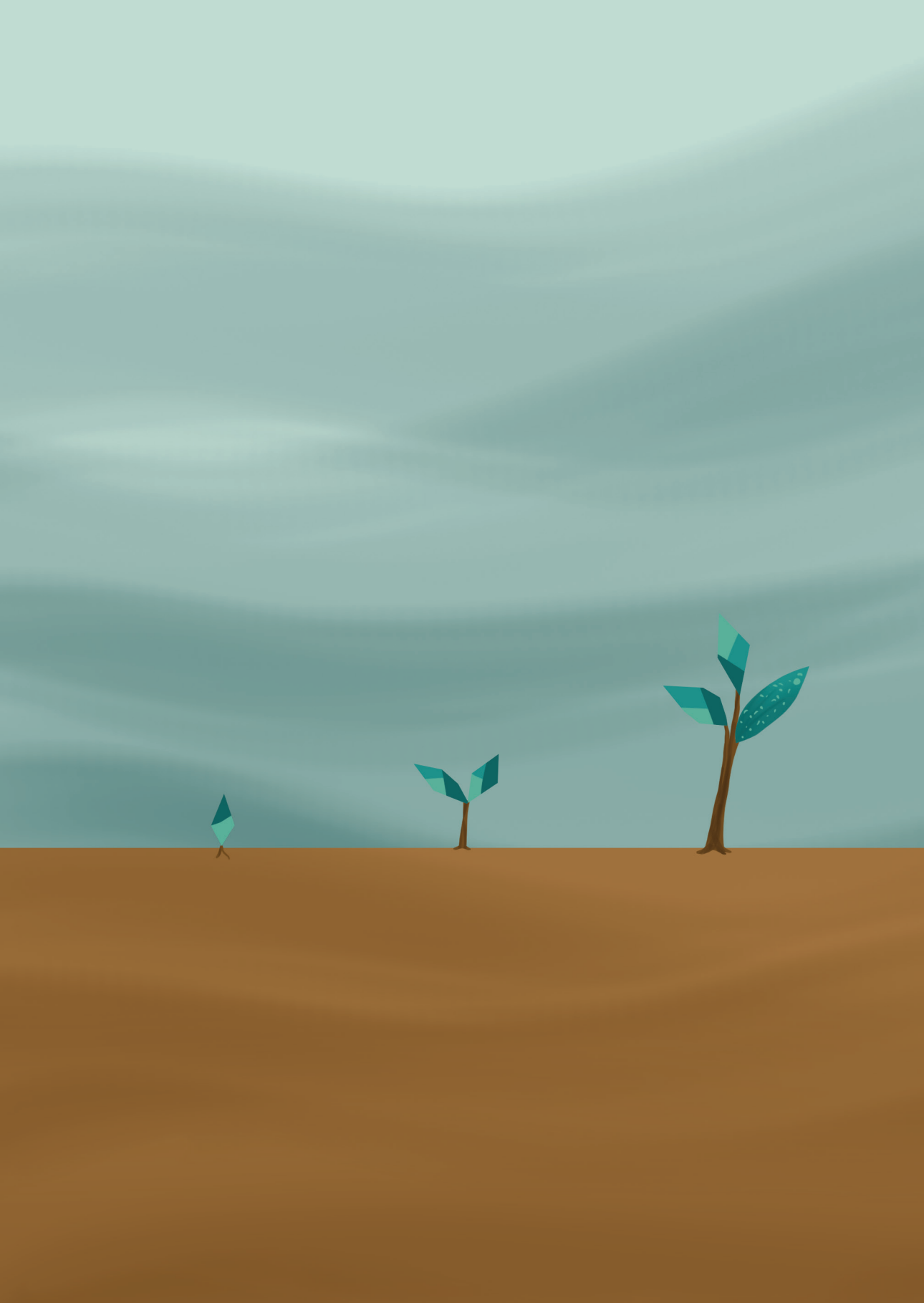
Supplementary Table 2. 'High' and 'low' sex-specific tertiles of FM%, FMI, FFMI, abdominal subcutaneous and visceral fat

		FM%	FMI (kg/m²)	FFMI (kg/m²)	ASF (cm)	Visceral fat (cm)
Age 3 months						
High	♂	> 24.00	> 3.90	> 12.95	> 0.46	> 2.65
	♀	> 25.23	> 4.02	> 12.31	> 0.45	> 2.58
Low	♂	< 20.57	< 3.26	< 12.14	< 0.36	< 2.14
	♀	< 20.43	< 3.05	< 11.61	< 0.35	< 2.04
Age 6 months						
High	♂	> 25.93	> 4.27	> 13.13	> 0.46	> 2.42
	♀	> 27.40	> 4.66	> 12.63	> 0.47	> 2.39
Low	♂	< 21.17	< 3.45	< 12.32	< 0.35	< 1.99
	♀	< 22.97	< 3.65	< 12.06	< 0.35	< 1.92
Age 2 years						
High	♂	> 18.50	> 3.03	> 13.63	> 0.37	> 2.39
	♀	> 19.82	> 3.20	> 13.15	> 0.36	> 2.39
Low	♂	< 15.79	< 2.42	< 12.79	< 0.29	< 1.87
	♀	< 15.67	< 2.45	< 12.50	< 0.29	< 1.96

Sex-specific 'high' and 'low' tertiles for FM%, FMI, FFMI, abdominal subcutaneous fat and visceral fat. Abbreviations: ASF= abdominal subcutaneous fat, FFMI = Fat Free Mass Index, FMI =Fat Mass Index, FM%= fat mass percentage. ♂ = boys, ♀= girls.

Part III

PFAS levels in infancy



Chapter 8

Longitudinal Poly- and Perfluoroalkyl substances (PFAS) levels in Dutch infants

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Abstract

Background: Per- and polyfluoroalkyl substances (PFAS) are a potential hazard for public health. These man-made chemicals are non-degradable with an elimination half-life of multiple years, causing accumulation in the environment and humans. Rodent studies demonstrated that PFAS are harmful, especially when present during the critical window in the first months of life. Because longitudinal data during infancy are limited, we investigated longitudinal plasma levels in infants aged 3 months and 2 years and its most important determinants.

Methods: In 369 healthy term-born Dutch infants, we determined plasma PFOS, PFOA, PFHxS, PFNA and PFDA levels at age 3 months and 2 years, using liquid chromatography-electrospray-ionization-tandem-mass-spectrometry (LC-ESI-MS/MS). We studied the associations with maternal and child characteristics by multiple regression models.

Results: At age 3 months, median plasma levels of PFOS, PFOA, PFHxS, PFNA and PFDA were 1.48, 2.40, 0.43, 0.23 and 0.07 ng/mL, resp. Levels decreased slightly until age 2 years to 1.30, 1.81, 0.40, 0.21 and 0.08 ng/mL, resp. Maternal age, first born, Caucasian ethnicity and exclusive breastfeeding were associated with higher infant's plasma levels at age 3 months. Levels at 3 months were the most important predictor for PFAS levels at age 2 years. Infants with exclusive breastfeeding during the first 3 months of life (EBF) had 2-3 fold higher levels throughout infancy compared to infants with exclusive formula feeding (EFF), with PFOA levels at 3 months 3.72 ng/mL versus 1.26 ng/mL and at 2 years 3.15 ng/mL versus 1.22 ng/mL, respectively.

Conclusions: Plasma PFAS levels decreased only slightly during infancy. Higher levels at age 3 months were found in Caucasian, first-born infants from older mothers and throughout infancy in EBF-infants. Our findings indicate that trans-placental transmission and breastfeeding are the most important determinants of PFAS exposure in early life.

Introduction

Per- and polyfluoroalkyl substances (PFAS) consist of >3000 man-made chemicals, produced since the 1950s. Because of their water-, dirt- and grease-repellent quality, they are used in a variety of consumer and industrial products, like outdoor clothing, baking paper, food-packing materials, non-stick coating in pans and fire-fighting foam, among other things [1, 2]. PFAS can simply migrate into the environment. Most PFAS are not biodegradable, can easily spread through the air and water and can be taken up by plants and animals. PFAS are hydrophobic and have an increased affinity with proteins in the human body. They have a tendency to accumulate in humans, because of their very long elimination half-life up to 8.5 years [1, 2].

A wide range of adverse effects of PFAS has been described in adults, like liver damage, increased risk of testicular and kidney cancer, thyroid disorders and changes in plasma lipid concentrations [1-3]. The most sensitive effect identified in children is a decreased response to tetanus, hepatitis B and diphtheria vaccinations due to prenatal PFAS exposure [4, 5]. Rodent studies show concerning developmental effects in offspring that was exposed to high levels of PFAS during pregnancy or in early life. Effects consist of a wide range of developmental effects, such as growth restriction, altered behavioral patterns and endocrine disruption [2, 6]. However, the very limited studies in infants to confirm or refute these findings show conflicting conclusions, mainly because of small study populations, cross-sectional designs or short follow-up periods [7-9].

PFAS exposure predominately takes place by inhalation of dust in air or by ingestion of PFAS in drinking water and food, particularly from fish, fruit and eggs [3]. PFAS have been detected in human plasma, cord blood and breast milk, so PFAS can migrate from a mother to her child during the pre- and postnatal period, potentially through trans-placental transmission and breastfeeding [10, 11]. However, knowledge about longitudinal exposure in infants and its most important determinants is limited. Based on data from cord blood and in infants aged > 6 months, it has been postulated that PFOS and PFOA levels increase during infancy and decrease thereafter to 1.5 ng/ml at age 10.5 years [12-14]. However, knowledge about longitudinal plasma levels and its effects in infants is lacking.

Because of the life-long negative effects of PFAS and the very limited data on longitudinal plasma PFAS levels in infants, we evaluated plasma PFOS, PFOA, PFHxS, PFNA and PFDA levels in a large cohort of healthy infants at 3 months and 2 years of age. Secondly, we determined which maternal and infant characteristics

were associated with infant PFAS plasma levels and if infant feeding type was associated with PFAS plasma levels. We hypothesized that infant PFAS plasma levels would decrease during the first 2 years of life and would be associated with maternal characteristics and infant feeding.

Material and Methods

Subjects

This study was embedded in the Sophia Pluto study, a birth cohort study in healthy infants aiming to provide detailed data on body composition trajectories and determinants from early life to childhood [15, 16]. Infants were recruited from several maternity wards in the Rotterdam area, The Netherlands. All participants met the following inclusion criteria: born singleton and term (≥37 weeks of gestation), with an uncomplicated neonatal period. Exclusion criteria were severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation, and any maternal disease or medication that could interfere with fetal growth, including maternal corticosteroids and diabetes mellitus, or known congenital or postnatal disease or intrauterine infection that could interfere with growth. For this study, infants with blood samples at age 3 months and/or 2 years were included. The Medical Ethics Committee of Erasmus Medical Center approved the study and written informed consent was given by all parents or caregivers with parental authority.

Data collection and measurements

Outpatient clinic visits were scheduled at age 1, 3, 6, 9, 12, 18 months and 2 years. Birth data were taken from hospital and midwife records. Maternal characteristics were obtained by interviews and questionnaires. Information about feeding type was recorded during every outpatient clinic visit and through questionnaires. Exclusive breastfeeding (EBF) was defined as receiving only breastfeeding until at least the age of 3 months. Exclusive formula feeding (EFF) was defined as starting exclusive formula feeding before the age of 1 month. Mixed feeding (Mix) was defined as starting with formula feeding next to breastfeeding between 1 and 3 months of age. Living area at birth was based on zip codes and subsequently arranged as east and west Rotterdam.

Blood samples

Between 2013 and 2018 a total of 276 blood samples at age 3 months and between 2015 and 2020 310 blood samples at age 2 years were collected in 369 infants. Capillary blood was collected in EDTA microtubes (BD Microtainer®) from the infant's finger or toe at the study location. Blood was then centrifuged at 4 °C, after which plasma was separated and frozen immediately. Samples were stored at -80°C until analysis.

PFAS analysis

PFAS analysis took place at the Diagnostic Laboratory of Endocrinology, an ISO-15189 certified laboratory, at the Erasmus University Medical Center in Rotterdam, The Netherlands.

Sample preparation

The PFAS plasma levels were measured in EDTA-plasma. 50 µl of plasma, calibrator or QC was mixed with 100 µl of methanol including the internal standard of each PFAS and 150 µl of 50% formic acid. Thereafter, the samples were subjected to an offline solid phase extraction using a WAX µ-elution plate (Waters™, the Netherlands) which was conditioned with methanol and water. After loading of the samples the plate was washed with 50% formic acid and 5% methanol. The PFAS were eluted with 1% of ammonia in methanol and subsequently 1:1 diluted with 10 mM ammonium acetate before analysis.

LC-ESI-MS/MS

After the sample preparation, the different PFAS were measured using liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS) (Acquity UPLC liquid chromatograph and a Xevo-TQ-S Mass Spectrometer (Waters™, the Netherlands)). An Acquity CSH Phenyl-Hexyl column was used for separation with a gradient utilizing 10 mM ammonium-acetate as solvent A and 10 mM ammonium acetate in methanol as solvent B. A 10-minute linear gradient was used with 50%A:50%B as initial condition, leading to 37%A:63%B after 10 minutes before re-equilibrating the column for the next injection. Mass spectrometer setting were: capillary voltage 1.00 kV in the negative mode, cone voltage 40 V, desolvation temperature 450˚C at a gasflow of 750 L/hr and cone gasflow of 200 L/hr. Argon was used as collision gas at a flowrate of 0.19 ml/min. The targeted PFAS were total and linear Perfluorooctane sulfonic acid (PFOS & LinPFOS), total and linear Perfluorooctanoic acid (PFOA & LinPFOA), total and linear Perfluorohexane sulfonic acid (PFHxS & LinPFHxS), Perfluorononanoic acid (PFNA) and Perfluorodecanoic acid (PFDA). Two mass transitions were used for each PFAS, whereby the result of samples with an ion-ratio deviating more than 10% from the mean ion-ratio were discarded. Quantification of the PFAS was performed using separate calibration curves and a ^{13}C-labeled internal standard for each PFAS and Masslynx software was used to determine the levels. Between run precision of the assay was ensured by using quality control samples in each batch of samples along with reagent blanks. Between run precision was between 1.9% and 5.1% for all tested PFAS. Absolute- and relative matrix effects were negligible (<5%) and lower limit of

quantification (LLoQ) was set at 0.05 ng/mL for PFNA and PFDA, 0.10 ng/mL for PFHxS and 0.15 ng/mL for PFOS and PFOA. If PFAS level was below the specific LLoQ, the plasma level was considered to be LLoQ/√2.

Statistical analysis

Of all 369 infants participating in the Sophia Pluto study, 276 had a blood sample at age 3 months and 310 at age 2 years, with 267 infants having blood samples at both time points. Reason for absent blood samples was no parental permission for blood sampling or child's resistance. SD-scores for birth length and birth weight and for length, weight and BMI were calculated at every visit were calculated using Dutch references [17] by Growth Analyser RCT software (http://www.growthanalyser.org). Baseline characteristics and body composition measurements are expressed as mean (SD). Not normally distributed values are expressed as median [interquartile range]. Independent student's *t* test was used to determine differences in the baseline characteristics between boys and girls and Wilcoxon signed rank test for differences in plasma PFAS levels between age 3 months and 2 years. Correlations between plasma PFAS levels were determined with Spearman's correlation coefficient.

Multiple linear regression analysis was used to determine associated maternal and child characteristics. PFAS plasma levels at 3 months and 2 years were the dependent variables. Maternal characteristics were ethnicity, education level, living area at delivery, fertility treatment, amenorrhea duration (AD), delivery mode, first born baby, age at delivery, BMI before pregnancy, breastfeeding of any previous children and food intake during pregnancy (amount of vegetables, fruit, meat, fish and eggs per week). Infant variables were sex and infant feeding mode until age 3 months. For PFAS levels at 2 years, ethnicity of the infant, total duration of breastfeeding and PFAS plasma level at 3 were added. Using backward elimination, non-significant characteristics were removed from the model. Since PFAS accumulation changes with time, all multiple linear regression analysis were corrected for blood collection date. Repeated measures ANOVA analysis and Mann-Whitney *U* tests were used to determine differences between infant feeding types.

All statistical tests were performed with SPSS statistical package version 25.0 (SPSS Inc. Chicago, Illinois). Tests were performed two-sided and results were regarded statistically significant if the *p*-value was < 0.05.

Results

Child characteristics are presented in Table 1. Of all infants, 57.7 % was male and 68.8% Caucasian. Characteristics were not different between boys and girls.

PFAS plasma levels during infancy

Median [IQR] plasma levels of PFOS, PFOA, PFHxS, PFNA and PFDA are presented in Table 2 and Figure 1. PFOS, PFOA and PFHxS levels decreased from age 3 months to 2 years, while levels of PFNA and PFDA remained similar (Table 2 and Figure 1). There were no differences in PFAS levels between boys and girls. Because total PFOA and PFHxS levels consisted mostly of the linear isomer, only analyses with total levels are presented from this point onwards.

Table 1. Clinical characteristics in 369 Dutch infants

	Boys	**Girls**	***p*-value**
N	213	156	
Child characteristics			
Ethnicity N(%)			0.158
Caucasian	142 (70.3%)	98 (63.2%)	
Non-Caucasian	60 (29.7%)	57 (36.8%)	
Birth weight SDS	0.33 (1.06)	0.13 (1.20)	0.087
Birth length SDS *	0.70 (1.17)	0.58 (1.21)	0.455
Infant feeding			0.628
EBF	81 (38.0%)	67 (42.9%)	
Mix	74 (34.7%)	49 (31.4%)	
EFF	58 (27.2%)	40 (25.6%)	
Total breastfeeding duration (months)	6.35 (8.3)	7.37 (9.0)	0.270
Pregnancy and delivery characteristics			
Fertility treatment	27 (13.2%)	19 (13.3%)	0.989
Parity	1.58 (1.6)	1.54 (0.77)	0.674
AD (weeks)	39.63 (1.3)	39.73 (1.2)	0.434
Delivery mode N(%)			0.908
Vaginal	148 (69.5%)	111 (71.2%)	
Caesarian section	63 (29.6%)	44 (28.2%)	
Missing	2 (0.9%)	1 (0.6%)	

Table 1. Continued

	Boys	Girls	*p*-value
N	213	156	
Maternal characteristics			
Age (years)	32.80 (4.6)	32.49 (4.7)	0.527
Pre-pregnancy BMI	24.41 (4.6)	24.13 (4.2)	0.569
Ethnicity N(%)			0.515
Caucasian	162 (77.1%)	115 (74.2%)	
Non-Caucasian	48 (22.9%)	40 (25.8%)	
Living area			0.210
East Rotterdam	132 (62.3%)	87 (55.8%)	
West Rotterdam	80 (37.7%)	69 (44.2%)	
Dietary habits (≥ 3 times/ week)			
Vegetables	173 (89.6%)	122 (89.1%)	0.954
Fruit	175 (91.2%)	124 (90.5%)	0.593
Meat	170 (88.5%)	121 (87.7%)	0.259
Fish	5 (2.6%)	9 (6.7%)	0.165
Eggs	24 (12.5%)	24 (17.6%)	

*Data expressed as mean (SD). *Available in 122 boys and 86 girls. Abbreviations: AD = amenorrhea duration BMI = body mass index, N = number of subjects, SDS = standard deviation score.*

Table 3. Multiple linear regression analysis for PFAS plasma levels at age 3 months and 2 years

	PFOS			PFOA		
	B±SE	β	*p*-value	B±SE	β	*p*-value
Age 3 months						
EBF_{3mo}	0.805± 0.08	0.52	<0.001	1.352± 0.13	0.51	<0.001
First born	0.531± 0.12	0.21	<0.001	1.333± 0.21	0.31	<0.001
Maternal age	0.059± 0.01	0.22	<0.001	0.065± 0.02	0.14	0.005
Maternal BMI				-0.055± 0.02	-0.12	0.020
Mother Caucasian	0.644± 0.14	0.21	<0.001			
Living area				0.53± 0.21	0.12	0.013
Fertility treatment	-0.373± 0.18	-0.10	0.040			
AD						
Constant	84.13± 17.4			101.1± 30.0		
Adjusted R^2 (p-value)	0.421 (<0.001)			0.413 (<0.001)		
Age 2 years						
$PFAS_{3mo}$	0.435± 0.04	0.58	<0.001	0.570± 0.03	0.68	<0.001
EBF_{3mo}						
Breastfeeding duration	0.033± 0.05	0.33	<0.001	0.065± 0.01	0.36	<0.001
Child Caucasian				0.392± 0.13	0.11	0.003
Constant	45.90± 12.4			40.4± 18.3		
Adjusted R^2 (p-value)	0.573 (<0.001)			0.734 (<0.001)		

Results of Multiple linear regression analysis, corrected for sample collection date. B±SE = Unstandardized coefficient B and Standard Error. β = standardized coefficient Beta Abbreviations: PFOS = Total Perfluorooctane sulfonic acid, PFOA = Total Perfluorooctanoic acid, PFHxS= Total Perfluorohexane sulfonic acid, PFNA= Perfluorononanoic acid,

	PFHxS			PFNA			PFDA		
	B±SE	β	*p*-value	B±SE	β	*p*-value	B±SE	β	*p*-value
	0.205± 0.04	0.32	0.008	0.104± 0.01	0.53	<0.001	0.029± 0.00	0.44	<0.001
	0.171± 0.06	0.16	0.019	0.065± 0.02	0.20	<0.001	0.013± 0.01	0.12	0.031
	0.015± 0.01	0.14	0.019	0.004± 0.00	0.13	0.010	0.002± 0.00	0.14	0.011
	0.187± 0.07	0.15	0.008						
							0.005± 0.00	0.11	0.041
	8.27± 8.74			10.6± 2.26			3.02± 0.83		
	0.142 (<0.001)			0.357 (<0.001)			0.262 (<0.001)		
	0.388± 0.02	0.69	<0.001	0.392± 0.04	0.47	<0.001	0.254± 0.07	0.21	<0.001
	0.071± 0.02	0.18	0.001						
	0.008± 0.00	0.23	<0.001	0.006± 0.00	0.47	<0.001	0.003± 0.00	0.41	<0.001
	0.082± 0.03	0.12	0.003	0.027± 0.01	0.11	0.021			
	10.2± 4.73			7.54± 1.66			5.07± 0.95		
	0.713 (<0.001)			0.579 (<0.001)			0.326 (<0.001)		

PFDA= Perfluorodecanoic acid. EBF3mo: 1= EFF, 2= mix, 3=EBF. First born: 0= no, 1= yes, Maternal age in years, Maternal BMI in kg/m2, Mother Caucasian: 0=no, 1=yes, Living area: 1= West Rotterdam, 2= East Rotterdam, Fertility treatment: 0= no, 1 = yes. AD= amenorrhea duration in weeks, Breastfeeding duration: total duration of breastfeeding in months (with 2 decimals), Child Caucasian: 0=no, 1=yes.

Table 4. PFAS plasma level and infant feeding

	3 months		
	EBF	**mix**	**EFF**
N	112	93	71
PFOS (ng/mL)	2.44 (0.12)	1.80 (0.13)	0.97 (0.14)
LinPFOS (ng/mL)	1.32 (0.06)	0.91 (0.07)	0.46 (0.08)
PFOA (ng/mL)	3.72 (0.19)	2.97 (0.20)	1.26 (0.22)
LinPFOA (ng/mL)	3.66 (0.18)	2.92 (0.20)	1.22 (0.22)
PFHxS (ng/mL)	0.70 (0.06)	0.47 (0.07)	0.31 (0.07)
LinPFHxS (ng/mL)	0.68 (0.06)	0.45 (0.07)	0.29 (0.07)
PFNA (ng/mL)	0.32 (0.01)	0.25 (0.02)	0.13 (0.02)
PFDA (ng/mL)	0.11 (0.01)	0.09 (0.01)	0.06 (0.01)

Data are presented as estimated marginal means (SD). Levels are presented in ng/mL. N = number of infants. Difference between infant feeding trajectories is the differences between feeding types in PFAS trajectories between age 3 months and 2 years. Abbreviations: PFOS = Total Perfluorooctane sulfonic acid, LinPFOS = linear Perfluorooctane sulfonic acid,

Association with maternal and child characteristics

The results of multiple linear regression analysis are shown in Table 3. At 3 months of age, the models explained 14.2% - 42.1% of variance in PFAS plasma levels. All PFAS levels were higher with increasing maternal age and when the child was first born. For example, when maternal age increased with 1 year, PFOS levels increased with 0.059 ng/mL. Additionally, PFOS levels were higher in case the pregnancy was conceived spontaneously instead of with fertility treatment. Besides, PFOS and PFHxS levels were higher in children of Caucasian mothers. PFOA levels were higher in children of mothers with lower pre-pregnancy BMI. Also, PFOA levels were associated with living area, with infants born in the east of the Rotterdam area having the highest plasma PFOA levels. Lastly, PFDA levels were higher with increasing gestational age. As determinants of linPFOS were similar to those of PFOS, data of only the latter were presented. Maternal education level, breastfeeding of a previous child, delivery mode and infant sex were no determinants of PFAS plasma levels. When studying maternal dietary habits, with correction of the aforementioned characteristics, only the PFDA levels at age 3 months were higher when the mother consumed more meat (β:0.121, p = 0.037). Other PFAS levels were not influenced by maternal dietary habits.

24 months			Difference between infant feeding trajectories from 3 – 24 months		
			p-value		
EBF	**mix**	**EFF**	**EBF vs EFF**	**EBF vs mix**	**EFF vs mix**
120	104	86			
2.08 (0.09)	1.27 (0.09)	0.94 (0.10)	**0.037**	0.326	**<0.001**
1.05 (0.05)	0.62 (0.05)	0.50 (0.06)	**0.001**	0.913	**<0.001**
3.15 (0.16)	1.99 (0.17)	1.22 (0.19)	**0.002**	**0.036**	**<0.001**
3.09 (0.16)	1.94 (0.17)	1.18 (0.19)	**0.001**	**0.033**	**<0.001**
0.66 (0.03)	0.38 (0.03)	0.29 (0.04)	0.314	**0.008**	0.067
0.64 (0.03)	0.36 (0.03)	0.27 (0.04)	0.313	**0.007**	0.074
0.31 (0.01)	0.21 (0.01)	0.16(0.01)	**0.006**	0.138	**<0.001**
0.11 (0.01)	0.09 (0.01)	0.08 (0.01)	**0.002**	0.978	**<0.001**

PFOA = Total Perfluorooctanoic acid, LinPFOA = linear Perfluorooctanoic acid, PFHxS = Total Perfluorohexane sulfonic acid, LinPFHxS = Linear Perfluorohexane sulfonic acid, PFNA = Perfluorononanoic acid, PFDA = Perfluorodecanoic acid. EBF = exclusive breastfeeding until 3 months of age, EFF = exclusive formula feeding until 3 months of age, mix = mixed feeding during the first 3 months of age.

At age 2 years, the models explained 32.6% - 73.4% of the variance in PFAS plasma levels (Table 3). PFAS levels were highly correlated with plasma levels at age 3 months, with correlation coefficients for PFOS, PFOA, PFHxS, PFNA and PFDA being 0.76, 0.82, 0.82, 0.68 and 0.46, resp. (all, p <0.001). PFOA, PFHxS and PFNA levels were higher in Caucasian infants.

Infant feeding

Infants with exclusive breastfeeding until age 3 months (EBF) had 2-3 fold higher PFAS levels throughout infancy compared to infants with exclusive formula feeding (EFF), with PFOA levels at 3 months being 3.72 ng/mL versus 1.26 ng/mL and PFOS levels being 2.44 ng/mL versus 0.97 ng/mL. At 2 years, PFOA levels were 3.15 ng/mL versus 1.22 ng/mL and PFOS levels were 2.08 ng/mL versus 0.94 ng/mL (Table 4). Even when corrected for other characteristics by multiple linear regression analysis , EBF-infants had 1.35 ng/mL higher PFOA level compared to mix-fed infants and 2.70 ng/mL compared to EFF-infants (Table 3) and at age 2 years, PFOA levels were 0.065 ng/mL higher with each month longer duration of breastfeeding (Table 3).

All PFAS plasma levels at age 3 months were highest in EBF-infants and lowest in EFF-infants (all, $p<0.001$) (Figure 1 and Table 4). At age 2 years, this difference had persisted (all, $p<0.04$) (Figure 1 and Table 4). The PFAS trajectories were different between infant feeding types (Figure 1 and Table 4). Plasma PFOA and PFOS levels decreased between age 3 months and 2 years in infants with EBF and mix feeding, but remained low and similar in EFF-infants, while PFNA and PFDA levels remained higher and similar in infants with EBF and increased in infants with EFF.

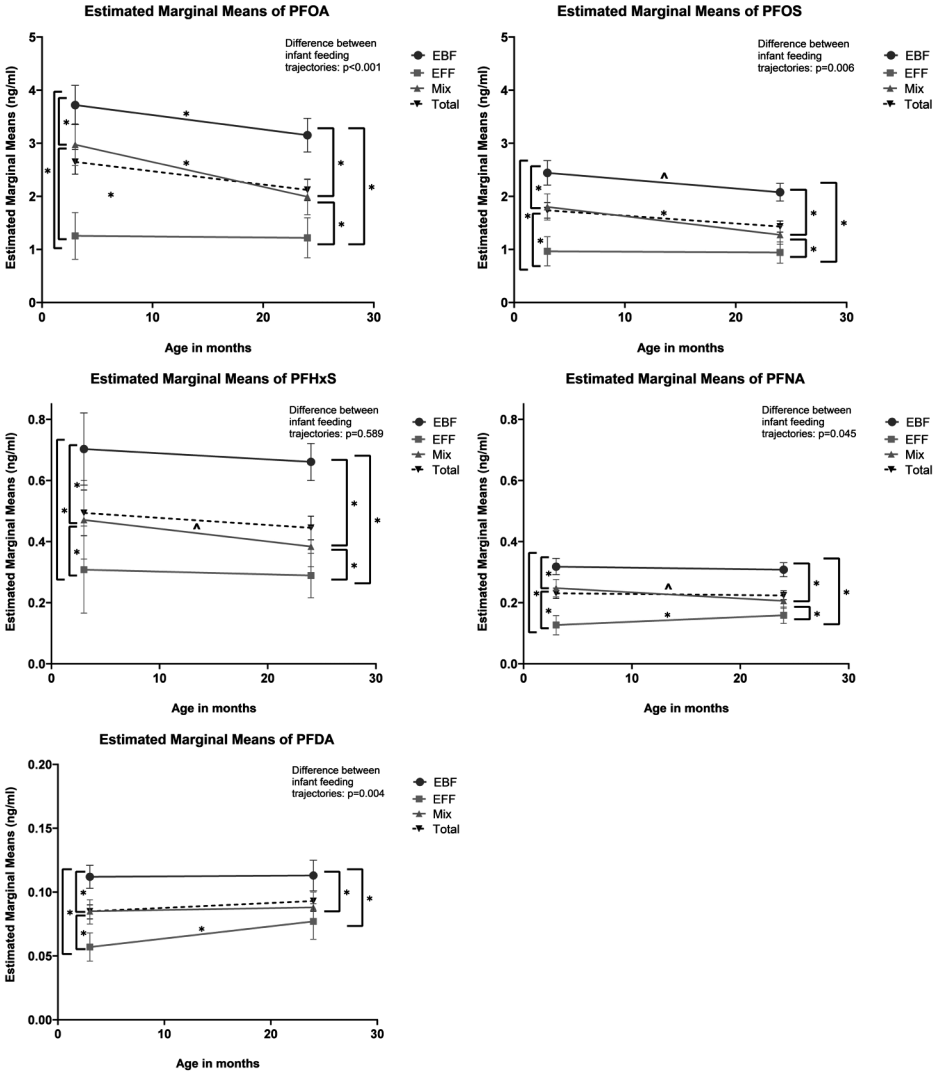

Figure 2 Figure 1. PFAS plasma levels during infancy. *Estimated marginal means with 95% confidence interval of infant plasma level of PFOS = Total Perfluorooctane sulfonic acid, PFOA = Total Perfluorooctanoic acid, PFHxS= Total Perfluorohexane sulfonic acid, PFNA= Perfluorononanoic acid, PFDA= Perfluorodecanoic acid at 3 and 24 months for the total group and categorized by infant feeding type., * p<0.001, differenceas between infant feeding types at 3, 24 and* Δ 3-24 months. *^ p<0.05, differences between infant feeding types at 3, 24 and* Δ 3-24 months. Difference between infant feeding trajectories is the differences between feeding types in PFAS trajectories between age 3 months and 2 years.

Discussion

We present longitudinal PFAS plasma levels at 3 months and 2 years of age in a large group of healthy Dutch infants. Older maternal age, first born, Caucasian ethnicity, exclusive breastfeeding and longer total breastfeeding duration were associated with higher infant's PFAS plasma levels at age 3 months. PFAS plasma levels decreased during infancy, but levels at 3 months of age were an important predictor for PFAS levels at age 2 years. Infants with exclusive breastfeeding during the first 3 months of life had the highest PFAS concentrations throughout infancy.

Our longitudinal PFAS levels until age 2 years show a decrease, which is in line with the very limited reported longitudinal data [12, 13]. In The Netherlands, PFAS plasma levels have only been reported for adults. The median PFOA level of an average Dutch adult was 3.4 ng/mL in 2016 [18], which is almost two-fold higher compared to the median level we measured in infants at age 2 years. Our findings show that PFAS plasma levels decrease during infancy, but might eventually increase later in life.

At 3 months of age, PFAS plasma levels were associated with several maternal characteristics. To our knowledge, we are the first to describe the association between maternal characteristics and infant PFAS plasma levels in early life. Infant plasma levels were higher with increasing maternal age and in first-born infants. Additionally, we found that PFOS and PFHxS levels at age 3 months were higher in children from Caucasian mothers. It has been described that gestational PFAS levels increase with maternal age [19] and that ethnicity was a major determinant of PFAS levels in midlife women [20]. Also, PFAS levels were reported to be lower in multiparous women, suggesting child-birth is an important PFAS elimination route [19]. We now show that these factors are determinants of PFAS levels in early life, potentially because older primiparous Caucasian mothers had more PFAS accumulation and subsequently more trans-placental transmission, resulting in higher PFAS levels in their infants.

In children, adolescents and adults, associations between PFAS plasma levels and high BMI have been inconsistent, with some describing positive associations and others no association [9, 21, 22]. However, the association between maternal BMI and infant PFOA levels have not yet been reported. Our findings show that infant PFOA levels were lower with increasing maternal pre-pregnancy BMI. High levels of several PFAS were thought to influence male and female fertility, but evidence has been inconsistent [23]. Our findings suggest that those who required fertility treatment, did not have infants with higher PFOS levels. Although, pregnancy duration was previously

not associated with PFAS plasma levels in mothers [24], our findings show that a longer pregnancy duration (AD) was slightly associated with a higher PFDA level in infants. This could be the result of a longer trans-placental transmission period, from mother to her child during pregnancy, with eventually more accumulation in the infant, resulting in higher infant plasma levels. It is, however, unclear why this association was only found for PFDA. It warrants further research.

We found that infant sex and maternal education levels or dietary habits were not associated with infant PFAS levels. In pregnant women, results about the influence about education level and dietary habits on their PFAS levels have been inconsistent. Some described an association between lower education levels and higher PFAS levels [25], while others reported the opposite, partly based on dietary habits [26]. PFAS can be ingested by the consumption of several products, especially fish, fruit, vegetables and eggs [3]. However, in pregnant women the change in PFAS levels due to the consumption of these products resulted in only small and non-trivial changes in PFAS levels [26], which might have led to attenuated trans-placental transmission and could, therefore, explain why we did not find an association between maternal dietary habits and infant PFAS levels.

Lastly, infants born in the eastern area of Rotterdam had higher PFOA levels compared to infants born in the west. Rotterdam is a large city, located near a large industrial and harbor region in The Netherlands. In Dordrecht, about 20 km south-east from Rotterdam, adult residents have higher median plasma levels of PFOA (10.2 ng/mL), compared to those in other regions (3.4 ng/mL), because of a local PFAS-producing chemical company, which produced PFOA until 2012 [18]. In a radius of 50 km around this company PFOA remained present in higher levels in soil and groundwater compared to 150 other areas in The Netherlands [27, 28]. The higher PFOA levels in infants born in East Rotterdam might potentially be the result of more PFAS accumulation in mothers living closer to the chemical company with subsequently more trans-placental transmission, resulting in higher PFAS levels in their infants. Despite the discontinuation of PFOA production by this company in 2012, our findings suggest that PFOA levels in infants born closer to this company remain higher. As this could have potentially negative consequences throughout life, further research is mandatory.

At age 2 years, PFAS plasma levels were highly associated with levels at age 3 months. This can be explained by the very long elimination half-life in humans, ranging from 1.7-3.2 years for PFNA, 3.8 years for PFOA, 5.4 years for PFOS and up to 8.5 years for PFHxS [29, 30]. Our findings show that PFAS plasma levels are measurable in considerable amounts in early life.

Our multiple linear regression models, at the age of 3 months primarily consisting primarily of maternal characteristics and at age 2 years mainly of the PFAS plasma levels at 3 months, explained a maximum of 42.1% and 73.4% of the variance in infant's PFAS levels at age 3 months and 2 years, resp. These findings suggest that PFAS levels in infants are also determined by maternal PFAS levels during pregnancy and their vertical transmission from mother to her child by migrating through the placenta. It was postulated that linear isomers are less likely to cross the placental barrier [31], but our findings show that PFOA and PFHxS levels in early life consist predominately of linear isomers. As these levels were not different between breastfed and formula fed infants, this suggests that linear isomers are capable of crossing the placental barrier.

We found exclusive breastfeeding until age 3 months and total duration of breastfeeding to be important determinants for PFAS levels throughout infancy. This is an important finding, as exclusive breastfeeding is highly recommended by the World Health Organization, due to its health benefits, in terms of growth outcomes, protection against obesity, infections and allergies [32-34]. Exclusive breastfeeding has been associated with higher PFAS plasma levels in children aged 3 and 8 years [19] and a longer duration of breastfeeding was associated with higher PFAS levels at 5 years of age [12]. We now add that PFAS plasma levels at 3 months are already 2-3 times higher in exclusively breastfed infants compared to mixed fed and EFF-infants and that this remains present until at least 2 years of age. In fact, infants with EBF had similar PFAS plasma levels as Dutch adults [18]. These findings show that breastfeeding is an important PFAS exposure pathway in infants. This is particularly concerning because the first months of life are known to be a critical window for adiposity programming and an important period for the programming of growth, neurodevelopment and immune system. Our findings could, therefore, indicate that infants with exclusive breastfeeding in the first months of life and those with a longer total duration of breastfeeding are more prone to the potential adverse developmental effects of PFAS in early life, such as growth restriction, increased adiposity, altered behavioral patterns, endocrine disruption and decreased vaccination response [2, 4-6], which could limit the health benefits of breastfeeding [32-34]. However, currently the knowledge about safe infant PFAS levels is lacking. Further research is, therefore, mandatory.

In contrast, EFF-infants had the lowest PFAS plasma levels, which remained low until 2 years of age. Only one research group measured PFAS in infant formula and reported low PFAS levels [35]. It has been calculated that approximately 5% of the total oral PFAS exposure can be attributed to drinking water in The Netherlands [36].

Our findings suggests that exposure to PFAS due to formula feeding is negligible during infancy and that exclusive formula feeding contributes to only minor PFAS accumulation between age 3 months and 2 years, especially compared with exclusive breastfeeding.

The PFOS and PFOA levels in our cohort were lower compared to 3 other studies conducted in Scandinavia and Germany, who reported these levels in infants aged 6 – 19 months, ranging from 3.0 – 24.0 ng/mL and 4.6 – 8.2 ng/mL, resp. [12-14]. However, these study populations were smaller compared to ours, consisted predominantly of breastfed infants and were conducted between 1997 and 2010. Since several PFAS have been enlisted as Persistent Organic Pollutants (POPs) by the Stockholm convention in 2009, guidelines regarding the reduction of these chemicals have been installed and production of PFOS and PFOA have been phased out from industrial production lines in Europe ever since [37, 38]. Also, the European Food Safety Authority (EFSA) adjusted the safety thresholds for PFAS intake through food [3]. We collected blood samples between 2013-2020, which could explain why we measured lower plasma levels compared to other research groups.

The strength of our study is the large collection of longitudinal blood samples in a prospective cohort of healthy Dutch infants in which PFAS plasma analyses were performed, using a high quality measuring method. We acknowledge some limitations, the blood samples were collected during two 5-year periods between 2013-2020. Over the last years, health concerns with regard to PFAS have been risen, guidelines to phase out the production of several PFAS have been set up and, therefore, PFAS levels are likely to decrease with time. We, therefore, have adjusted the multiple linear regression analyses for collection date. Also, plasma level trajectories between age 3 months and 2 years were not different in infants born in the first and last year of the study period and the time between PFAS sampling in the same child is shorter than the elimination half-life. We, therefore, consider the influence of the sampling period on our findings on PFAS trajectories in infancy and its determinants to be small. Also, we did not collect maternal blood samples, which could have potentially given more detailed insight in the trans-placental transmission of PFAS. However, we measured the infant's PFAS levels already at 3 months of age.

Conclusion

In conclusion, Dutch infants aged 3 months have considerable PFAS plasma levels, which only slightly decrease until age 2 years. PFAS levels at age 3 months and 2 years were highly correlated. Higher PFAS levels were especially found in first-born infants of older primiparous Caucasian mothers. Infants with exclusive breastfeeding for the first 3 months of life had 2-3 fold higher longitudinal PFAS levels compared to exclusive formula fed infants. Our findings indicate that trans-placental and breastfeeding transmission are the most important determinants of PFAS exposure in early life, with potentially life-long effects, which require further research.

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Author contributions: AHK was in charge of designing the study. IvB, KdF and AHK were in charge of the cohort, design, and collecting of the data and samples. BvZ and SvdB were in charge of the design of the laboratory method. BvZ conducted all PFAS analysis. IvB performed the statistical analysis. Drafting the manuscript was primarily done by IvB under supervision of AHK. All authors were involved in writing the manuscript and had final approval of the submitted version.

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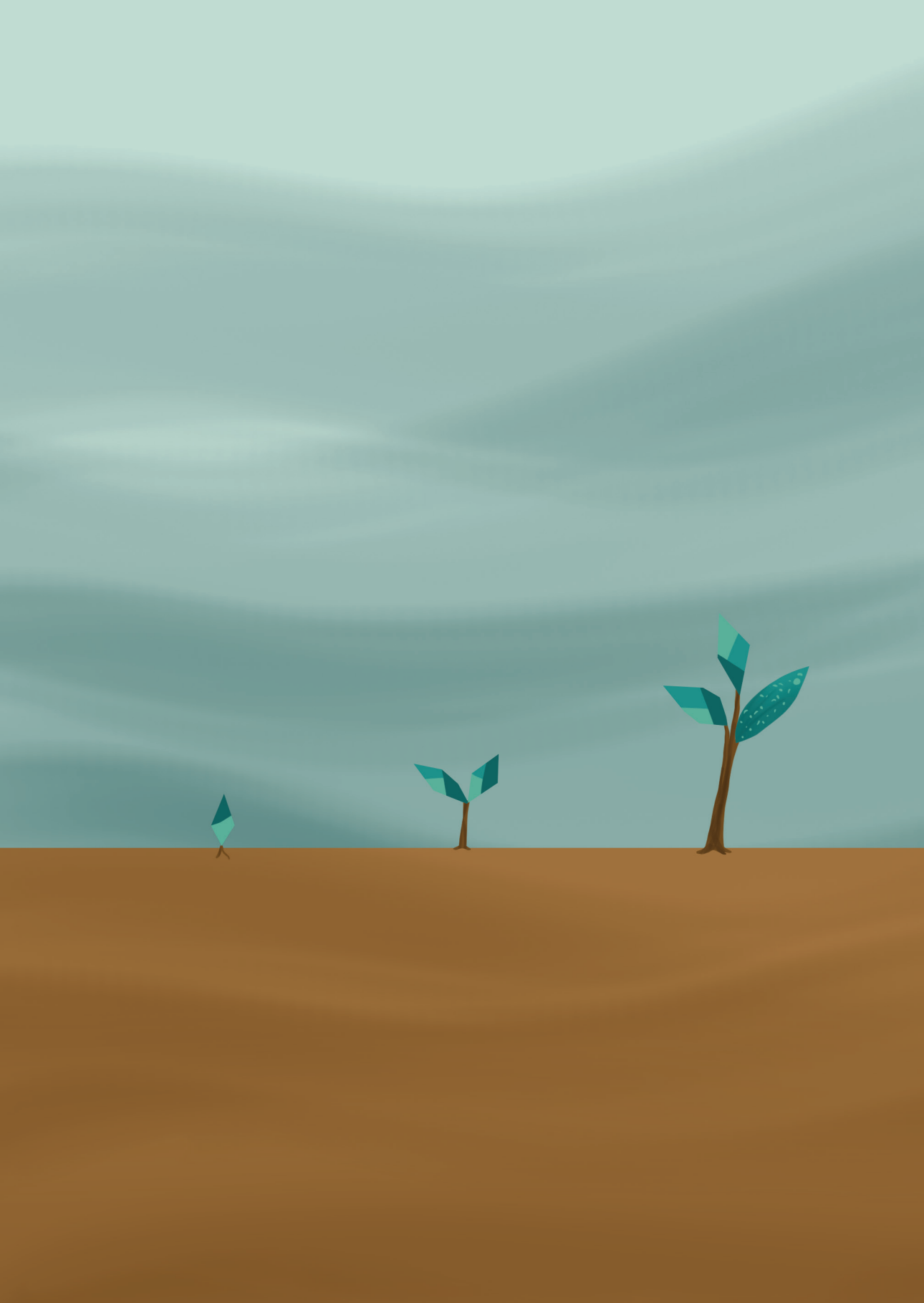

Chapter 9

Poly- and Perfluoroalkyl substances (PFAS) exposure through infant feeding in early life

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Abstract

Background: Per- and polyfluoroalkyl substances (PFAS) are non-degradable, man-made chemicals with an elimination half-life of multiple years, causing accumulation in the environment and humans with potential harmful effects. However, longitudinal PFAS levels in human milk, daily PFAS intake and the association with infant plasma PFAS levels have never been reported. We investigated longitudinal PFOA and PFOS levels in human milk and the daily PFAS intake through infant feeding in the first 3 months of life, the most important determinants and the correlation with PFAS plasma levels at age 3 months and 2 years.

Methods: In 372 healthy term-born Dutch infants, we determined PFOA and PFOS levels in human milk given at age 1 and 3 months, in 6 infant formula brands and in infant plasma at 3 months and 2 years, using Liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS). We studied the associations between daily PFAS intake and predictive characteristics by multiple regression models.

Results: PFOA and PFOS levels in human milk decreased between 1 and 3 months after delivery, regardless whether breastfeeding was given exclusively (EBF) or in combination with formula feeding. PFOA and PFOS could not be detected in any formula feeding. Daily PFAS intake (ng/kg) at age 3 months was highest in EBF-infants. Higher amount of human milk, older maternal age, lower parity and first-time breastfeeding were associated with higher daily intake. Daily PFAS intake in early life was strongly correlated with PFAS plasma levels at age 3 months and 2 years ($R=0.642$ - 0.875, $p<0.001$).

Conclusions: Human milk contains PFOA and PFOS, in contrast to formula feeding. Daily PFOA and PFOS intake in early life is highest in exclusively breastfed infants and it is highly correlated with infant's plasma levels throughout infancy. Our findings show that breastfeeding is an important PFAS exposure pathway in the first months of life, with unknown but potential adverse effects. This warrants further research, lowering of thresholds for PFAS intake and a further phase out and preferably a ban on PFAS production.

Introduction

Endocrine disrupting chemicals (EDCs) are non-degradable chemicals, which can accumulate in humans. They interfere with endocrine processes and can cause adverse effects on perinatal, neurodevelopmental, metabolic and reproductive outcomes. Especially when exposure occurs during susceptible periods of human development, such as early life [1]. Multiple EDCs have been thoroughly studied, such as Bisphenol A and phthalates. However, human data about another class of EDCs, per- and polyfluoroalkyl substances (PFAS), are very limited.

PFAS are a group of >3000 man-made chemicals, produced since the 1950s. They are used in a variety of consumer and industrial products. Because of their water-, dirt- and grease-repellent quality, they are used in food-packing materials and non-stick coating in pans, among other things. PFAS can easily migrate into the environment. Most PFAS are not degradable and can spread through the air and water. They have a tendency to accumulate in humans, because of their very long elimination half-life up to 8.5 years [2, 3]. Rodent studies show concerning effects in offspring that was exposed to high levels of PFAS during pregnancy or in early life. These adverse effects consist of a wide range of developmental effects, such as growth restriction, altered behavioral patterns and endocrine disruption [3, 4]. Besides for a decreased vaccination response [5], human studies to confirm or refute other adverse effects are scarce and have conflicting conclusions, partly because of small study populations and short or even lack of follow-up periods [6-8].

In contrast to other EDCs, PFAS are hydrophobic and have an increased affinity with proteins in the human body. PFAS have been detected in human serum and cord blood, so PFAS can migrate from mother to child during the prenatal period [9, 10]. Later in life, PFAS can be taken up by inhalation of dust or by ingestion of PFAS in drinking water and food, particularly from fish, fruit and eggs [11]. To avoid potential adverse effects, the European Food Safety Authority (EFSA) lowered the tolerable weekly intake (TWI) through food in 2020 to 4.4 ng/kg for PFOA, PFOS, PFHxS and PFNA combined [11]. However, data on PFAS exposure in infancy through infant feeding are very scarce. In infant formula feeding, PFAS levels are reported to be low [12]. In human milk, PFAS are probably transferred over the mammary epithelial membrane through both protein binding and direct membrane transport mechanisms [13]. The highest PFAS levels have been measured in human milk of primiparous woman who lactate for the first time, especially in highly industrialized countries [14, 15]. The influence of other factors,

such as maternal diet and macronutrient composition of the milk have not been elucidated yet. Also, longitudinal PFAS levels in human milk and daily PFAS intake through infant feeding and their association with infant plasma PFAS levels have never been reported in a large cohort of healthy infants.

We, therefore, evaluated PFAS levels in formula feeding and longitudinally in human milk and plasma in a large group of healthy infants aged 1 months, 3 months and 2 years. Secondly, we studied the intake of PFOA and PFOS per kilograms body weight per day through infant feeding and if maternal (dietary) characteristics and human milk macronutrients were associated with infant's daily PFOA and PFOS intake. Lastly, we investigated the correlation between daily PFAS intake and plasma levels during infancy. We hypothesized that PFAS levels in formula feeding would be lower compared to human milk and that the daily PFAS intake in early life is, therefore, highest in infants with exclusive breastfeeding. In addition, we hypothesized that PFAS intake through infant feeding would be correlated with infant's plasma levels at age 3 months and 2 years.

Material and Methods

Subjects

This current study was embedded in the Sophia Pluto study, a birth cohort study in healthy infants, aiming to provide detailed data on body composition trajectories and determinants from early life to childhood [16, 17]. Infants were recruited from several maternity wards in the Rotterdam area, The Netherlands. All participants met the following inclusion criteria: born singleton and term (≥37 weeks of gestation). Exclusion criteria were: known congenital or postnatal disease or intrauterine infection that could interfere with growth, severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation, and any maternal disease or medication use that could interfere with fetal growth, including maternal corticosteroids and diabetes mellitus. For this sub-study, infants with blood samples at 3 months and/or at 2 years were included. The Medical Ethics Committee of Erasmus Medical Center approved the study and written informed consent was given by all parents or caregivers with parental authority.

Data collection and measurements

Study visits were scheduled at age 1, 3, 6, 9, 12, 18 months and 2 years. Birth data were taken from hospital and midwife records. Maternal characteristics and dietary characteristics were obtained by interviews and questionnaires. Information about feeding type was recorded during every visits and through questionnaires. Exclusive breast feeding (EBF) was defined as receiving solely breastfeeding until at least the age of 3 months. Exclusive formula feeding (EFF) was defined as starting solely infant formula before the age of 1 month. Mix feeding (mix) was defined as starting with formula feeding between 1 and 3 months of age. Living area at birth was based on zip codes.

Sample collections

Between 2013-2018, a total of 124 and 133 human milk samples were collected at infant's age 1 and 3 months, resp. Mothers were instructed to collect hindmilk, immediately store the sample at -20˚C and transport the sample to the study location in an isolated bag containing cooling elements. During the study visits, a total of 276 and 310 infant blood samples were collected at age 3 months and 2 years, resp. Capillary blood was collected in EDTA microtubes (BD Microtainer®) from the infant's toe or finger. Blood was then centrifuged at 4 °C, after which plasma was separated and frozen immediately. All human milk and blood samples were stored at -80˚C until analysis.

Infant formula

PFAS analysis was also performed in standard formula for infants aged 0-6 months of the 6 most used brands in The Netherlands; *Nutrilon, Hero, Kruidvat, Etos, Albert Heijn* and *Holle* (biologic goat's milk). Thirty mL of each brand of formula was prepared twice according to the enclosed prescription, with bottled water and tap water.

PFAS analysis

PFAS analysis took place at the Diagnostic Laboratory of Endocrinology, an ISO-15189 certified laboratory, at the Erasmus University Medical Center in Rotterdam, The Netherlands.

Sample preparation

The PFAS plasma levels were measured in human milk, infant formula, and EDTA-plasma. 500 µl of milk, calibrator or QC was mixed with 1000 µl of methanol including the internal standard of each PFAS and 100 µl of 100% formic acid. Or 50 µl of plasma, calibrator or QC was mixed with 100 µl of methanol including the internal standard of each PFAS and 150 µl of 50% formic acid. Thereafter, the samples were subjected to an offline solid phase extraction using a WAX µ-elution plate (Waters™, the Netherlands) which was conditioned with methanol and water. After loading of the samples the plate was washed with 5% methanol plus 10% formic acid for milk and 50% formic acid for plasma. The PFAS were eluted with 1% of ammonia in methanol and subsequently 1:1 diluted with 10 mM ammonium acetate before analysis.

LC-ESI-MS/MS

After the sample preparation, the different PFAS were measured using liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS) (Acquity UPLC liquid chromatograph and a Xevo-TQ-S Mass Spectrometer (Waters™, the Netherlands)). An Acquity CSH Phenyl-Hexyl column was used for separation with a gradient utilizing 10 mM ammonium-acetate as solvent A and 10 mM ammonium acetate in methanol as solvent B. A 10-minute linear gradient was used with 50%A:50%B as initial condition, leading to 37%A:63%B after 10 minutes before re-equilibrating the column for the next injection. Mass spectrometer setting were: capillary voltage 1.00 kV in the negative mode, cone voltage 40 V, desolvation temperature 450˚C at a gasflow of 750 L/hr and cone gasflow of 200 L/hr. Argon was used as collison gas at a flowrate of 0.19 ml/min. The targeted PFAS were total and linear Perfluorooctane sulfonic acid (PFOS & LinPFOS), total and linear Perfluorooctanoic acid (PFOA & LinPFOA), total and linear Perfluorohexane sulfonic

acid (PFHxS & LinPFHxS), Perfluorononanoic acid (PFNA) and Perfluorodecanoic acid (PFDA). Two mass transitions were used for each PFAS, whereby the result of samples with an ion-ratio deviating more than 10% from the mean ion-ratio were discarded. Quantification of the PFAS was performed using separate calibration curves and a ^{13}C-labeled internal standard for each PFAS and Masslynx software was used to determine the levels. Between run precision of the assay was ensured by using quality control samples in each batch of samples along with reagent blanks. Absolute- and relative matrix effects were negligible (<5%). The lower limit quantification (LLoQ) for PFOA and PFOS was set at 0.015 ng/mL in human milk and 0.15 ng/mL in infant plasma. Other PFAS could not be detected (levels < LLoQ) in (human) milk. Precision in plasma was between 1.9% and 2.9% for both tested PFAS. Precision in human milk was between 7.6% and 10.4% for both PFAS. If PFAS level was below the specific LLoQ, the plasma level was considered to be LLoQ/√2.

Human milk macronutrient analysis

Analysis of human milk macronutrients was performed in 180 samples at Erasmus University Medical Center in Rotterdam, The Netherlands [18]. Samples were warmed to 40°C and homogenized using an ultrasonic processor (MIRIS, Uppsala, Sweden). Analysis of the concentrations of fat, carbohydrate, true protein and energy was performed in three-fold, using a Human Milk Analyzer (HMA, MIRIS, Uppsala, Sweden). All samples were analyzed with the same device by one person. The device was used, cleaned and calibrated according to the manufacturer's protocol. The intra-assay coefficients of variance (mean (95% confidence interval)) were 1.1 (1.0 - 1.2)% for fat, 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. Samples with protein values of <0.5g/100 mL were considered of poor quality and were excluded from further analysis. Samples were divided in tertiles based on containing 'high', 'middle' and 'low' concentrations of macronutrients.

Daily intake

For each child, PFAS intake per kilogram body weight per day (daily PFAS intake), through infant feeding was calculated as the product of the concentration of PFAS (ng/mL) and the amount of infant feeding per day (mL) divided by the weight of the child (kg). When the amount of infant feeding was unknown, the average daily amount of infant feeding was assumed (150 ml/kg/day).

Statistical analysis

SD scores for birth length and birth weight were calculated to correct for AD and sex [19] and calculated by Growth Analyser RCT software http://growthanalyser.org. Baseline characteristics are expressed as mean (SD). Independent student's *t* test

was used to determine differences in the baseline characteristics . Not normally distributed values are expressed as median [interquartile range]. Mann-Whitney *U* test was used to determine differences in concentrations between high and low macronutrient content. Correlations between daily PFAS intake and infant plasma level were determined with Spearman's correlation coefficient.

Multiple linear regression analysis was used to determine associated maternal, child and intake characteristics. Daily PFAS intake (ng/kg/day) at 1 and 3 months were the dependent variables. Maternal characteristics were ethnicity, education level, living area at childbirth, fertility treatment, amenorrhea duration (AD), parity, delivery mode, maternal age at birth, maternal BMI before pregnancy, history and total duration of breastfeeding in a previous child, dietary habits during pregnancy (amount of vegetables, fruit, meat, fish and eggs per week) and human milk macronutrients (fat, protein and carbohydrates). Child and intake variables were sex and amount of formula feeding at age 1 and 3 months. Using backward elimination, non-significant characteristics were removed from the model.

All statistical tests were performed with SPSS statistical package version 25.0 (SPSS Inc. Chicago, Illinois). Tests were performed two-sided and results were regarded statistically significant if the *p*-value was < 0.05.

Results

Infant characteristics are presented in Table 1. Of all infants, 57.7 % was male and 68.8% Caucasian. Besides a small difference in AD, there were no differences in clinical characteristics between infants with exclusive breast-, formula or mixed feeding.

PFAS levels in infant feeding

Median [IQR] human milk of PFOA and PFOS are presented in Table 2. Other targeted PFAS could not be detected in human milk (levels < LLoQ). At age 1 month, the median [IQR] PFOA and PFOS levels in human milk were 0.043 [0.03 - 0.06] ng/mL and 0.035 [0.02 - 0.05] ng/mL, resp. PFOA and PFOS levels decreased to 0.036 [0.03 - 0.05] ng/mL and 0.031 [0.02 - 0.04] ng/mL at age 3 months, resp. (all, p <0.001). The total level of PFOA consisted almost entirely of the linear isomer, while of the total PFOS 2/3 consisted of the linear isomers. There were no differences in PFAS levels between boys and girls (data not shown).

We also analyzed PFAS levels in the 6 most commonly used infant formula brands, but all PFAS levels were below the LLoQ of 0.015 ng/mL, so PFOA and PFOS could not be measured in any of the infant formula brands. There was no difference in PFAS levels when the milk was prepared with bottled or tap water (data not shown).

In plasma, median [IQR] PFAS levels at age 3 months and 2 years are presented in Table 2. Since PFOA and PFOS could not be measured in formula feeding, only analyses with plasma levels of EBF- and mixed fed infants are presented from this point onwards. Median [IQR] plasma levels of PFOA and PFOS at age 3 months were 3.080 [1.97 - 4.44] ng/mL and 1.829 [1.26 - 2.89] ng/mL, resp. PFOA and PFOS levels decreased to 2.360 [1.57 - 3.28] ng/mL and 1.667 [1.04 - 2.34] ng/mL at age 2 years (all, p <0.001). There were no differences in PFAS plasma levels between boys and girls.

Human milk macronutrients

Human milk samples with high protein content had higher PFOA levels compared to samples with low protein content (Figure 2). PFOA levels were not different between samples containing low and high fat or carbohydrate concentrations. PFOS levels were not different between samples containing low and high fat, protein or carbohydrate concentrations.

Table 1. Clinical characteristics

	EBF	Mix	EFF	*p*-value
N	150	125	97	
Maternal characteristics				
Ethnicity N(%)				0.854
Caucasian	101 (67.3%)	82 (65.6%)	72 (74.2%)	
Black	10 (6.7)	11 (8.8%)	6 (6.2%)	
Asian	1 (0.7%)	1 (0.8%)	1 (1.0%)	
Latin-American	1 (0.7%)	0	1 (1.0%)	
Other & mixed	33 (22.0%)	27 (21.6%)	13 (13.4%)	
Missing	4 (2.7%)	4 (3.2%)	4 (4.1%)	
Delivery mode N(%)				0.726
Vaginal	111 (74.0%)	84 (67.2%)	65 (67.0%)	
Caesarian section	38 (25.3%)	40 (32.0%)	31 (32.0%)	
Missing	1 (0.7%)	1 (0.8%)	1 (1.0%)	
Primiparous N(%)				0.052
Yes	83 (55.3%)	84 (67.2%)	51 (52.6%)	
No	67 (44.7%)	41 (32.8%)	46 (47.4%)	
First-time breastfeeding N(%)				0.074
Yes	83 (55.3%)	84 (67.2%)	57 (58.8%)	
No	62 (41.3%)	37 (29.6%)	26 (26.8)	
Missing	5 (3.3%)	4 (3.2%)	14 (14.4%)	
Child characteristics				
AD (weeks)	39.87 (1.16)	39.60 (1.23)	39.40 (1.30)	**0.011**
Sex N(%)				0.729
Boys	83 (55.3%)	74 (59.2%)	58 (59.8%)	
Girls	67 (44.7%)	51 (40.8%)	39 (40.2%)	
Birth weight SDS	0.26 (1.08)	0.16 (1.17)	0.32 (1.15)	0.569
Birth length SDS *	0.78 (1.10)	0.51 (1.31)	0.54 (1.23)	0.111

*Data expressed as mean (SD). *Available in 92 EBF-, 62 mix- and 56 EFF-infants. Abbreviations: AD= amenorrhea duration, EBF = exclusive breastfeeding until age 3 months, EFF= exclusive formula feeding until age 3 months, Mix: mixed feeding until age 3 months, N=number of subjects, SDS= standard deviation score.*

Table 2. PFAS levels in human milk and infant plasma during infancy

	PFOA	*LinPFOA*	PFOS	*LinPFOS*
Age 1 month				
Human Milk				
N	123	121	118	97
% Detected	99.2%	97.6%	95.2%	78.2%
Level (ng/mL)	0.043 [0.03-0.06]	0.041 [0.03-0.06]	0.035 [0.02-0.05]	0.023 [0.02-0.03]
Daily intake (ng/kg)	6.150 [4.67-9.15]	5.850 [4.35-8.81]	4.875 [3.24-6.90]	3.300 [1.59-4.50]
Age 3 months				
Human Milk				
N	129	127	123	101
% Detected	97.0%	95.5%	92.5%	75.9%
Level (ng/mL)	0.036 [0.03-0.05]	0.034 [0.03-0.05]	0.031 [0.02-0.04]	0.021 [0.02-0.03]
Daily intake (ng/kg)	4.950 [3.28-6.90]	4.650 [3.15-6.60]	4.053 [2.70-5.78]	2.550 [1.59-4.05]
Infant plasma				
N	205	205	205	205
% Detected	100%	100%	100%	100%
Level (ng/mL)	3.080 [1.97-4.44]	3.020 [1.93-4.37]	1.829 [1.26-2.89]	0.931 [0.63-1.45]
Age 2 years				
Infant plasma				
N	224	224	224	224
% Detected	100%	100%	100%	100%
Level (ng/mL)	2.360 [1.57-3.28]	2.306 [1.53-3.23]	1.667 [1.04-2.34]	0.769 [0.50-1.17]

Data are presented as median [IQR]. N= number of measurements above the lower limit of detection. % detected = amount of samples with PFAS level above the lower limit of detection. Abbreviations: PFOA = Total Perfluorooctanoic acid, LinPFOA = linear Perfluorooctanoic acid, PFOS = Total Perfluorooctane sulfonic acid, LinPFOS = linear Perfluorooctane sulfonic acid.

At age 1 and 3 months, the PFAS levels in human milk were not different whether breastfeeding was given exclusively or in combination with formula feeding (mixed feeding) (Figure 1A).

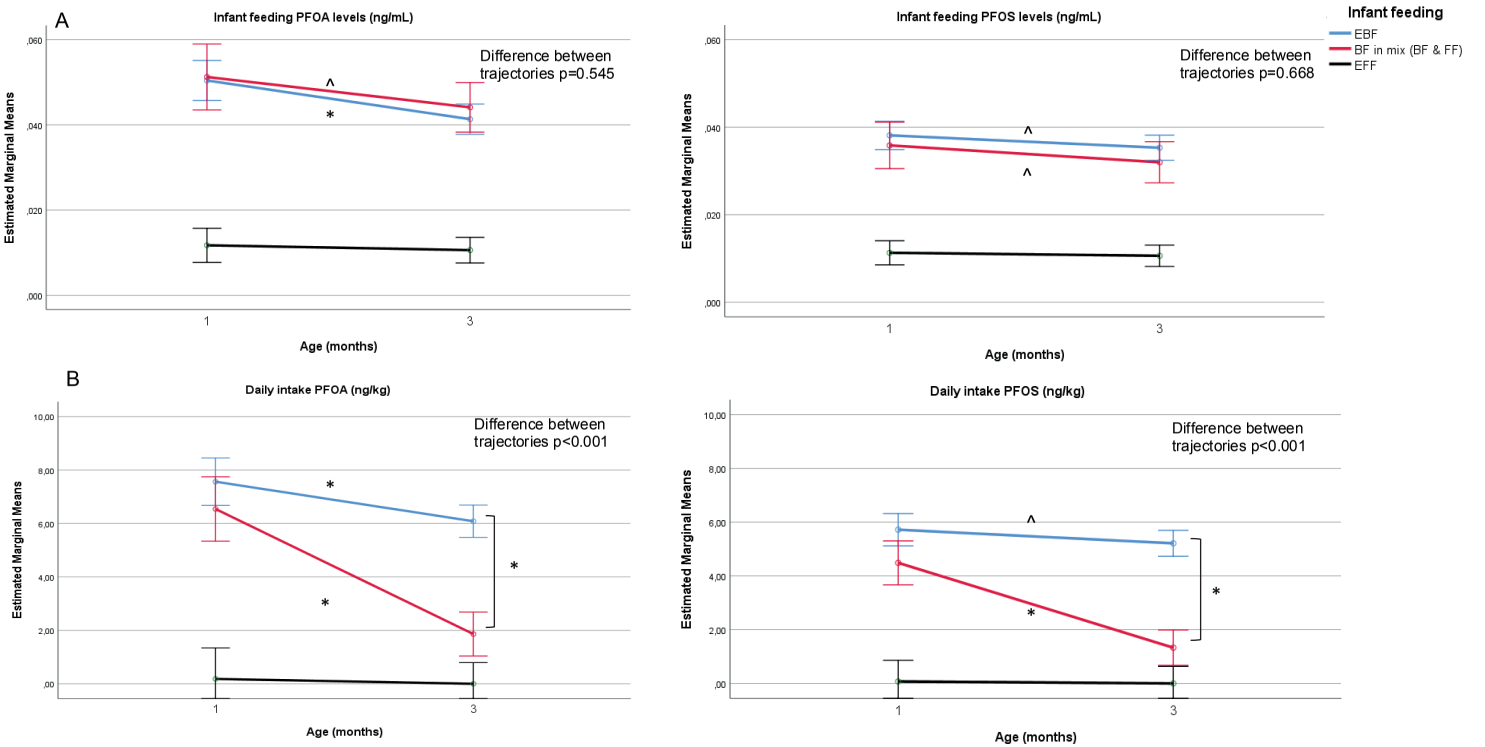

Figure 1. PFAS in early life. A. Estimated marginal means with 95% confidence interval of human milk level of at age 1 and 3 months categorized by infant feeding type. B. Estimated marginal means with 95% confidence interval of daily PFOS and PFOA intake categorized by infant feeding type. *Differences between infant feeding types at 1, 3 and Δ 1-3 months; * p<0.001, ^ p<0.05. Difference between trajectories is the differences between PFAS trajectories in infants with EBF and BF in mix between age 1-3 months. Abbreviations: PFOS = Total Perfluorooctane sulfonic acid and PFOA = Total Perfluorooctanoic acid, EBF = exclusive breastfeeding until age 3 months, EFF= exclusive formula feeding until age 3 months, EBF in mix: breast- and formula feeding until age 3 months*

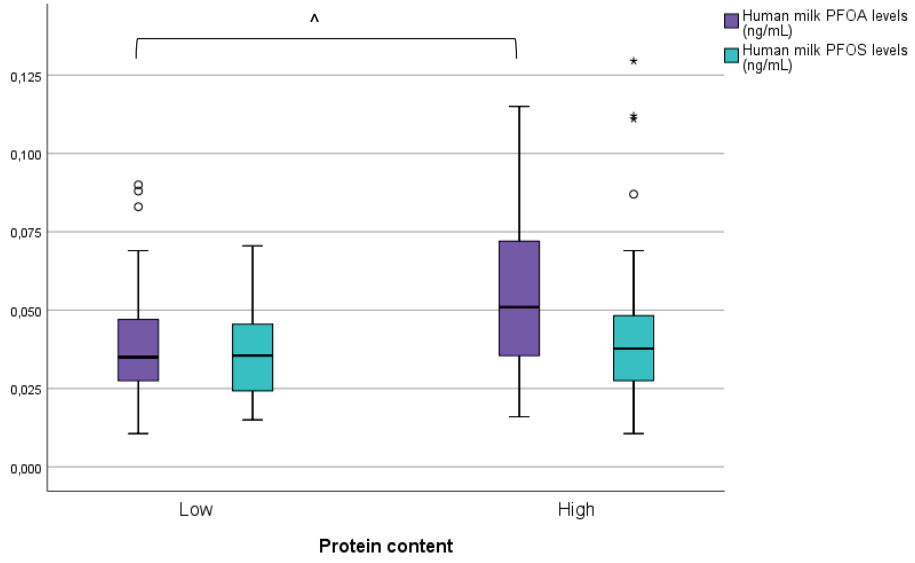

Figure 2. Differences in PFAS levels in human milk samples with low and high protein content.

^= p-value <0.01. *PFOA = Total Perfluorooctanoic acid, PFOS = Total Perfluorooctane sulfonic acid.*

Daily PFAS intake

Intake per kg per day (daily intake) of PFOA and PFOS was highest in EBF-infants (Figure 1B). At age 1 month, estimated marginal mean daily intake of PFOA and PFOS was, 7.56 ng/kg and 5.72 ng/kg, resp., which declined to 6.08 ng/kg and 5.21 ng/kg, resp. at age 3 months.

In formula feeding, PFOA and PFOS could not be detected, so daily PFAS intake through formula was negligible.

In infants with mixed feeding, the mean (SD) daily formula intake increased from 36.36 (57.6) ml/kg at age 1 month, to 109.8 (56.0) ml/kg at age 3 months. As a result of the relatively higher intake of formula compared to human milk, the estimated marginal mean daily PFOA and PFOS intake in mixed fed infants decreased, from 6.54 ng/kg and 4.49 ng/kg, resp. at age 1 month to 1.86 ng/kg and 1.33 ng/kg, resp. at age 3 months.

Table 3. Multiple linear regression analysis for daily PFAS intake by human milk at age 1 and 3 months

	PFOA			*LinPFOA*		
	B±SE	β	*p*-value	B±SE	β	*p*-value
Age 1 month						
Amount of formula/day	-0.009 ± 0.00	-0.73	<0.001	-0.009 ± 0.00	-0.72	<0.001
Parity	-1.005 ± 0.26	-0.18	<0.001	-0.984 ± 0.26	-0.18	<0.001
Maternal age						
Constant	9.057 ± 0.50			8.728 ± 0.50		
Adjusted R^2 (p-value)	0.562 (<0.001)			0.546 (<0.001)		
Age 3 months						
Amount of formula/day	-0.007 ± 0.00	-0.83	<0.001	-0.006 ± 0.00	-0.83	<0.001
Living area	0.696 ± 0.33	0.09	0.037	0.674 ± 0.32	0.10	0.039
Breastfeeding previous child	-1.316 ± 0.36	-0.17	<0.001	-1.337 ± 0.35	-0.18	<0.001
Maternal age						
Constant	5.561 ± 0.56			5.297 ± 0.55		
Adjusted R^2 (p-value)	0.625 (<0.001)			0.619 (<0.001)		

Results of multivariate linear regression. B±SE = Unstandardized coefficient B and Standard Error. β = standardized coefficient Beta. Amount of formula / day = amount of formula in ml per 24 hours, breastfeeding of previous child: 0= no, 1 = yes. Maternal age in years, weeks, living area: 1= West Rotterdam, 2= East Rotterdam, PFOA = Total Perfluorooctanoic acid, PFOS = Total Perfluorooctane sulfonic acid.

Associations with daily intake

The results of multiple linear regression analysis are shown in Table 3. At age 1 month, the model explained 54.5% - 56.2% of variance in the daily PFAS intake. As formula did not contain PFAS, daily PFAS intake was inversely associated with the daily amount of formula, so when the infant received 100 ml of formula feeding in combination with breastfeeding, the daily PFOA and PFOS intake was resp. 0.9 and 0.7 ng/kg lower than in EBF-infants. This shows indirectly that a higher intake of breastfeeding is associated with a higher daily PFOA and PFOS intake. Daily PFOA and PFOS intake was highest in infants of primiparous mothers. With each previously born child, the daily PFOA and PFOS intake via human milk was resp. 1.005 and 0.577 ng/kg lower. For PFOS, daily intake increased also with increasing maternal age, when maternal age increased with 1 year, the daily PFOS intake of the infant increased with 0.086 ng/kg.

	PFOS			***LinPFOS***	
B±SE	β	*p*-value	B±SE	β	*p*-value
-0.007 ± 0.00	-0.73	<0.001	-0.005 ± 0.00	-0.72	<0.001
-0.577 ± 0.20	-0.14	0.024	-0.349 ± 0.14	-0.13	0.040
0.086 ± 0.04	0.11	0.013	0.054 ± 0.03	0.10	0.012
3.663 ± 1.25			2.467 ± 0.86		
0.566 (<0.001)			0.545 (<0.001)		
-0.005 ± 0.00	-0.74	<0.001	-0.004 ± 0.00	0.77	<0.001
0.088 ± 0.03	0.12	0.007	0.066 ± 0.02	0.13	0.005
2.293 ± 1.08			1.308 ± 0.77		
0.586 (<0.001)			0.558 (<0.001)		

At age 3 months, the model explained 55.8% - 62.5% of variance in the daily PFAS intake. Daily PFOA and PFOS intake was inversely associated with the daily amount of formula feeding. When the infant received 100 ml formula, the daily PFOA and PFOS intake was resp. 0.7 and 0.5 ng/kg lower compared to EBF-infants. Daily PFOA intake was higher in infants of mothers who had never breastfed a previous child. Also, infants born in the east of Rotterdam had a higher daily PFOA intake compared to infants born in the west of Rotterdam and daily PFOS intake was higher with increasing maternal age.

We found no associations between PFAS intake at age 1 and 3 months and maternal dietary habits, including consumption of fish and eggs.

Correlation with infant plasma levels

At age 3 months, daily PFOA and PFOS intake through breastfeeding was highly correlated with infant's plasma levels, with correlation coefficients of 0.836 and 0.875 ($p < 0.001$), resp. Furthermore, daily PFOA and PFOS intake at age 3 months was also highly correlated with infant's plasma levels at age 2 years, $R=0.642$ and 0.692 ($p < 0.001$), resp.

Discussion

We present longitudinal daily PFAS intake through human milk and formula feeding in a large group of healthy Dutch infants. In human milk, both PFOA and PFOS levels decreased between 1 and 3 months after delivery and PFOA levels were higher in human milk with a higher protein content. The PFAS levels in human milk were not different whether breastfeeding was given exclusively or in combination with formula feeding (mixed feeding). As PFAS could not be detected in any formula feeding, the daily PFAS intake (ng/kg) was significantly higher in infants with exclusive breastfeeding compared to infants with mixed feeding or exclusive formula feeding at age 3 months. Older maternal age, lower parity, first-time breastfeeding and lower amount of formula feeding were associated with higher daily PFOA and PFOS intake. Daily PFAS intake in early life was strongly correlated with PFAS plasma levels at age 3 months and 2 years.

To our knowledge, we are the first to present longitudinal data of PFAS levels in human milk and their associations with longitudinal plasma levels in infancy. A few European studies reported cross-sectional data about PFAS levels in human milk, mostly using < 50 pooled samples [13]. They reported divergent results in human milk with mean PFOA and PFOS levels ranging from 0.05 - 0.18 ng/mL and 0.05 – 0.32 ng/mL, resp., which are higher compared to the levels we found. These study populations were, however, smaller compared to ours and consisted predominantly of samples collected between 1996 and 2010. Both PFOA and PFOS have been enlisted as Persistent Organic Pollutants (POPs) by the Stockholm convention in 2009 and, therefore, guidelines to phase out the production of PFOA and PFOS from industrial production lines in Europe have been set up [11]. We have collected samples between 2013-2020, which could explain why we measured lower PFAS levels in human milk.

PFOA and PFOS could not be detected in the 6 most commonly used Dutch brands of infant formula, indicating that levels were below 0.015 ng/mL. In The Netherlands, PFAS has never been measured in infant formula. Globally, only 2 research groups measured PFAS in infant formula and reported low levels [12, 20], which is in line with our results. Our findings show that PFOA and PFOS exposure in infancy through infant formula is negligible.

Median daily PFOA and PFOS intake decreased between 1 and 3 months of age. EBF-infants had about 6-8 times higher daily intake compared to EFF-infants. Besides, we found that a higher amount of formula feeding, and thus a lower human

milk intake, was associated with a lower daily PFOA and PFOS intake. This is in line with our previous finding that EBF-infants have 2-3 fold higher serum PFAS levels compared to EFF-infants up until the age of 2 years [21]. We have now evidence that breastfeeding is a major contributor to PFAS exposure in early life. In 2020, EFSA revised their safety thresholds for PFAS intake by food for children and adults, to avoid potential adverse effects [22]. By setting this threshold, the scarce data on pre- and postnatal transmission from mother to her child were taken into account and it was chosen to prevent a lower vaccination response in children [5, 11]. Altogether, the tolerable intake for PFOA, PFOS, PFHxS and PFNA combined was assumed to be 4.4 ng/kg per week [22]. Our findings show that breastfeeding exceeds this safety threshold solely by PFOA and PFOS intake in only 1 day instead of 1 week. This is concerning, because the first months of life are known to be a critical window for adiposity programming and a vulnerable period for the programming of growth and neurodevelopment. Our findings might, therefore, indicate that especially infants with exclusive breastfeeding in the first months of life are potentially more prone to not only decreased vaccination response, but also other potential adverse developmental effects of PFAS in early life, such as growth restriction, increased adiposity, altered behavioral patterns and endocrine disruption as has been shown in rodent studies [3, 4]. Such adverse effects would reduce the health benefits of breastfeeding [23-25]. It is, therefore, crucial to study if early life PFAS exposure will result in adverse effects later in childhood. Based on the recommendation of the World Health Organization to give exclusive breastfeeding for the first 6 months of life [23] and our present findings, guidelines to lower the thresholds for PFAS intake and further phase out and preferably ban all PFAS production are highly recommended.

Our results show that daily PFOA and PFOS intake in early life does not only correlate with infant's plasma levels at age 3 months, but also at age 2 years. This can be explained by the very long elimination half-life in humans, 3.8 years for PFOA and 5.4 years for PFOS [2, 3]. Our findings show that not only breastfeeding is an important PFAS exposure pathway, but also that PFAS exposure in early life has lasting effect on plasma levels throughout infancy. This could have potentially negative consequences throughout life, but further research to the effects of PFAS exposure in early life is needed.

Our findings show that PFOA, and not PFOS, levels are higher in milk with higher protein content. The exact mechanism of PFAS secretion in and excretion through breastfeeding is not well elucidated yet. It has been postulated that transmission occurs via transfer over the mammary epithelial membrane through binding on

protein, mainly albumin or via direct membrane transport mechanisms, because the chemical structure mimics fatty acids [13]. Our findings suggest that only PFOA and not PFOS excretion is associated with protein binding.

In contrast to PFOA, the linear PFOS isomer accounted for about 2/3 of the total PFOS levels. The only other research group who reported the level of linear PFOS in human milk also described a 2/3 contribution of the linear isomer to the total PFOS level [20]. This is in line with adult plasma, in which the contribution of linear to total PFOS levels is described to be around 59 - 68% [26] and the total PFOA plasma levels predominately consist of linear isomer [27]. Since the ratio of linear to total PFOA and PFOS levels in human milk and plasma in present study are similar compared to literature, our findings suggest that both the linear and branched isomers can be equally transported in and excreted by the mammary glands.

We found parity to be associated with PFOA and PFOS intake, with the highest daily intake through human milk of primiparous mothers. Also, daily PFOA intake was higher when mothers had not breastfed a previous child. Lastly, daily PFOS intake increased with increasing maternal age. Our findings suggest that older primiparous mothers have higher PFAS plasma levels and that these could lead to higher excretion through the mammary glands and subsequently to higher levels in human milk.

Daily PFOA intake of infants born in the eastern area of Rotterdam was higher compared to infants born in the western area. Rotterdam is a large city, located near a large industrial and harbor region in The Netherlands. In Dordrecht, about 20 km south-east from Rotterdam, adult residents have higher median plasma levels of PFOA (10.2 ng/mL), compared to those in other regions (3.4 ng/mL) [28], because of the local PFAS-producing chemical company, which produced PFOA until 2012. In a radius of 50 km around this company, PFOA remained present in higher levels in soil and groundwater compared to 150 other areas in The Netherlands [29]. Our findings are potentially the result of more PFAS accumulation in mothers living closer to the chemical company resulting in higher PFAS levels in their breastmilk.

Maternal dietary habits were not associated with daily PFAS intake of the infants. PFAS can be ingested by the consumption of several products, especially by fish, fruit, vegetables and eggs [11]. Because PFOA and PFOS have a very long elimination half-life [2, 3] and have different affinity to accumulate in organs and tissues [9], dietary

habits could possibly have long-term effects on PFAS levels in human milk. We did not find any association with maternal dietary habits, which might be explained by the fact that we could only determine short-term dietary habits during pregnancy and the first months after delivery.

The strengths of our study are the longitudinal PFAS analyses in both human milk and infant plasma in a large prospective cohort of healthy infants. PFAS analysis were performed using a high quality measuring method. We acknowledge some limitations. As the exact amount of human milk intake cannot be measured, we used an average human milk intake per day of 150 ml/kg as an estimation. Besides, we did not collect maternal blood samples, which could have given more detailed insight in the plasma to human milk transmission of PFAS, which could be of interest in future research.

Conclusion

In conclusion, human milk contains PFOA and PFOS, in contrast to formula feeding. Daily PFAS intake was highest in EBF-infants of older primiparous women. Furthermore, daily PFAS intake in early life was highly correlated with infant's PFAS plasma levels throughout infancy. Our findings show that breastfeeding is an important exposure pathway in the first months of life, with potential adverse effects. Therefore, international guidelines to lower the thresholds for PFAS intake and further phase out and preferably even ban all PFAS production are highly recommended.

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Author contributions: AHK designed the study. IvB, KdF and AHK were in charge of the cohort, design, and collecting of the data and samples. BvZ and SvdB designed the laboratory method. BvZ conducted all PFAS analysis. KdF conducted all human milk macronutrient analysis. IvB performed the statistical analysis. Drafting the manuscript was primarily done by IvB under supervision of AHK. All authors were involved in writing the manuscript and had final approval of the submitted version.

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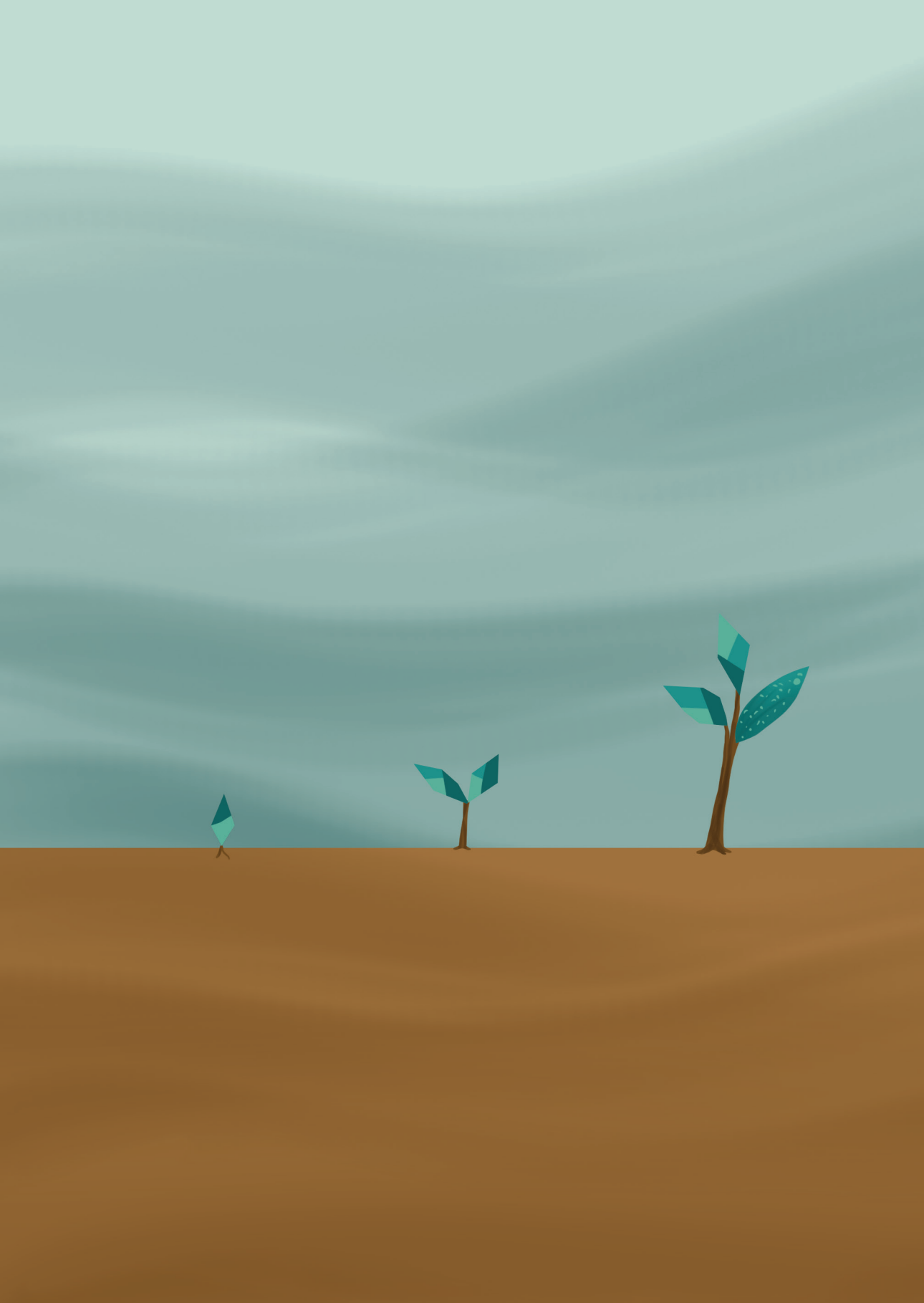

Chapter 10

General Discussion

General Discussion

In 2012, the Sophia Pluto study was initiated based on the outcomes of the PROGRAM study, which showed that especially accelerated weight gain during the first months of life is an important risk factor for unfavorable metabolic health and body composition outcomes in young adulthood [1]. The Sophia Pluto study is a prospective birth cohort study aiming to determine early growth and body composition trajectories in term-born children and identify determinants of adiposity programming and metabolic profile in early life. Since the start of the Sophia Pluto study, our research group presented methods to accurately assess longitudinal body composition in infancy [2]. We showed that especially the first 6 months of life are a critical window for adiposity programming, and identified infant feeding, rapid gain in fat mass and appetite regulating hormone trajectories as determinants [3-5]. The ability to identify infants at risk of obesity at an early stage is clinically highly relevant, since it will provide the opportunity to develop more targeted preventive strategies. It is, therefore, important to identify all determinants that influence adiposity programming in early life.

With the studies presented in this thesis, we fill the current gap of assessing body composition longitudinally in infancy and early childhood, we provide insights in several (potential) determinants of adiposity programming in early life and we explore PFAS exposure routes during infancy. In this chapter, the results of these studies are discussed in view of recent literature. Furthermore, the clinical implications are addressed, as well as future directions for research.

Part I - Assessing longitudinal body composition in infancy and early childhood

It has been demonstrated that fat mass is a more adequate indicator of adiposity than anthropometric measures such as weight or body mass index (BMI) [6, 7]. Although BMI is often used to assess adiposity, in infants and children, it is poorly associated with true body composition. Also, BMI SD-scores have low specificity to identify changes in fat mass percentage (FM%) [8, 9]. Longitudinal body composition monitoring during infancy and childhood is important to identify children who are at risk for developing excess adiposity.

DXA

Dual energy X-ray Absorptiometry (DXA) is known as a reference method to evaluate bone density [10] and is often used as a reference method to determine body composition in children, both in research and clinical practice [11]. Besides, it is the only widely used method which provides, not only results for total body fat and lean body mass, but also regional (trunk, arms, legs) estimates [12]. DXA uses a three

component method (3-C method), meaning that its software differentiates bone, fat mass and lean body mass by the attenuation of X-ray energy passing different types of tissue [13]. This differentiation is sensitive for movement artifacts [13], so especially in infants and young children obtaining accurate DXA results is a major challenge. Mainly because of this, reference values, that are needed to identify young children with increasing adiposity, were very scarce and based on very small populations [14, 15]. As a result, it was impossible to monitor body composition longitudinally from birth to young childhood. In **Chapter 2**, we describe our new sex-specific reference data and charts for body composition and bone mineral density for children aged 2-5 years, based on almost 600 DXA measurements.

We show that FM and lean body mass (LBM) increase from age 2 to 5 years, but that body size-corrected FM% and fat mass index (FMI) decrease during that period. Lean body mass index (LBMI) remained similar between age 2 and 5 years. Girls had higher FM, FM% and FMI and lower LBM and LBMI compared to boys. Both bone mineral content (BMC) and bone mineral density (BMD_{TBLH}) increased between 2 and 5 years and were similar for boys and girls.

Subsequently, we found that SD-scores based on these new reference values are comparable with SD-scores based on our previously published reference values for infants aged 0-2 years, in which a vacuum cushion was used to avoid movement artifacts [2]. Our new reference values can, therefore, be used longitudinally in young children aged 2-5 years and in combination with our previously published reference values for children from birth to age 2 years [2].

In conclusion, we developed DXA references for children aged 2-5 years, which, together with our previous references from birth – 2 years, makes it possible to longitudinally monitor DXA measurements from birth to age 5 years and identify children at risk for excess adiposity.

ADP

Air Displacement Plethysmography (ADP) is also a method to measure body composition. It is based on a two component method (2-C method), which only differentiates between fat mass en fat free mass. Next to DXA, ADP is increasingly used to assess body composition during infancy and childhood. However, due to practical limitations ADP cannot be used longitudinally from birth to adolescence. ADP (PEA POD) can be used to assess body composition in infants aged ≤6 months and ADP (BODPOD) in children aged >2 years, but in our experience BODPOD is only feasible from age 3 years onwards. It would, therefore, be favorable if ADP

and DXA could be used interchangeably to monitor longitudinal body composition throughout the first years of life. Our research group previously assessed that results from ADP (PEA POD) were comparable with DXA (with vacuum cushion to avoid movement artifacts) in infants aged 6 months [2]. **Chapter 3** describes the first comparison of results by ADP (BODPOD) with those of DXA in young full-term and very preterm born children, aged 3-5 years.

We found significant fixed and proportional bias between FM, FM% and FFM results measured by both methods. A fixed bias means that the mean difference between results is significantly larger than 0 and proportional bias means that the difference between results assessed with ADP and DXA increase when FM, FM% and FFM deviate from the mean. These differences were even larger in very preterm children compared to full-term born children. We, therefore, had to conclude that ADP results are neither comparable nor interchangeable with DXA results in early childhood.

In clinical practice, especially in young children with deviant body composition, such as preterm born children, we noticed that ADP results were often clinically questionable (e.g. extremely low values of fat mass percentage, <5%). As ADP measures body volume based on the change in air pressure in a secluded space with a known volume. ADP-software calculates fat mass percentage (and following from this fat mass and fat free mass) using certain constant values of fat mass (Dfm) and fat free mass density (Dffm); the density models [16]. These density models are based on multiple assumptions. Fat mass is thought to not contain water, and have a constant density throughout life [17]. In contrast, fat free mass hydration is presumed to decrease with age, which increases its density. The Dffm-model according to Lohman, used as default in the ADP-software, was based on data of very small study groups aged 0-1 and 8-30 years measured in the 1980's [17-19]. Sex- and age-specific Dffm-estimates were then extrapolated to other pediatric age categories per 2-year-interval [19]. Therefore, these Dffm-estimates may not be valid in young children, which could explain the inter-method differences we observed between ADP and DXA. Wells *et al* developed novel Dffm-estimates for healthy children aged ≥5 years [18], which have been reported to be superior to the default estimates in children aged 5 years [20]. However, improved Dffm-estimates for children aged <5 years were not yet available.

We, therefore, revised the Dffm-model based on our large group of healthy full-term born children, using a 3-compartment-model as reference [21]. By doing so, the inter-method differences, in both term and very preterm born children decreased. However, although smaller, both fixed and proportional bias remained.

It has been reported that Dffm can vary in children with different nutritional status, physical activity level, ethnicity and disease status, but these variations were not included in the default Lohman Dffm-model used in ADP-software [19, 22]. Besides, children born preterm show a different pattern of body composition and Dffm over childhood. While FM in preterm children was found to be higher around term age, studies later in childhood reported lower FM as compared to full-term children [23, 24]. It was also reported that a leaner body type is accompanied with a lower FFM hydration and consequently higher Dffm [22]. Furthermore, recent studies showed that bone mineral content and density were also lower in preterm born children as compared to full-term born children [25, 26]. Besides, incorrect assumptions about thoracic gas volume could lead to incorrect body composition estimates by ADP [16]. In addition, preterm born children, with or without bronchopulmonary dysplasia (BPD), have more often reduced lung volumes or impaired lung function [27]. Altogether, caution is needed when interpreting ADP-results of this specific patient group. In fact, it warrants further research to compose separate Dffm-estimates for children with deviant body composition trajectories, such as preterm born children.

In conclusion, despite revised Dffm-estimates, results of ADP and DXA remained not comparable and should not be used interchangeably in the longitudinal assessment of body composition in children aged 3-5 years, especially not in preterm born children.

Anthropometry-based equations

Both DXA and ADP are expensive and non-portable techniques. Therefore, several research groups have developed anthropometric-based equations to calculate or predict fat mass (percentage) in absence of a measuring device. The equations of Slaughter *et al* [28], Aris *et al* [29] and Catalano *et al* [30] are most commonly used in pediatric research and general practice. However, the Slaughter equations have been developed in 1988 in children and adults aged 8-29 years [28]. The Aris and Catalano equations have both been developed in 1-3 day old infants in 2013 and 1995, resp. [29, 30]. All equations have been validated against a reference method, but all have random and systematic errors, especially when used in children of other age categories [31].

In **Chapter 4**, we describe newly developed anthropometry-based prediction equations for FM and FFM in healthy term-born UK infants aged 5-16 weeks using ADP by PEA POD as the criterion method. These equations were validated in infants aged 3 and 6 months participating in our independent Dutch cohort (Sophia Pluto cohort). We showed that the new anthropometry-based equations are more

accurate in calculating fat mass (FM) and fat free mass (FFM) compared to the 3 previously published equations [28-30] for infants aged ≤ 6 months. We found a small proportional bias for FM and FFM. This means that the difference between the outcomes, when calculated with the equation or measured by ADP, increases when deviating from the mean, resulting in overestimation, (in subjects with low FM or FFM) and underestimation (in subjects with high FM or FFM) of outcomes when calculated with the new anthropometry-based equations. However, the proportional bias in our equations was much smaller compared to the 3 previously published equations.

Recently, an American research group also developed anthropometry-based equations using both ADP by PEA POD and Infant quantitative magnetic resonance (Infant-QMR) as criterion methods [32]. They developed 3 separate equations, for ages 3 days and 15 weeks, based on skinfolds, weight and head circumference, and for age 54 weeks, also including length. Their equations were stronger correlated with fat mass compared to ours, however, they did not validate the equations in an independent cohort. The stronger correlation could possibly be explained by the use of multiple equations for different age categories.

In general, body composition undergoes multiple changes throughout childhood [2, 6, 18], the use of multiple adjoined prediction equations for different age categories, would potentially reduce proportional bias and systematic errors, when used longitudinally. This could make anthropometry-based equations more reliable for calculating body composition longitudinally in both infancy and childhood.

In conclusion, the use of our anthropometry-based equations are preferred over the use of previously published equations [28-30] to calculate FM and FFM, in infants aged ≤ 6 months in research and general practice when a measuring device, such as DXA or ADP is not available.

General conclusions and clinical implications of part I

Based on our findings, we can conclude that multiple methods can be used to assess body composition in young children. In order to monitor body composition longitudinally during infancy and childhood, we advise to use DXA, which is a reliable and accurate method, that not only assess body composition, but also bone mineral density. Our sex-specific reference values and charts can be used longitudinally from birth to age 5 years to identify those at risk for excess adiposity, provided that the same equipment and software is used (DXA by Lunar Prodigy with Encore software (V14.1)).

When DXA is not available or allowed because of the very small dose of radiation (0.0002 mSv), we advise to use ADP with our revised age- and sex-specific estimates for Dffm. However, caution is needed when interpreting ADP-results, especially in those at risk of altered adiposity trajectories and thus with FM% that deviates from the mean, such as very preterm born or overweight children.

Lastly, anthropometry-based equations can be used to calculate FM and FFM when DXA or ADP is not available. In that case, our new anthropometry-based equations are preferred over previously published equations for infants aged ≤ 6 months.

Part II – (Potential) determinants of adiposity programming in early life

Multiple determinants of adiposity programming in early life have been described. However, the exact mechanism has not been elucidated yet. In this thesis, several additional factors have been identified that influence body composition development. These are presented in Figure 1 as bold continuous lines.

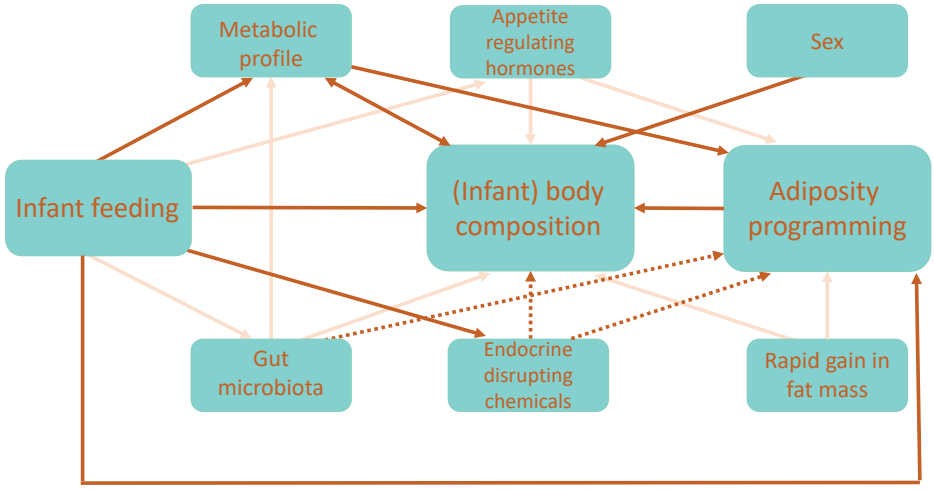

Figure 1. Visual summary of factors (potentially) influencing body composition development. Light continues lines represent previously described associations, bold continues lines represent described associations in this thesis. Dashed lines represent hypotheses about potential associations, which warrant further research.

Infant body composition

It has been reported that adiposity in infancy or early childhood is predictive for adiposity and metabolic outcomes in adulthood [33]. BMI is predominately used as a proxy to determine tracking of adiposity, however it has been demonstrated that

fat mass is a more adequate indicator of adiposity [6, 7]. In 2020, our research group reported that infants with rapid rise in fat mass in the first 6 months of life have higher fat mass trajectories until 2 years of age [4], but data about tracking of body composition from infancy to childhood were not yet available.

In **Chapter 5**, we studied the tracking of high FM%, FM-index, abdominal subcutaneous and visceral fat and FFM-index (FFMI) from early life to age 4 years in 224 healthy term-born children. We showed that infants in the highest tertile of FM%, FMI and abdominal subcutaneous fat in the first 6 months of life, had high odds to remain in the highest tertile at 4 years of age. These finding support the presence of a critical window for adiposity programming in the first 6 months of life. Also, we showed that high FFM-index tracked already from age 1 month to 4 years. This could indicate that the first months of life are not only a critical window for adiposity programming, but also for fat free mass programming. In addition, we showed that tracking of FM% was different between infants with exclusive breastfeeding for the first 3 months of life (EBF) and infants without exclusive breastfeeding (non-EBF). EBF-infants had no significant tracking of FM% from early life, whereas non-EBF infants with high FM% at age 3 and 6 months had a high odds ratio (OR) to track to 4 years.

It has been described that exclusive breastfeeding is protective for the development of childhood obesity [34]. Our findings suggest that infant feeding influence adiposity programming in early life and support that EBF in early life has a potential protective effect on adiposity programming later in life. Although body composition is different between boys and girls from early age onwards (**Chapter 3**), our findings show that tracking of body composition until age 4 is not subject to sex-differences. This suggest that different hormone levels, including endogenous testosterone production in boys versus girls [35], probably cause sex-difference in body composition from birth onwards, but are not likely to influence adiposity programming.

In conclusion, our longitudinal data support the presence of a critical window of adiposity and fat free mass programming in the first 6 months of life.

Metabolic profile

In order to identify infants at an early age who are at risk for developing excess adiposity, the metabolic profile could potentially serve as metabolic biomarker. Ideally, these biomarkers should, at an early age, be able to predict body composition later in life. However, eligible biomarkers have not been identified yet. In **Chapter 6** we present plasma metabolites at age 3 months that modestly predicted body

composition, measured as truncal-to-peripheral skinfold ratio (T:P-ratio), at age 2 years. Of the 15 plasma metabolite variables that were most strongly associated with T:P-ratio at age 2 years, 11 were also associated with visceral fat. In addition, in **Chapter 7** we present plasma metabolites at age 3 months that associate with FM-percentage, FM-index, FFM-index and visceral fat at age 2 years. We are the first to describe plasma metabolite variables in early life in association with future body composition. Our findings support again the presence of a critical window for adiposity programming in the first months of life.

Several of these identified metabolites (dimethylarginine, LysoPA, LysoPS, LysoPE and LysoPG) are known to be associated with pro-inflammatory pathways in preadipocytes [36, 37] and/or with inflammatory processes [38-40], mainly via interaction with Toll-like receptor dimers [41] and peroxisome proliferator-activated receptor gamma (PPAR-γ) [42]. Our findings might suggest that the identified metabolites are involved in adiposity development and systematic low grade inflammatory processes from early age onwards.

In addition, we identified distinct metabolites, predominately several classes of Lyso-phospholipids, that associated with high FM-percentage, FM-index, FFM-index and visceral fat in EBF-infants and not in EFF-infants. Differences in metabolic profile between EBF- vs EFF-infants aged 3 months have been previously described [43-46]. We now add that these differences in early life, can potentially lead to differences in future body composition. Together, these findings suggest that phospholipids are involved in adiposity programming and that the supply and incorporation of phospholipids is different between EBF- and EFF-infants. Potentially, this could be influenced by the milk fat globule membrane (MFGM), which coats fat droplets in human milk, but is absent in formula feeding [47, 48]. The MFGM is associated with neurodevelopmental outcomes and microbiome development [48]. Our findings suggest the MFGM could potentially also be involved in adiposity programming.

In conclusion, our findings indicate that plasma metabolites at age 3 months are associated with future body composition outcomes and that these association were predominately found in infants with exclusive breastfeeding.

General conclusions and clinical implications of part II

Our findings show additional support for the presence of a critical window of adiposity programming in the first 6 months of life. Furthermore, we have identified infant feeding and metabolic profile as additional determinants for adiposity programming.

Since the first 6 months of life are a critical window for adiposity programming, this could be an optimal time for obesity prevention in early life. The identified determinants could be used for early identification of infants at risk for developing obesity. Based on the findings in this thesis, infants with high FM% in early life should be monitored closely, especially when they are exclusively formula fed. Specific plasma metabolites could potentially be used to develop more targeted preventative strategies. These might be different in infants with exclusive breastfeeding vs exclusive formula feeding.

Part III - PFAS levels in infancy

Per- and polyfluoroalkyl substances (PFAS) exposure in early life might influence adiposity programming. Rodent studies have shown concerning effects in offspring that was exposed to high levels of PFAS during pregnancy or in early life. These adverse effects consist of a wide range of developmental effects, such as growth restriction, altered behavioral patterns and endocrine disruption [49, 50]. Besides for a decreased vaccination response [51], human studies to confirm or refute other adverse effects are scarce and have conflicting conclusions, partly because of small study populations and short or even lack of follow-up periods [52-54].

PFAS plasma levels

In order to study these adverse effects in humans, it is important to have insight in the PFAS plasma levels during infancy. However, knowledge about longitudinal exposure and plasma levels in infants was lacking.

In **Chapter 8**, we describe the longitudinal plasma PFOS, PFOA, PFHxS, PFNA and PFDA levels in 369 healthy and term-born infants, at age 3 months and 2 years. Infants aged 3 months had considerable PFAS plasma levels, which only slightly decreased until age 2 years. PFAS levels at age 3 months and 2 years were highly correlated, which could be explained by the very long elimination half-life in humans, ranging from 1.7-3.2 years for PFNA up to 8.5 years for PFHxS [50, 55].

Higher PFAS levels were especially found in first-born infants of older primiparous Caucasian mothers. Potentially because these mothers had more PFAS accumulation and subsequently more trans-placental transmission, resulting in higher PFAS levels in their infants [56, 57].

Other identified determinants for high infant plasma PFAS levels were longer total breastfeeding duration and exclusive breastfeeding during the first 3 months of life. In fact, at age 3 months, PFAS plasma levels in EBF-infants were 2-3 times higher compared to EFF-infants and mixed fed infants. This difference remained present until at least 2 years of age.

In conclusion, our findings indicate that trans-placental and breastfeeding transmission are the most important determinants of PFAS exposure in early life, which could potentially have life-long effects.

PFAS exposure through infant feeding

Infant feeding could be an exposure pathway for Persistent Organic Pollutants (POPs) that can act as endocrine disrupting chemicals (EDCs), with potential adverse effects on adiposity programming. However, (longitudinal) PFAS levels in infant formula and human milk and daily PFAS intake through infant feeding had never been reported in a large cohort of healthy infants.

Our data in **Chapter 9** show that PFOA and PFOS could not be detected in the 6 most used Dutch brands of infant formula. Indicating that the PFOA and PFOS levels were lower than 0.015 ng/mL. These findings suggest that PFOA and PFOS exposure through infant formula is negligible. On the other hand, we did detect PFOA and PFOS in human milk. These levels in human milk decreased between 1 and 3 months after delivery, regardless whether breastfeeding was given exclusively (EBF) or in combination with formula feeding (mixed feeding) and especially PFOA levels were higher in human milk with a high protein content. Daily PFAS intake (ng/kg) at age 3 months was highest in EBF-infants. Higher amount of human milk, older maternal age, lower parity and first-time breastfeeding were associated with higher daily intake. Besides, we confirmed the importance of breastfeeding for PFAS exposure in early life, as we found that daily PFAS intake through breastfeeding is highly correlated with infant's plasma levels throughout infancy.

Our findings are of particularly concern, because breastfeeding is known to have multiple health benefits, such as mother and child bonding, and protection against obesity, infections, asthma, eczema and allergies [58-60] and the first months of life are known to be a critical window for adiposity programming. Thus, PFOA and PFOS in human milk could negate the protective effect of breastfeeding and our findings could indicate that especially infants with exclusive breastfeeding in the first months of life are more prone to potential adverse developmental effects of PFAS in early life, such as decreased vaccination response, increased adiposity and endocrine disruption [49-51, 61].

In conclusion, our findings confirm that breastfeeding is an important determinant of PFAS exposure in early life, which warrants further research.

General conclusions and clinical implications of part III

Our findings indicate that trans-placental and breastfeeding transmission are the most important determinants of PFAS exposure in early life. Since PFAS exposure in early life could have developmental and long lasting adverse effects, our findings warrant further research.

The novel insights in PFAS transmission and exposure during infancy presented in this thesis, could be used to improve guidelines regarding PFAS production and safety thresholds for PFAS ingestion. Especially, to prevent potentially adverse developmental effects and reduction of health benefits of breastfeeding.

Directions for future research

Long-term follow-up of body composition development in healthy term-born children of the Sophia Pluto study is warranted in order to further elucidate the consequences of adiposity programming in early life. During this long-term follow-up the effect of the determinants, described in this thesis, on body composition in mid-childhood should be evaluated, as well as the investigation of additional factors that might influence adiposity programming in early life, such as gut microbiota, (epi-)genetics and content, origin and preparation of complimentary food intake. These insights could further add to the development of individualized and more targeted preventative strategies in the battle against (childhood) obesity.

Long-term follow-up of these children would have more advantages. Firstly, it would give the opportunity to develop or improve methods to assess body composition longitudinally from birth to adolescence in order to increase early identification of children with risks for adiposity. When using DXA, the existing reference values and charts could be extended from age 5 to 18 years, based on a large number of children. Also, the agreement of ADP and DXA could be evaluated in children aged >5 years and new Dffm-models should be developed for older children and specific patient-groups, such as children with undernutrition or (genetic) obesity. For situations where DXA and ADP are not available or desirable, the anthropometry-based equations should be developed and validated for older age categories, for ages >6 months.

More research is needed to elucidate the influence of infant plasma metabolomics, whether or not in combination with gut microbiota on adiposity programming and body composition. This knowledge would not only be beneficial for preventive

strategies, but could also be a window of opportunity for early nutritional intervention, for example, through addition of pre- or probiotics, or other promising substances to infant formula feeding.

Lastly, it is important to use the follow-up in the Sophia Pluto cohort to evaluate the potential adverse developmental effects of PFAS exposure in early life. Special attention should be paid to the effects on body composition, endocrine disruption, vaccination response, cognitive functioning and altered behavioral patterns.

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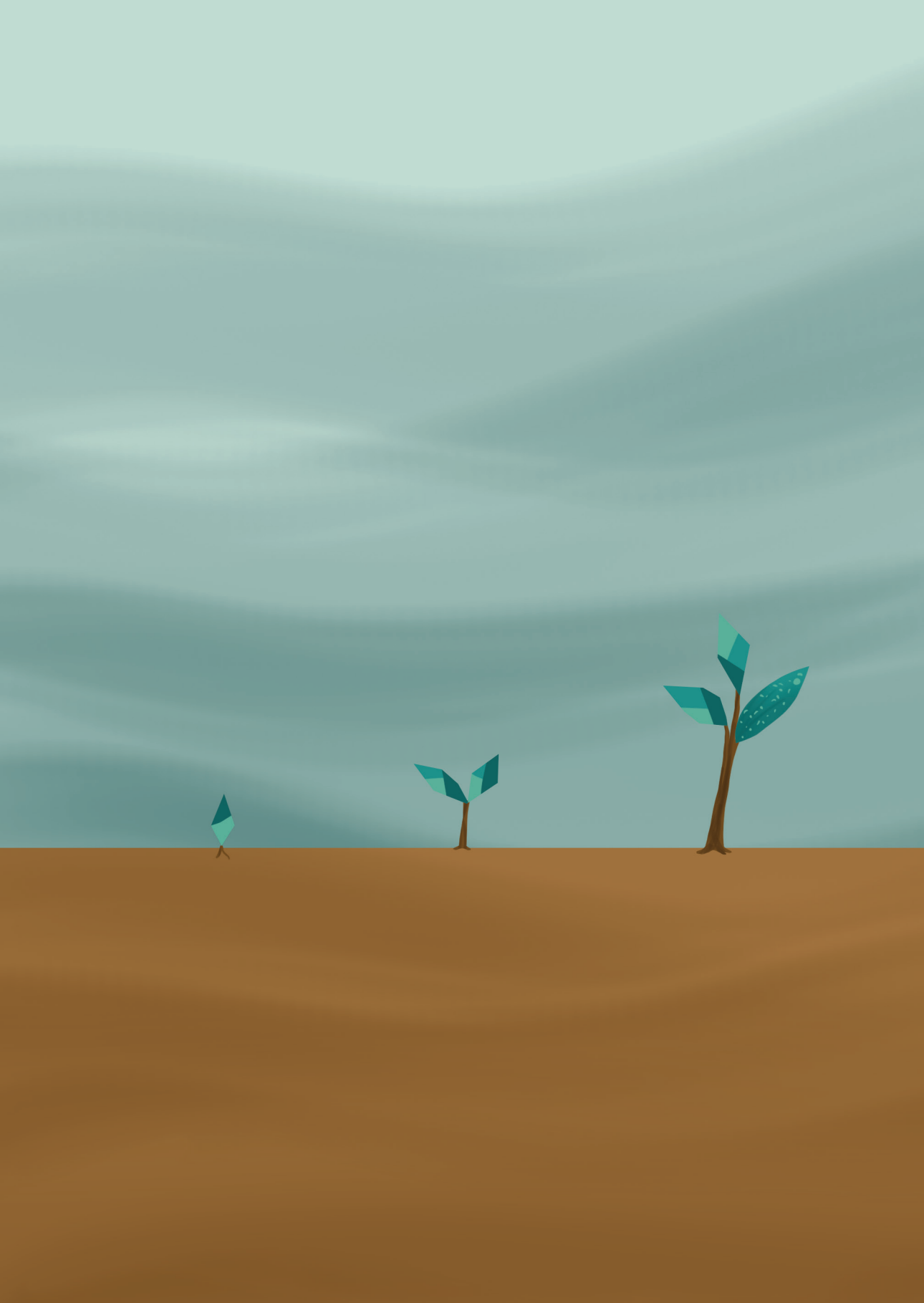

Chapter 11

Summary - Samenvatting

Summary

This thesis reports 8 studies performed in the last few years in a large cohort of healthy children aged 0-5 years. The main aims of these studies were to provide detailed data on the longitudinal assessment of body composition from early life to childhood (part I), to improve knowledge about early determinants of adiposity programming (part II) and to provide new insights in a potential group of hazardous substances; PFAS. Especially about their longitudinal exposure and plasma levels in infancy (part III). This chapter summarizes these studies and their most important outcomes.

Chapter 1 provides an summary of the importance of assessing body composition in infancy and childhood. It discusses the possibilities, the difficulties and the knowledge-gaps in the existing methods to assess body composition. Chapter 1 also provides a general introduction of the influence of early life on adult body composition and metabolic health. It gives an summary of multiple known determinants of early body composition development and it provides an overview of potential factors that could influence adiposity programming in early life, which were not well elucidated yet. All to provide an introduction on parameters addressed in the studies in this thesis. Finally, the aims and outlines of this thesis are presented.

Part I - Assessing longitudinal body composition in infancy and early childhood
In order to identify excess adiposity in infancy and childhood, it is important to accurately assess fat mass. Dual-energy X-ray Absorptiometry (DXA) is often used as a reference method to assess body composition. However, reference values for age 2-5 years, needed to interpret measurements and identify young children at risk, were lacking. In **Chapter 2** we aimed to fill the existing gap in reference values by constructing age- and sex-specific body composition reference values and charts for fat mass (FM), fat mass percentage (FM%), fat mass index (FMI), lean body mass (LBM), lean body mass index (LBMI) and total body less head bone mineral density (BMD_{TBLH}) for children aged 2-5 years using DXA.

Using almost 600 accurate DXA-measurements from 340 term-born healthy children aged 2-5 years we constructed age- and sex-specific reference values and charts for body composition and bone mineral density. Girls had higher FM, FM% and FMI and lower LBM and LBMI compared to boys. Bone mineral content (BMC) and bone mineral density (BMD_{TBLH}) both increased between 2 and 5 years and were similar for boys and girls.

In a random sample of 2 year old children, we found SD-scores based on these novel reference values, to be similar to SD-scores calculated using previously published references values for infants ≤2 years (using a vacuum cushion to prevent movement artifacts).

These novel references can, therefore, be used for clinical practice and research purposes to monitor body composition and bone development longitudinally from birth to age 5 years and identify infants and young children at risk for excess adiposity.

Multiple methods are available to assess body composition. Next to DXA, Air Displacement Plethysmography (ADP) is often used. However, due practical limitations, it is not possible to use ADP longitudinally from birth to adolescence. ADP by PEA POD can be used to assess body composition in infants aged ≤6 months and ADP by BODPOD in children aged > 2 years. It would be favorable if ADP and DXA could be used interchangeably to monitor longitudinal body composition throughout the first years of life, especially in those at risk of altered adiposity trajectories, such as children born very preterm. We, therefore, studied in **Chapter 3** if body composition results from ADP by BODPOD are comparable with DXA (Lunar Prodigy with EnCore V14.1) in young children born full-term and very preterm (GA <30 weeks).

In 154 and 67 healthy full-term and very preterm born children, resp., aged 3-5 years, we found FM, FM% and FFM outcomes from ADP and DXA to be significantly different, with both fixed and proportional bias. In full-term born children we found mean differences [LoA] of -1.08 [-2.92 - 0.76] kg, -5.78 [-16.25 - 4.69] % and 0.90 [-1.00 - 2.80] kg, resp. Differences between ADP and DXA were even larger in very preterm born children, with mean differences [LoA] of -1.89 [-4.10 - 0.32] kg, -9.79 [-20.92 - 1.34] % and 1.64 [-0.63 - 3.91] kg, resp.

In addition, the algorithm in ADP software uses default constant values (estimates for fat free mass density (Dffm)) in order to calculate FM%. These constants are based on outdated and small studies in which results of healthy older children and adults were extrapolated to children aged <8 years, per 2-year intervals and may, therefore, not be valid in younger children. In fact, especially in young children with deviant body composition, such as preterm-born children, we noticed that ADP results were often clinically questionable (e.g., extremely low values of FM% <5%). We, therefore, aimed to improve the ADP algorithm by revising sex- and age specific Dffm-estimates for children aged 3-5 years. After revising these estimates,

agreement between ADP and DXA improved, but a small fixed and proportional bias remained. In very preterm born children the differences between ADP and DXA remained larger.

In conclusion, despite revised and improved sex- and age-specific Dffm-estimates, results of ADP and DXA remained not comparable and should not be used interchangeably in the longitudinal assessment of body composition in children aged 3-5 years, especially not in very preterm born children.

When advanced techniques, such as ADP and DXA are not available (for example in general practice), anthropometry-based equations are commonly used to estimate fat mass. However, existing equations were designed in newborns or adolescents and are often biased or have random and systematic errors when used in other age categories. **Chapter 4** describes the development and validation of new anthropometry-based prediction equations for FM and FFM and the comparison with previous published equations.

New anthropometry-based equations (consisting of sex, age, weight, length and skinfold measurements) to calculate FM and FFM were developed using data of infants aged 6 weeks and 3 months (N=55 and N=64), using ADP by PEA POD as the criterion. Sixty-five percent of the variance in FM and 79% in FFM were explained by the equations.

Subsequently, these equations were validated in an independent cohort (Sophia Pluto) at age 3 and 6 months (N=571 and N=447). They showed smaller bias compared to 3 previously published equations. With mean difference [LoA] of 0.008 [-0.49 - 0.51] kg and 0.084 [-0.55 - 0.71] kg at age 3 and 6 months, resp. calculating FM and 0.099 [-0.39 - 0.59] kg and -0.021 [-0.66 - 0.62] kg at age 3 and 6 months, resp. calculating FFM.

In conclusion, our new anthropometry-based equations showed better validity compared to existing equations and can, therefore, be used to calculate FM and FFM in infants, when ADP or DXA are not available.

Part II - Determinants of adiposity programming in early life

The first months of life are a critical window for adiposity programming later in life. This programming seems to be influenced by several factors.

Infants with an high BMI have high risk to have overweight or obesity as a (young) adult. However, programming of body composition in early life is not well elucidated yet. In **Chapter 5**, we evaluated the tracking of high FM%, FMI, FFMI and high abdominal subcutaneous and visceral fat from early life until the age of 4 years in 224 healthy term-born children. We also investigated whether this tracking would be different between boys and girls or between types of infant feeding.

During 4 years of follow-up, healthy term-born infants with high FM% tracked from age 3 and 6 months to 4 years (odds ratio (OR): 2.62 – 6.54, p <0.027). If infants had high abdominal subcutaneous fat, they tracked from 6 months to 4 years of age (OR: 2.30, p = 0.035). Infants with high FFMI at age 1, 3 and 6 months continued to have high FFMI at age 4 years (OR: 3.36-4.16, p <0.019). We found no differences in tracking between boys and girls. However, in contrast to infants with exclusive breastfeeding during the first 3 months of life, high FM% at age 3 and 6 months in infants with exclusive formula feeding or mixed feeding, tracked to age 4 years (OR: 4.00-7.33, p <0.006).

In conclusion, our longitudinal data support the presence of a critical window of adiposity and FFM programming in the first 6 months of life, as well as a protective effect of exclusive breastfeeding on adiposity programming.

An unfavorable body composition with excessive body fat and more visceral fat is associated with an adverse lipoprotein profile, especially with high LDL cholesterol levels. However, not only the standard lipoproteins, but also several hundred lipid species were found in infant plasma. Metabolic profile in early life may reflect adiposity programming and correlate with later life body composition. These could, therefore, potentially be early biomarkers for unfavorable metabolic outcomes later in life. In **Chapter 6**, we investigated if metabolic profile at age 3 months is predictive for body composition at 2 years and if there are differences between boys and girls and between infant feeding types.

In 318 healthy term-born infants, we found that plasma metabolites (including LysoPS(22:2), dimethylarginine and 13 other metabolite variables) at age 3 months can modestly predict body composition at age 2 years, based on truncal : peripheral skinfold ratio (T:P-ratio). This metabolite profile model had a predictive value of 75.8%, sensitivity of 100% and specificity of 50%. Eleven of the 15 most strongly associated metabolite variables were also associated with visceral fat at age 2 years.

Furthermore, we found the predictive value of the model to be higher in boys compared to girls. The prediction model seemed independent of infant feeding type, since we only found a difference between the relative abundance of 2 of the 15 metabolite variables between infants with exclusive breastfeeding and formula feeding.

Notably, 5 of the identified metabolites are known to be associated with inflammatory processes and/or pro-inflammatory pathways in preadipocytes. Our findings could, therefore, suggest that the identified metabolites are involved in adiposity development and systematic low grade inflammatory processes from early age onwards.

In conclusion, we found plasma metabolites at age 3 months that can modestly predict body composition at 2 years of age, measured as T:P-ratio. Of the 15 highest associated plasma metabolite variables, 11 were also associated with visceral fat at 2 years and 5 are known to be involved in (pro)inflammatory processes. Our findings contribute to the insight into the adiposity programming in the first months of life.

Furthermore, in **Chapter 7** we investigated if plasma metabolites at age 3 months are associated with fat mass and fat free mass measured by DXA, as well as subcutaneous and visceral fat, measured by abdominal ultrasound at age 2 years. In addition, we studied if these associations are different between infants with exclusive breastfeeding for the first 3 months of life and those with exclusive formula feeding.

In 318 healthy term-born infants aged 3 months, we identified distinct plasma metabolites that were associated with high FMI, FM%, FFMI and visceral fat at age 2 years. These metabolites include several classes of lyso-phospholipids. Furthermore, we found that most association were found in infants with exclusive breastfeeding for the first 3 months of life, in contrast to those with exclusive formula feeding.

These finding suggest that phospholipids are involved in adiposity programming in early life and that the supply and incorporation of these phospholipids is different between infants with breast- and formula feeding. Furthermore, these findings suggest that infant feeding type in early life is associated with differences in metabolic profile and can potentially be involved in differences in adiposity programming between infants with exclusive breast- and formula feeding.

In conclusion, associations between plasma metabolites at age 3 months and high body fat at age 2 years are dependent on infant feeding type. These findings contribute to our insight into the importance of infant feeding on adiposity programming in early life.

Part III – PFAS levels in infancy

Per- and polyfluoroalkyl substances (PFAS) are man-made-chemicals. They are non-degradable with an elimination half-life of multiple years, causing accumulation in the environment and humans. Rodent studies demonstrated that PFAS are associated with concerning developmental and metabolic effects, especially when exposure is present during the first months of life. Because longitudinal human data during infancy are lacking, we investigated, in **Chapter 8,** the longitudinal plasma levels during infancy and their most important determinants.

In 369 healthy term-born infants, we determined plasma PFOA, PFOS, PFHxS, PFNA and PFDA levels at age 3 months and 2 years, using liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS). We found that median PFAS levels at age 3 months were especially higher in first born infants from older Caucasian mothers, potentially because of higher PFAS accumulation in these mothers and subsequently more trans-placental transmission to the infant. Plasma PFAS levels at age 3 months and 2 years were highly correlated (R = 0.46-0.82) and only decreased slightly during this period.

We also found that Infants with exclusive breastfeeding during the first 3 months of life (EBF) had 2-3 fold higher plasma levels throughout infancy compared to infants with exclusive formula feeding (EFF), with PFOA levels at 3 months of 3.72 ng/mL versus 1.26 ng/mL and at 2 years of 3.15 ng/mL versus 1.22 ng/mL, respectively.

Thus, our findings indicate that not only trans-placental transmission, but also breastfeeding are the most important determinants of PFAS exposure in early life.

Although breastfeeding is a potential major contributor to PFAS exposure in early life, longitudinal PFAS levels in human milk and daily PFAS intake through infant feeding have never been reported in a large cohort of healthy infants before. In **Chapter 9**, we determined PFOA and PFOS levels in the human milk of 372 infants at age 1 and 3 months, and in 6 infant formula brands, using LC-ESI-MS/MS. We also studied the associations between daily PFAS intake, infant feeding and other important determinants and their correlation with PFAS plasma levels throughout infancy.

Although, PFOA and PFOS could not be detected in formula feeding (levels below lower limit of detection), human milk did contain PFOA and PFOS. Levels decreased between 1 and 3 months of age, regardless of breastfeeding was given exclusively or mixed with formula feeding.

At age 3 months, daily PFAS intake (ng/kg) was 6 times higher in EBF-infants compared to EFF- infants. In infants with breast- and formula feeding, especially a lower amount of formula feeding was associated with a higher daily PFAS intake. Also daily PFAS intake through infant feeding was higher through breastfeeding of older mothers who were primiparous or had no history of breastfeeding.

Lastly, we found that daily PFAS intake at age 3 months was not only strongly correlated with PFAS plasma levels at the same age ($R = 0.836 - 0.875$, $p < 0.001$), but also with plasma levels at age 2 years ($R = 0.642 - 0.692$, $p < 0.001$).

So, our findings suggest that PFAS exposure through infant formula is negligible and our finding confirm that breastfeeding is an important exposure pathway in the first months of life, with potentially lasting effects.

In **Chapter 10**, we discuss the most important findings of our studies in a broader context, and compared to recent literature. We emphasize the clinical implications and provide directions for further research.

Samenvatting

Dit proefschrift geeft een overzicht van 8 studies die we de afgelopen jaren hebben verricht in een groot cohort van gezonde kinderen in de leeftijd van 0-5 jaar. De voornaamste doelen van deze studies waren om gedetailleerde data te verzamelen over het longitudinaal bepalen van lichaamssamenstelling, vanaf zeer jonge leeftijd tot in de kindertijd (deel I), om de kennis te verbeteren over vroege determinanten van de programmering van lichaamssamenstelling en (over-) gewicht (deel II) en om nieuwe inzichten te verschaffen over een groep chemische verbindingen die potentieel schadelijk zijn; PFAS. In het bijzonder, over de longitudinale blootstelling en plasma concentratie in jonge kinderen (deel III). Dit hoofdstuk vat deze studies en hun belangrijkste uitkomsten samen.

Hoofdstuk 1 geeft een samenvatting over het belang van de bepaling van lichaamssamenstelling bij baby's en jonge kinderen. De mogelijkheden, moeilijkheden en hiaten in kennis van de bestaande methodes om lichaamssamenstelling te bepalen worden bediscussieerd (deel I). Hoofdstuk 1 geeft ook een algemene introductie over de invloed van het vroege leven op de lichaamssamenstelling en metabole gezondheid op volwassenleeftijd. Het geeft tevens een samenvatting van verschillende bekende determinanten van deze programmering van lichaamssamenstelling en (over-) gewicht, als mede een overzicht van factoren die potentieel ook invloed zouden kunnen hebben hierop, maar nog niet eerder zijn opgehelderd. Dit alles om een introductie te geven over de parameters die besproken worden in de studies van dit proefschrift. Tenslotte worden de doelstellingen en hoofdlijnen van dit proefschrift beschreven.

Deel I – Bepaling van longitudinale lichaamssamenstelling bij baby's en jonge kinderen

Om baby's en kinderen met overmatig vet te herkennen, is het belangrijk om de hoeveelheid lichaamsvet accuraat te kunnen bepalen. Dual energy X-ray Absorptiometry (DXA) wordt meestal gebruikt als referentiemethode bij het bepalen van lichaamssamenstelling. Echter, referentiewaarden voor kinderen tussen de 2 – 5 jaar, die nodig zijn om de metingen te kunnen interpreteren, bestonden nog niet. Het doel van **Hoofdstuk 2** was om dit hiaat in de referentiewaarden op te vullen, door leeftijd- en geslacht-specifieke referentiewaarden en grafieken op te stellen voor vetmassa, vetpercentage, vetmassa-index, magere massa, magere massa-index, en botdichtheid voor kinderen van 2 – 5 jaar.

Door gebruik te maken van bijna 600 accurate DXA-metingen, afkomstig van 340 a-term geboren, gezonde kinderen tussen de leeftijd van 2 – 5 jaar, hebben we leeftijd- en geslacht-specifieke referentiewaarden en grafieken voor lichaamssamenstelling en botdichtheid geconstrueerd. Meisjes hadden een hogere vetmassa, vetpercentage en vetmassa-index, en een lagere magere massa en magere massa-index vergeleken met jongens. Het mineraalgehalte en de dichtheid van de botten steeg tussen 2 – 5 jaar en was gelijk bij jongens en meisjes.

In een aantal willekeurig gekozen 2-jarigen, vonden we dat de SD-scores, gebaseerd op deze nieuwe referentiewaardes, vergelijkbaar waren met SD-waardes als ze gebaseerd waren op onze eerder gepubliceerde referentiewaarden voor kinderen ≤2 jaar (in combinatie met een vacuümkussen om bewegingsartefacten te voorkomen).

Deze nieuwe referenties kunnen dus gebruikt worden in de gezondheidszorg en voor onderzoeksdoeleinden om lichaamssamenstelling en botontwikkeling longitudinaal te monitoren vanaf de geboorte tot op 5-jarige leeftijd. Hiermee kunnen baby's en jonge kinderen herkend worden die risico lopen om overmatig vet te ontwikkelen.

Meerdere methodes kunnen worden gebruikt om lichaamssamenstelling te bepalen. Naast DXA, wordt Air Displacement Plethysmography (ADP) hiervoor vaak gebruikt. Vanwege praktische beperkingen is het echter niet mogelijk om ADP longitudinaal te gebruiken vanaf de geboorte tot de adolescentie. Met de PEA POD kan ADP gebruikt worden bij baby's tot en met de leeftijd van 6 maanden. Met de BODPOD kan ADP gebruikt worden in kinderen vanaf 2 jaar. Het zou gunstig zijn als ADP en DXA uitwisselbaar gebruikt zouden kunnen worden om lichaamssamenstelling longitudinaal te monitoren tijdens de eerste jaren van het leven, met name in kinderen met een veranderde vetontwikkeling, zoals extreem prematuur geboren kinderen. In **Hoofdstuk 3** hebben we de meetresultaten afkomstig van ADP gemeten met de BODPOD vergeleken met de resultaten van de DXA (Lunar Prodigy met EnCore V14.1) in jonge, a-term en extreem prematuur (AD < 30 weken) geboren kinderen.

In 154 en 67 a-term en extreem prematuur geboren kinderen, resp. in de leeftijd van 3-5 jaar, vonden we dat vetmassa, vetpercentage en vet vrije massa resultaten van ADP en DXA significant verschillend waren, met zowel gefixeerde als geproportioneerde bias. In a-term geboren kinderen vonden we gemiddelde verschillen [LoA] van -1.08 [-2.92 - 0.76] kg, -5.78 [-16.25 - 4.69] % en 0.90 [-1.00 - 2.80] kg, resp. De verschillen tussen ADP en DXA waren zelfs groter in de prematuur

geboren groep, met gemiddelde verschillen [LoA] van -1.89 [-4.10 - 0.32] kg, -9.79 [-20.92 - 1.34] % en 1.64 [-0.63 - 3.91] kg, resp.

Het algoritme van de ADP software gebruikt standaard constante waardes (als schatting voor de dichtheid van de vetvrije massa (Dffm) om vetpercentage te berekenen. Deze constanten zijn gebaseerd op verouderde en kleine studies, waarin resultaten van gezonde oudere kinderen en volwassenen zijn geëxtrapoleerd naar kinderen jonger dan 8 jaar, gebruikmakend van 2-jaar-intervallen. Daardoor zouden ze mogelijk niet valide zijn in jongere kinderen. In de praktijk merkten we dat, met name kinderen met een afwijkende lichaamssamenstelling, zoals vroeggeboren kinderen, klinisch twijfelachtige ADP resultaten hadden (extreem lage waarden van vetpercentage, <5%). We hebben daarom het ADP algoritme verbeterd door de geslacht- en leeftijd specifieke Dffm-waardes te reviseren voor kinderen van 3-5 jaar. Met het gebruik van deze gereviseerde waardes kwamen de resultaten van ADP en DXA beter overeen, echter een kleine gefixeerde en geproportioneerde bias bleef bestaan. In extreem prematuur geboren kinderen bleven de verschillen tussen ADP en DXA groter.

Concluderend, ondanks het reviseren en verbeteren van de geslacht- en leeftijd specifieke Dffm-waardes, bleven de resultaten van ADP en DXA niet vergelijkbaar. Ze zouden daarom niet uitwisselbaar gebruikt mogen worden in de longitudinale bepaling van lichaamssamenstelling in kinderen in de leeftijd van 3-5 jaar, vooral niet in extreem prematuur geboren kinderen.

Wanneer geavanceerde technieken, zoals ADP en DXA niet beschikbaar zijn (bijvoorbeeld in de eerstelijns gezondheidszorg), worden vaak formules, gebaseerd op antropometrie, gebruikt om de vetmassa te berekenen. De bestaande formules zijn echter gebaseerd op gegevens van pasgeborenen en adolescenten en zijn daarom vaak gebiast en bevatten zowel willekeurige, als systemische fouten, wanneer ze gebruikt worden in andere leeftijdscategorieën. **Hoofdstuk 4** beschrijft de ontwikkeling en validatie van nieuwe formules om vetmassa en vetvrije massa te berekenen en de vergelijking met eerder gepubliceerde formules.

De nieuwe antropometrie-gebaseerde-formules (met daarin geslacht, leeftijd, gewicht, lengte en huidplooi-metingen) om vetmassa en vetvrije massa te berekenen werden ontwikkeld op basis van gegevens afkomstig van baby's van 6 weken en 3 maanden (N=55 en N=66). Hierbij werd ADP door PEA POD gebruikt als de maatstaaf. Vijfenzestig procent van de variantie in vetmassa en 79% van de variantie in vetvrije massa kon worden verklaard door deze formules.

Vervolgens werden deze formules gevalideerd in een onafhankelijk cohort (Sophia Pluto) op de leeftijd van 3 en 6 maanden (N=571 en N=447). Hierbij werd een kleinere bias gevonden vergeleken met 3 eerder gepubliceerde formules. Met een gemiddeld verschil [LoA] van 0.008 [-0.49 - 0.51] kg en 0.084 [-0.55 - 0.71] kg op de leeftijd van 3 en 6 maanden, resp. voor de berekening van vetmassa en 0.099 [-0.39 - 0.59] kg en -0.021 [-0.66 - 0.62] kg op de leeftijd van 3 en 6 maanden, resp. voor de berekening van vetvrije massa.

Concluderend, onze nieuwe antropometrie-gebaseerde formules laten een betere validiteit zien vergeleken met de bestaande formules en kunnen daarom worden gebruik om vetmassa en vetvrije massa te berekenen bij baby's, als ADP en DXA niet beschikbaar zijn.

Deel II – Determinanten in de vroege programmering van lichaamssamenstelling en (over-)gewicht

De eerste levensmaanden zijn een essentiële periode in de programmering en ontwikkeling van lichaamssamenstelling en (over-)gewicht later in het leven. Dit proces lijkt beïnvloed te worden door meerdere factoren.

Baby's met een hoog BMI hebben een hoog risico op het krijgen van overgewicht of obesitas als (jong-)volwassene. Het proces van de ontwikkeling van lichaamssamenstelling in het vroege leven is nog niet voldoende opgehelderd. In **Hoofdstuk 5** hebben we bij 224 kinderen geëvalueerd of degene met een hoog vetpercentage, vetmassa-index, vetvrije massa-index en abdominaal subcutaan en visceraal vet in de eerste maanden van het leven dat nog steeds hadden op de leeftijd van 4 jaar. We onderzochten ook of dit verschillend was tussen jongens en meisjes of tussen kinderen met verschillende soorten babyvoeding.

Gedurende de 4 jaar lange opvolging bleek dat gezonde a-term geboren baby's met een hoog vetpercentage op de leeftijd van 3 en 6 maanden, een hoog vetpercentage hielden op de leeftijd van 4 jaar (odds ratio (OR): 2.62 – 6.54, p <0.027). Als baby's veel abdominaal subcutaan vet hadden op de leeftijd van 6 maanden, dan was de kans groot dat ze dit op 4-jarige leeftijd nog steeds hadden (OR: 2.30, p = 0.035). Baby's met een hoge vetvrije massa op 1, 3 en 6 maanden, hadden dat nog steeds als 4-jarigen (OR 3.36 - 4.16, p <0.019). We vonden geen verschillen tussen jongens en meisjes. In tegenstelling tot kinderen die gedurende eerste 3 levensmaanden exclusief borstvoeding kregen, vonden we dat kinderen die dat niet hadden gekregen en een hoog vetpercentage hadden op de leeftijd van 3 en 6 maanden, een grote kans hadden om op 4 jaar nog steeds een hoog vetpercentage te hebben (OR: 4.00 - 7.33, p <0.006).

Dus, onze longitudinale gegevens ondersteunen zowel de aanwezigheid van een essentiële periode, in de eerste 6 levensmaanden, voor de programmering van zowel vetmassa als vetvrije massa later in het leven, als het beschermende effect dat exclusieve borstvoeding heeft op dit proces.

Een ongunstige lichaamssamenstelling met overtollig lichaamsvet en veel visceraal vet is geassocieerd met een ongunstige lipoproteïne profiel, met voornamelijk veel LDL cholesterol. Niet alleen de standaard lipoproteïnen, maar ook honderden andere lipide soorten kunnen worden gevonden in plasma van baby's. Dit metabool profiel, zou op jonge leeftijd al een afspiegeling kunnen zijn voor de ontwikkeling van (over-)gewicht en de lichaamssamenstelling op latere leeftijd. Dit zouden, daarom, potentiele biomarkers kunnen zijn voor de ontwikkeling van ongunstige metabole uitkomsten in de toekomst. In **Hoofdstuk 6** onderzochten we of het metabool profiel op 3 maanden voorspellend was voor lichaamssamenstelling op 2 jaar en of er verschillen waren tussen jongens en meisjes of tussen kinderen met verschillende soorten babyvoeding.

We vonden een plasma metabolieten profiel (inclusief LysoPS(22:2), dimethylarginine en 13 andere metabolieten) op 3 maanden dat in enige mate voorspellend was voor de lichaamssamenstelling op 2 jaar, gemeten als romp : perifere huidplooi-ratio (T:P-ratio). Dit model had een voorspellende waarde van 75.8%, een sensitiviteit van 100% en een specificiteit van 50%. Elf van de 15 meest geassocieerde metaboliet variabelen waren ook geassocieerd met visceraal vet op 2 jaar.

Daarnaast vonden we dat de voorspellende waarde van het model hoger was bij jongens dan bij meisjes. Het voorspelmodel was tevens onafhankelijk van voedingstype, want we vonden enkel verschil in relatieve aanwezigheid van 2 van de 15 metabolieten tussen baby's met exclusieve borst- en flesvoeding.

Opvallend was dat 5 van de geïdentificeerde metabolieten betrokken zijn bij inflammatoire processen en/of pro-inflammatoire cascades in pre-adipocyten. Onze bevindingen suggereren daarom dat de gevonden metabolieten betrokken zijn bij zowel de ontwikkeling van lichaamssamenstelling en (over-)gewicht, als bij laaggradig inflammatoire processen, al vanaf het vroege leven.

Concluderend vonden we dat het metabool profiel op 3 maanden in enige mate de lichaamssamenstelling, gemeten als T:P-ratio, op 2 jaar kan voorspellen. Elf van de 15 meest geassocieerde metabolieten waren ook geassocieerd met

visceraal vet op 2 jaar en van 5 is tevens bekend dat ze betrokken zijn bij (pro) inflammatoire processen. Onze bevindingen dragen bij aan de inzichten in de programmering van lichaamssamenstelling en (over-)gewicht tijdens de eerste maanden van het leven.

Vervolgens hebben we, in **Hoofdstuk 7**, onderzocht of plasma metabolieten op de leeftijd van 3 maanden ook geassocieerd waren met vetmassa en vetvrije massa gemeten met DXA en met subcutaan en visceraal vet gemeten middels abdominale echografie op de leeftijd van 2 jaar. Daarnaast hebben we bestudeerd of deze associaties verschillend zijn tussen kinderen met exclusieve borstvoeding gedurende de eerste 3 levensmaanden en kinderen met exclusieve flesvoeding.

In 318 gezonde a-term geboren kinderen hebben we, op de leeftijd van 3 maanden, onderscheidende plasma metabolieten geïdentificeerd die waren geassocieerd met een hoog vetmassa-index, vetpercentage, vetvrije massa-index en visceraal vet op de leeftijd van 2 jaar. Deze metabolieten bestonden uit verschillende klassen van lyso-fosfolipiden. Verder vonden we dat de meeste associaties aanwezig waren bij kinderen die exclusieve borstvoeding kregen gedurende de eerste 3 levensmaanden, in tegenstelling tot degene met exclusieve flesvoeding.

Deze bevindingen suggereren dat fosfolipiden betrokken zijn bij de vroege programmering van lichaamssamenstelling en dat de aanvoer en incorporatie van deze fosfolipiden verschillend is tussen kinderen met borst- en flesvoeding. Daarnaast suggereren deze bevindingen dat babyvoeding in het vroege leven geassocieerd is met verschillen in metabool profiel en dat het mogelijk ook betrokken is in verschillen in programmering van lichaamssamenstelling tussen kinderen met exclusieve borst- en flesvoeding.

Concluderend, associaties tussen plasma metabolieten op de leeftijd van 3 maanden en veel lichaamsvet op de leeftijd van 2 jaar zijn afhankelijk van de soort babyvoeding. Deze bevindingen dragen bij aan onze inzichten in het belang van babyvoeding op de programmering van lichaamssamenstelling in het vroege leven.

Deel III – PFAS concentraties in jonge kinderen

Per- en polyfluoralkylstoffen (PFAS) zijn door de mens ontwikkelde chemische verbindingen die niet-afbreekbaar zijn en een eliminatie halfwaardetijd hebben van meerdere jaren. Hierdoor veroorzaken ze stapeling in het milieu en in de mens. Knaagdierstudies laten zien dat PFAS zijn geassocieerd met zorgelijke effecten op metabole processen en de algehele ontwikkeling van kinderen, vooral wanneer

de PFAS blootstelling plaatsvond gedurende de eerste levensmaanden. Omdat er geen longitudinale gegevens in baby's bekend waren, hebben we, in **Hoofdstuk 8**, de longitudinale plasma concentraties in de eerste 2 levensjaren en de belangrijkste determinanten hiervan, onderzocht.

We bepaalden de plasma concentraties van PFOS, PFOA, PFHxS, PFNA en PFDA op de leeftijd van 3 maanden en 2 jaar, in 369 gezonde a-term geboren kinderen, middels liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS). We vonden dat op de leeftijd van 3 maanden, de PFAS concentraties hoger waren in eerstgeborenen van oudere Kaukasische moeders, mogelijk omdat deze moeders meer PFAS stapeling en daardoor meer trans-placentaire transmissie naar hun kind hadden. Plasma concentraties op 3 maanden en 2 jaar waren sterk gecorreleerd (R=0.46 - 0.82) en daalden slechts licht gedurende deze periode.

Als kinderen exclusief borstvoeding kregen gedurende de eerste 3 levensmaanden (EBF), dan hadden ze 2-3x hogere PFAS plasmaconcentraties dan kinderen die exclusief flesvoeding kregen (EFF), met PFOA concentraties van 3.72 ng/mL versus 1.26 ng/mL op de leeftijd van 3 maanden en 3.15 ng/mL versus 1.22 ng/mL op 2 jaar.

Dus, onze bevindingen wijzen erop dat niet alleen trans-placentaire transmissie, maar ook borstvoeding de belangrijkste determinanten voor PFAS blootstelling zijn tijdens de eerste levensjaren.

Ondanks dat borstvoeding mogelijk een grote bijdrage levert aan de PFAS blootstelling in het vroege leven, zijn longitudinale gegevens over PFAS concentraties in moedermelk en de dagelijkse PFAS inname via babyvoeding nog nooit eerder beschreven in een groot cohort van gezonde kinderen.

In **Hoofdstuk 9** hebben we PFOA en PFOS concentraties bepaald in moedermelk op de leeftijd van 1 en 3 maanden en in 6 verschillende merken kunstvoeding, middels LC-ESI-MS/MS. Ook hebben we de associaties tussen dagelijkse PFAS inname, babyvoeding en andere belangrijke determinanten bepaald, als ook de correlatie met plasma concentraties tot de leeftijd van 2 jaar.

Alhoewel PFOA en PFOS niet werden gedetecteerd in kunstvoeding, omdat de concentraties lager waren dan de onderste detectiegrens, bevatte moedermelk weldegelijk PFOA en PFOS. De concentraties daalden tussen de leeftijd van 1 en 3 maanden, ongeacht of de moedermelk exclusief werd gegeven of in combinatie met kunstvoeding.

De dagelijkse PFAS inname (ng/kg) op 3 maanden was zesmaal hoger in EBF-kinderen vergelijken met EFF-kinderen. Bij kinderen die zowel borst- als flesvoeding kregen, was met name een lagere hoeveelheid kunstvoeding geassocieerd met een hogere dagelijkse PFAS inname. Ook was de dagelijkse PFAS inname via moedermelk hoger wanneer deze afkomstig was van oudere moeders die niet eerder een kind hadden gekregen of borstvoeding hadden gegeven.

Tenslotte vonden we dat de dagelijkse PFAS inname op 3 maanden niet alleen sterk gecorreleerd was met plasma concentraties op dezelfde leeftijd (R=0.836 - 0.875, p <0.001), maar ook met plasma concentraties op 2-jarige leeftijd (R=0.642 - 0.692, p <0.001).

Dus, onze bevindingen suggereren dat PFAS blootstelling via kunstvoeding verwaarloosbaar is en ze bevestigen dat borstvoeding een belangrijke PFAS blootstellingsroute is gedurende de eerste levensmaanden, met potentieel blijvende effecten.

In **Hoofdstuk 10**, bediscussiëren we de belangrijkste bevindingen van onze studies in bredere context en vergelijken we deze met recente literatuur. Ook bespreken we de klinische implicaties en suggesties voor toekomstig onderzoek.

Appendices

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List of abbreviations

AD	Amenorrhea duration
ADP	Air Displacement Plethysmography
ANOVA	Analysis of variance
ASF	Abdominal subcutaneous fat
BCAA	Branched chain amino acid
BCCG	Box-Cox Cole and Green distribution
BD	Body density
BIA	Bioelectrical Impedance Analysis
BMC	Bone mineral content
BMD_{TBLH}	Total body less head bone mineral density
BMI	Body mass index
BPD	Bronchopulmonary dysplasia
BV	Body volume
BW	Body weight
CBGS	Cambridge Baby Growth Study
CI	Confidence interval
CV%	Coefficient of variance
Dffm	Fat free mass density
Dfm	Fat mass density
DG	Diacylglycerol
DXA	Dual energy X-ray Absorptiometry
EBF	Exclusive breastfeeding
EDC	Endocrine disrupting chemical
EFF	Exclusive formula feeding
EFSA	European Food Safety Authority
FDR	False discovery rate
FM	Fat mass
FM%	Fat mass percentage
FMI	Fat mass index
GA	Gestational age

GAMLSS	Generalized additive models for location, scale and shape
GLM	Generalized linear model
GPCR	G protein-coupled receptor
ICC	Intra-class correlation coefficient
IQR	Interquartile range
LBM	Lean body mass
LBMI	Lean body mass index
LC-ESI-MS/MS	Liquid chromatography-electrospray-ionization tandem mass spectrometry
LC-MS	Liquid chromatography with mass spectrometry
LDL	Low density lipoprotein
LloQ	Lower limit of quantification
LoA	Limits of agreement
LysoPA	Lysophosphosphatidic acid
LysoPE	Lysophosphatidylethanolamine
LysoPG	Lysophosphatidylglycerol
LysoPS	Lysophosphatidylserine
m/z	Mass-to-charge-ratio
MFGM	Milk fat globule membrane
N	Number
NCD	Non-communicable disease
OR	Odds ratio
PC	Phosphatidylcholine
PCA	Principal component analysis
PFAS	Per- and polyfluoroalkyl substances
PFDA	Perfluorodecanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PLS-DA	Partial least squares - discriminant analyses
POP	Persistent Organic Pollutants

PPAR-γ	Peroxisome proliferator-activated receptor gamma
QMR	Quantitative nuclear Magnetic Resonance
RMSE	Root mean squared error
Rt	Retention time
S:V-ratio	Subcutaneous : visceral fat ratio
SD	Standard deviation
SDS	Standard deviation score
SE	Standard error
SFT	skinfold thickness
SM	Sphingomyelin
T:P-ratio	Truncal : peripheral fat skinfold ratio
TLR	Toll-like receptor
TOBEC	Total Body Electrical Conductivity
TWI	Tolerable weekly intake
UV	Unit variance
WHO	World Health Organization

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PhD portfolio

Name: Inge Aloisia Lucia Petronella van Beijsterveldt

Promotor: Prof. dr. A.C.S. Hokken-Koelega

Copromotor: Dr. M. van der Steen

Affiliations: Department of Pediatrics, Subdivision of Endocrinology, Erasmus MC Sophia Children's Hospital, University Medical Center, Rotterdam, The Netherlands

PhD period: September 2017 – December 2021

Summary of PhD training:

	Year	WorkLoAd (ECTS)
General courses		
Medical Research Involving Human Subjects Act (BROK), WMO, Erasmus MC	2017	1.5
Biostatistical Methods I: Basic Principles, NIHES, Erasmus MC	2018	5.7
PhD-course on Scientific Integrity, Erasmus MC	2019	0.3
Specific courses		
Radiation protection 5R, Zorgacademie, Erasmus MC	2017	0.5
Basic Introduction to SPSS, Molmed, Erasmus MC	2017	1.0
Open Clinica, Erasmus MC	2018	0.1
Epigenetic regulation in health and disease, Boerhaave, LUMC	2018	0.8
Basic and translational Endocrinology, Molmed, Erasmus MC	2019	2.2
Course Microbiomics for PhD students, Molmed, Erasmus MC	2019	0.6
Basic Course on 'R' , Molmed, Erasmus MC	2020	2.0
Seminars and workshops		
Annual Research Day, Sophia Children's hospital	2019-2020	0.6
Weekly research meetings, department of Pediatric Endocrinology	2017-2021	4.0
International conferences		
5th International conference Nutrition & Growth, Paris, France (poster presentation)	2018	1.0
57th Meeting of the European Society of Pediatric Endocrinology (ESPE), Athens, Greece (poster presentation)	2018	1.0
6th International conference Nutrition & Growth, Valencia, Spain (oral presentation)	2019	1.0

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	Year	WorkLoAd (ECTS)
58th Meeting of the European Society of Pediatric Endocrinology (ESPE), Vienna Austria (poster presentation)	2019	1.0
5th JPI HDHL conference, Brussels, Belgium (vlog contest for young researchers)	2019	1.5
7th International conference Nutrition & Growth, online due to Covid-19 pandemic (poster presentation)	2020	1.0
8th International conference Nutrition & Growth, online due to Covid-19 pandemic (2x oral presentation)	2021	1.5
59th Meeting of the European Society of Pediatric Endocrinology (ESPE), online due to Covid-19 pandemic (oral and poster presentation)	2021	1.5
9th International conference Nutrition & Growth, online due to Covid-19 pandemic (oral presentation)	2022	1.0
Teaching activities		
IMC Weekend school 'Growth and Development', Rotterdam	2019	0.1
Supervising 3 Master Thesis students, Erasmus MC	2020-2021	4.0
Coaching Medical students during Bachelor, Erasmus MC	2020-2021	0.5
Other		
5th JPI HDHL conference, *Runner-up vlog contest*	2019	
Radiation protection supervisor	2020-2021	1.0
Peer review of manuscripts for scientific journal	2021	0.2
60th Meeting of the European Society of Pediatric Endocrinology (ESPE), *Awarded with Registration Grant*	2021	
9th International conference Nutrition & Growth *Awarded with Educational Grant for best abstract*	2022	

ETCS (European Credit Transfer and Accumulation System) are training credits.
One ECTS stands for approximately 28 working hours.

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About the author

Inge van Beijsterveldt was born on August 22nd, 1990, in Oosterhout (NB), The Netherlands. After graduating in 2008 from Mgr. Frencken College in Oosterhout, she moved to Leuven (Be) to study Biomedical sciences at KU Leuven. In 2009, she moved back to The Netherlands and studied Medicine at Erasmus University Rotterdam. In 2015 she obtained her medical degree, after which she worked as resident (ANIOS) at the department of pediatrics at Elisabeth-Tweesteden hospital in Tilburg and at the neonatal intensive care unit at Raboud University Medical Center – Amalia Children's Hospital in Nijmegen. In 2017 she took on a PhD position at the department of pediatric endocrinology of Erasmus University Medical Center – Sophia Children's Hospital in Rotterdam, under supervision of prof. dr. A.C.S. Hokken-Koelega and dr. M. van der Steen. During her PhD period, she was part of the research group conducting the Sophia Pluto study; a large prospective birth cohort study focusing on growth and body composition trajectories of healthy children aged 0-5 years in the Rotterdam area, The Netherlands. Furthermore, she worked on the BioFN project, an international consortium studying early biomarkers in adiposity programming. Inge particularly investigated methods to measure body composition in young children and early determinants of adiposity programming, which resulted in this thesis.

During her PhD period Inge also worked as a volunteer at Stichting de Kindertelefoon. Where she talked with children about 'everything' and trained new volunteers. Furthermore, especially during the COVID pandemic, she developed a peculiar interest in growing (house)plants.
Following this PhD period, Inge will continue to pursuit her dream to become a pediatrician.