Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Effect of changing the n-6/n-3 fatty acid ratio in the maternal diet during pregnancy and lactation on child body composition and neurodevelopment: long-term results from the INFAT study

Christina Brei

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"Life can only be understood backwards; but it must be lived forwards."

– Søren Kiergegard

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Abstract

During the periconceptional, prenatal and early postnatal periods, maternal and environmental factors can have an impact on health outcomes later in life. Evidence regarding the impact of n-3 long-chain polyunsaturated fatty acids (PUFAs) during pregnancy/lactation on offsprings' body composition and neurodevelopment is currently inconclusive.

The INFAT (Impact of Nutritional Fatty acids during pregnancy and lactation on early human Adipose Tissue development) study investigated the effect of a decreased n-6/n-3 FA ratio in maternal diet during pregnancy/lactation on infant adipose tissue (AT) growth and fat distribution up to 1 year (y) of age. Healthy pregnant women (n=208) were randomly assigned to an intervention (1.2 g n-3 long-chain PUFAs as fish oil supplements per day together with an arachidonic acid balanced diet from the 15th week (wk) of gestation to 4 months postpartum) or control group. Direct and indirect measuring techniques were applied, such as ultrasound (US). A follow-up study investigated long-term effects of the intervention on body composition and additional outcomes such as neurodevelopment up to 5 y of age. This thesis addressed the following issues: Verification of US as a feasible method for the assessment of abdominal fat distribution in early infancy ($\leq$ 1 y) and analysis of longitudinal sonographic data on abdominal AT growth (6 wk–5 y). Assessment of long-term effects of the intervention on offsprings' body composition (2–5 y) and neurodevelopment (4 and 5 y).

Strong inter- and intra-observer agreement (0.97–0.99) indicated that the measurements of fat areas were reproducible at 6 wk–1 y of life. Results suggested a differential growth of subcutaneous and preperitoneal fat depots. Compared to boys, girls had significantly higher subcutaneous fat areas from 6 wk onwards and significantly higher preperitoneal fat areas at 3, 4, and 5 y. The intervention had no significant impact on children's sum of 4 skinfold thickness measurements, consistent with other measured anthropometric parameters at any time point in the adjusted model. There were largely no significant differences by treatment group for the applied neurodevelopmental tests (child development inventory, mirror movement test).

In conclusion, the sonographic method was found to be feasible and reproducible in early infancy. Further, the analysis revealed age- and sex-dependent development of the fat compartments. The INFAT study provides no evidence that a dietary reduction of the n-6/n-3 long-chain PUFA ratio during pregnancy/lactation has long-term effects on body composition or clinically relevant effects on neurodevelopment in healthy, predominantly term-born preschool children.

Zusammenfassung

Mütterliche und umweltbedingte Faktoren in der perikonzeptionellen, prä- und frühen post-natalen Periode scheinen die Gesundheit im späteren Leben zu beeinflussen. Der Effekt langkettiger mehrfach ungesättigter n-3 Fettsäuren (PUFAs) während der Schwangerschaft/ Stillzeit auf die Körperzusammensetzung und die neurologische Entwicklung der Nachkommen ist nicht eindeutig.

Die INFAT (Impact of Nutritional Fatty acids during pregnancy and lactation on early human Adipose Tissue development) Studie untersuchte den Effekt einer diätetischen Reduktion des n-6/n-3 Fettsäurequotienten während der Schwangerschaft/Stillzeit auf die kindliche Fett-gewebsentwicklung bis zum ersten Lebensjahr (LJ). Gesunde, schwangere Frauen (n=208) wurden in eine Interventions- (Supplementation von 1,2 g langkettigen n-3 PUFAs/Tag sowie eine moderate Arachidonsäurezufuhr zwischen der 15. Schwangerschaftswoche (SSW) und dem 4. Monat postpartum) oder Kontrollgruppe randomisiert. Zur Fettgewebsbestimmung wurden direkte und indirekte Methoden eingesetzt, wie z.B. Ultraschall (US). Ein Follow-up bis zum fünften LJ untersuchte langfristige Auswirkungen auf die Körperzusammensetzung sowie die neurologische Entwicklung. Ziele dieser Arbeit waren: Verifizierung, ob US eine praktikable Methode zur Erfassung der abdominellen Fettverteilung ≤ 1. LJ ist sowie die longitudinale Analyse des Fettgewebswachstums (6. Woche–5. LJ). Bewertung der Langzeit-effekte der Intervention auf die Körperzusammensetzung (2.–5. LJ) und die neurologische Entwicklung (4. und 5. LJ) der Nachkommen.

Im ersten LJ wiesen die Inter- und Intraklassenkorrelationskoeffizienten ein hohes Maß an Übereinstimmung auf (0,97–0,99). Die Daten zeigten ein differenzielles Wachstum der subkutanen und präperitonealen Fettdepots. Mädchen hatten im Vergleich zu Jungen ab der 6. Woche signifikant mehr subkutanes sowie ab dem 3. LJ signifikant mehr präperitoneales Fettgewebe. Zu keinem Zeitpunkt hatte die Intervention einen signifikanten Effekt auf die Summe der vier Hautfalten, konform mit anderen anthropometrischen Parametern im adjustierten Modell. Die neurologischen Tests zeigten größtenteils keine signifikanten Gruppenunterschiede (Fragebogen zur kindl. Entwicklung, spiegelbildliche Mitbewegung).

Zusammenfassend erwies sich die US-Methode im frühen Kindesalter als praktikabel und reproduzierbar. Die Fettkompartimente entwickelten sich abhängig von Alter und Geschlecht. Mit einer diätetischen Reduktion des n-6/n-3 Fettsäurequotienten in der Schwangerschaft/ Stillzeit liefert die INFAT-Studie keine Evidenz für Langzeiteffekte auf die Körper-zusammensetzung sowie klinisch relevante Effekte auf die neurologische Entwicklung gesunder, überwiegend reifgeborener Vorschulkinder.

Abbreviations

AA	Arachidonic acid (C20:4n-6)
ALA	α-linolenic acid (C18:3n-3)
AT	Adipose tissue
BMI	Body mass index
CDI	Child development inventory
CT	Computer tomography
DHA	Docosahexaenoic acid (C22:6n-3)
DOMInO	DHA to Optimize Mother Infant Outcome
DXA	Dual energy x-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid (C20:5n-3)
FA	Fatty acid
FADS	Fatty acid desaturase
FAME	Fatty acid methyl esters
FKE	Forschungsinstitut für Kinderernährung, Research Institute of Child Nutrition
INFAT	Impact of Nutritional Fatty acids during pregnancy and lactation on early human Adipose Tissue development
ISSFAL	Congress of the International Society for the Study of Fatty Acids and Lipids
LA	Linoleic acid (C18:2n-6)
MM	Mirror movement
mo	Month
MRI	Magnetic resonance imaging
NAT	Nonadipose tissue
PA	Physical activity

PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RCT	Randomized controlled trial
SAT	Subcutaneous adipose tissue
SFT	Skinfold thickness
US	Ultrasound
VAT	Visceral adipose tissue
wk	Week
y	Year

1 Introduction

Given the high rates of childhood obesity (Ahrens et al. 2014), the importance of obesity prevention in early infancy and childhood has increasingly been recognized. There is strong evidence that obesity can persist from childhood to adolescence and into adulthood (Durmus et al. 2010; Péneau et al. 2011; Freedman et al. 2005). A recent meta-analysis with data from 23 studies (16 cohorts) confirmed the association; it estimates that obese children are five times as likely to become obese as adults compared to non-obese children (relative risk, 5.21; 95% CI: 4.50, 6.02) (Simmonds et al. 2015). At the same time, suitable, valid and easy to handle methods for the assessment of body composition and fat distribution in early infancy up to adolescence are required (Holzhauer et al. 2009).

The first 1000 days, defined as the period between conception and one's second birthday, are particularly relevant in the context of the Developmental Origins of Health and Disease hypothesis (Gillman et al. 2007). Research over the last 30 years (y) has shown that the prenatal and early postnatal phases are windows of opportunity, where the cornerstones for optimum health, growth, and neurodevelopment are set (Bay et al. 2016).

The quality and quantity of dietary intake of the expectant mother are considered one of the most influential factors for optimal fetal growth and development (Wood-Bradley et al. 2013). Previous research has established that an imbalanced intake of nutrients, leading either to fetal undernutrition (due to maternal nutritional imbalances or placental dysfunction) or fetal overnutrition (due to maternal obesity, excessive gestational weight gain, diet in pregnancy or gestational diabetes mellitus), can negatively impact on long-term offspring health. Both conditions are associated, for example, with an increased risk of overweight/obesity and other non-communicable diseases, such as coronary heart disease, hypertension, and diabetes mellitus (Alfaradhi and Ozanne 2011; Koletzko et al. 2011).

Therefore, the identification of the ideal nutrient composition of maternal diet for optimal offspring development is of strong interest (Blumfield et al. 2012a). Previous research has focused not only on the effect of maternal energy intake, macronutrient composition or their dietary patterns but also on individual nutritional components (Poston 2012; Murrin et al. 2015; Veena et al. 2016). In this context, specific emphasis has been placed on maternal and fetal/infant essential n-3 and n-6 long-chain polyunsaturated fatty acids (PUFAs) (Demmelmair and Koletzko 2015).

1.1   Long-chain PUFAs in the prenatal and early postnatal period

The n-3 long-chain PUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), as well as the n-6 long-chain PUFA arachidonic acid (AA, 22:5n-6), are critical nutrients for humans. Due to the lack of delta-12 and -15 desaturases in mammals, cells are not able to synthesize them de novo. They are derived from their precursors, α-linolenic acid (ALA, C18:3n-3) and linoleic acid (LA, C18:2n-6) (Innis 2005; Lee et al. 2016) and are generated through alternate elongation and desaturation steps by competing for the same set of enzymes. Conversion rates are estimated to be low, ranging from 0.1–10 %. They also depend on polymorphisms in the fatty acid desaturase (FADS) 1 and FADS2 gene cluster, which encode these enzymes (Xie and Innis 2008). Thus, dietary intake of pre-formed long-chain PUFAs are the primary determinant and are therefore essential (Koletzko et al. 2014; Gibson et al. 2011). Long-chain PUFAs have multiple functions and are required e.g. for energy storage/supply, oxygen transport, and cell membrane function. They act further as precursors to eicosanoids, which are important regulatory signals involved in several processes, such as immune response, inflammation and cell proliferation (Lunn and Theobald 2006). Particularly DHA serves as a precursor for docosanoids that are mainly known to evoke anti-inflammatory mechanisms (Serhan 2014). Due to being major constituents of membrane phospholipids, essential for growth, eye and the central nervous system regarding function and development, a sufficient supply, particularly during the pre- and postnatal period, is necessary (Gibson et al. 2011).

During pregnancy, long-chain PUFAs are transmitted from the expectant mother to the fetus across the placenta. It is suggested that the amount primarily depends on the maternal habitual diet before and during pregnancy. To prevent the human fetus from deficiency and to further optimize the delivery, various adaptive mechanisms have been identified, emphasizing the relevance of these fatty acids (FAs). Discussed mechanisms include the mobilization of maternal stores, long-chain PUFA synthesis and a preferential placental uptake and transfer of long-chain PUFAs from the mother to the fetus (Haggarty 2010; Gil-Sanchez et al. 2011). They are predominantly stored in skeletal muscle, skin, liver and brain tissue. However, in term infants, LA, AA, and DHA are mostly located in adipose tissue (AT), accounting for 68, 44 and 50 %, respectively (Kuipers et al. 2012). These concentrations are much higher than found in adults, and therefore AT is considered an important postnatal mobilizable storage (Haggarty 2014).

Postnatally, FA supply is compensated by FAs coming from infant formulas enriched in AA and DHA (Brenna et al. 2007) or from breastmilk. Breastmilk contains a mean ($\pm$ SD) concentration of DHA of 0.37 $\pm$ 0.11 % (weight percentage of total FAs) and AA of 0.55 $\pm$ 0.14 % (Fu et al. 2016).

Recommendations for fetal requirements for long-chain PUFAs during pregnancy are mainly based on observed accretion rates during gestation. Before the 25th week (wk) of gestation, lipid accumulation is relatively low, but it then rapidly increases with maximal accretion rates of about 7 g/day. Concerning DHA, estimated requirements rise from approximately 100 mg/day at 25th wk to 300 mg/day just before term (Haggarty 2014). Daily intake recommendations differ slightly between national and international organizations and societies (FAO 2010; Koletzko et al. 2013; Koletzko et al. 2014; GOED 2014; EFSA Panel on Dietetic Products Nutrition and Allergies (NDA) 2010).

In Germany, an average daily supply of at least 200 mg DHA during pregnancy and lactation is advised. The recommendation is achieved by a balanced and varied diet along with two portions fish per week, one of them oily. An exception being specific target groups, such as vegetarians or vegans, who do probably not meet the recommended amount. In this case, supplements are alternatively suggested (Koletzko et al. 2013; D-A-CH 2015).

Today's scientific interest on the fetal requirement of n-3 long-chain PUFAs is more focused on the optimization of health outcomes than on demand, which is necessary to avoid symptoms of deficiency (Haggarty 2014). The first evidence that the perinatal FA status is positively related to infant development came from observational studies. In one such study, Olsen et al. linked n-3 supplementation during pregnancy with better pregnancy outcomes via a prolonged gestation (Olsen et al. 1986). At present, research focuses primarily on three fields, namely atopic disease, the impact on growth and body composition/prevention of obesity, and neurological development (Demmelmair and Koletzko 2015).

This work aimed to address the two latter aspects, which will be briefly introduced.

1.2   Long-chain PUFAs and AT development

1.2.1   The origins of AT growth

Adipose tissue growth is characterized by dynamic changes and a sexual dimorphism in the distribution pattern (Ailhaud et al. 2006) and involves two mechanisms: firstly, an increase in cell number (hyperplasia) and secondly, an increase in cell volume (hypertrophy) (Jo et al. 2009). Both hyperplasia and hypertrophy seem to determine human fat mass (Spalding et al. 2008). Hyperplasia is mainly attributed to the two periods of life with the highest proliferation and differentiation capacity, namely the first year of life and the stages of pre-puberty (9–13 y) (Hauner et al. 1989; Salans et al. 1973). Changes in fat mass in adults can be primarily attributed to changes in fat cell volume. Even following weight loss in adulthood, fat cell number remains constant (Spalding et al. 2008). When it comes to AT expansion in adulthood, however, an *in vitro* study could show that the number of stroma cells and adipocyte number increase, albeit, to a lesser extent than fat cell size (van Harmelen et al. 2003).

Data indicated that AT development in utero first appears between 14–16th wk of gestation and progressively develops in fat cell number up to the 23rd wk of gestation. Thus, the time between the 14th and 23rd wk of gestation has been suggested as a susceptible period for early programming in fat lobule development (Poissonnet et al. 1983; Poissonnet et al. 1984).

1.2.2   Rationale for the impact of fatty acids on early AT growth

In 2004, Ailhaud and Guesnet introduced the hypothesis that a reduced dietary n-6/n-3 FA ratio in the fetal and early postnatal period may limit AT growth and thereby might constitute a primary prevention strategy against childhood obesity (Ailhaud and Guesnet 2004). The biological basis for the hypothesis came from several *in vitro* and animal studies, accompanied by epidemiological findings.

In the preadipocyte stage*, in vitro* studies in rodents and humans have identified AA through the precursor for prostacyclin as an adipogenic component. Prostacyclin activates through the IP/PC system and the protein kinase A pathway the upregulation of the expression of the transcription factors CCAAT/enhancer binding protein β and C/EBPδ, both stimulatory determinants for the peroxisome proliferator-activated receptor (PPAR) γ. PPARγ is a transcription factor critically required for adipogenesis. Further, prostacyclin triggers the expression of PPARβ/δ, which also results in an upregulation of PPARγ (Madsen et al. 2005; Ailhaud et al. 2006). Compared to this, EPA and DHA act by inhibiting the stimulatory effect

at multiple steps (Simopoulos 2016). These data were confirmed in an animal model investigating the impact of two different n-6/n-3 ratios (59:1 vs. 2:1) in the maternal diet during pre-pregnancy up to suckling period as well as in the diet of the offspring mice up to 22 wk of age on offspring body composition. The study showed less body weight and total body fat mass in the n-3-rich diet group up to adulthood, suggesting that a balanced n-6 to n-3 FA ratio in critical phases of AT development is of importance (Massiera et al. 2003). To date, several animal studies supplementing n-3 long-chain PUFAs during pregnancy and/or lactation have been carried out that have consistently observed a reduction in offspring fat mass (Oosting et al. 2010; Korotkova et al. 2002; Wyrwoll et al. 2006). However, in contrast, other studies have observed no effect (Ibrahim et al. 2009) or negative implications (Muhlhausler et al. 2011b). The substantial disparity in study design among these studies might be accountable for this inconsistency; thus, definitive evidence from animal studies is still lacking (Muhlhausler et al. 2011a).

Epidemiological data from westernized countries show that the n-6/n-3 FA ratio has progressively increased via a high supply of LA/AA-rich food items, whereas the content of n-3 FAs has not changed in a simultaneous way (Blasbalg et al. 2011; Sanders 2000). This shift is also reflected in breast milk, a parameter that is considered to give a good reflection of dietary FA intakes (Ailhaud et al. 2006; Sanders 2000).

Based on these findings along with the considerable rise of obesity, the authors concluded that these changes in dietary patterns lead to an altered n-6/n-3 FA ratio. This modification could enhance the development of AT growth in early infancy and, thus, result in obesity in the adult age (Ailhaud and Guesnet 2004).

In recent years, several observational studies and prospective human randomized controlled trials (RCTs) have been carried out to investigate the cause-and-effect relation of a dietary intervention of long-chain PUFA supplementation during pregnancy and/or lactation on offspring fat mass (Hauner and Brunner 2015). However, this includes post-hoc analyses of studies that were primarily designed to examine other outcomes such as infant neurodevelopment (Asserhoj et al. 2009; Campoy et al. 2011; Escolano-Margarit et al. 2011; Helland et al. 2008; Rytter et al. 2011; Donahue et al. 2011; Moon et al. 2013). Based on several reviews and one meta-analysis, there is currently not conclusive evidence to support or refute a favorable effect of n-3 long-chain PUFAs on AT growth (Muhlhausler et al. 2010; Stratakis et al. 2014; Voortman et al. 2015; Rodriguez et al. 2012; Hauner et al. 2013). Due to high heterogeneity between RCTs and methodology limitations, such as small sample sizes or

selective attrition rates, the authors from the first meta-analysis in 2014 claimed that there is a need for additional high-quality studies with a particular focus on long-term effects (Stratakis et al. 2014).

1.2.3 Assessment of body composition during infancy and childhood

There is a broad range of body composition measuring techniques in infancy and early childhood available, with all approaches having specific advantages and limitations. Thus, the choice of measurement technique depends mostly on the research question, study group, setting as well as technical and financial criteria (Weber et al. 2012; Toro-Ramos et al. 2015; Horan et al. 2015).

In brief, simple anthropometry includes length/height, body weight, several circumferences and skinfold thickness (SFT) measurements. From the latter, a predictive technique, body fat and lean body mass can be indirectly predicted via equations. Further approaches to quantify the amount and relative proportions of body tissue compartments include for example bioelectrical impedance analysis, air displacement plethysmography via Bodpod/Peapod and dual energy x-ray absorptiometry (DXA) (Wells and Fewtrell 2006; Wells 2012).

Beyond the measurement of body fatness, the distribution of AT is of interest, as differences among fat deposits are observed. Especially visceral fat, located in the trunk, is related to adverse health effects such as cardiovascular disease and type 2 diabetes mellitus (He et al. 2007; Dencker et al. 2012). While waist circumference is used as an indirect and crude alternative for the assessment of body fat distribution, a distinction between subcutaneous and visceral fat is thereby not possible (Toro-Ramos et al. 2015). For quantifying fat distribution, magnetic resonance imaging (MRI) and computer tomography (CT) as direct measures are considered as gold standards (Wells 2012). Further approaches include the estimation of visceral fat mass via DXA (Micklesfield et al. 2012) or ultrasonography for the assessment of visceral and preperitoneal fat mass (Horan et al. 2015). The latter provides a good approximation of visceral fat mass (Mook-Kanamori et al. 2009).

1.3 Long-chain PUFAs and brain development

The dry weight of the human adult brain consists of 50–60 % of lipids. With 20–25 % of lipids, long-chain PUFAs account for the largest proportion, with highest levels of DHA and AA (Lauritzen et al. 2001; Nyaradi et al. 2013). Long-chain PUFAs are accumulated in the neuron-rich cortical gray matter and to a lesser extent in the white matter. Diau et al. (2005) report that

in baboon neonates the highest concentrations are located near the brain stem and diencephalon, mainly in the basal ganglia, limbic regions, thalamus, and midbrain.

During the last trimester and up to the first 18 months (mo) after birth, a 10-fold increase in brain size (beginning of the third trimester, 100 g; 18 mo postpartum, 1100 g) along with a 30-fold increase of total amount of DHA (25 wk of gestation, 3000 nmol/g; 2 y postpartum, 10000 nmol/g) takes place. This stage is termed brain growth spurt (Lauritzen et al. 2001). It is assumed that the accretion of AA is greater during the first two trimesters, resulting in higher concentrations of AA compared to DHA in the brain at term. Conversely, the human brain accumulates DHA gradually over time up to about 18 y of age (Carver et al. 2001), being the major constituent of long-chain PUFAs in the adult brain (Martinez 1992). However, knowledge on this issue is still limited (Hadders-Algra 2011). Long-chain PUFAs (particularly DHA) serve as a major determinant for brain functions, including for example membrane fluidity and volume. They are further involved in the production/activity of several neurotransmitters (i.e. dopamine, serotonin), synaptic transmission, and gene expression (Heaton et al. 2013). Based on these findings, the literature suggests the critical role of essential FAs in this period of life for proper brain development and function (Koletzko et al. 2008; Bazinet and Laye 2014).

Animal studies have mainly been conducted in rodents and have focused primarily on the effect of severe restriction to DHA. Results have shown poorer test performance concerning cognitive and behavioral outcomes, assessed for example with the Morris water maze test (Luchtman and Song 2013; McCann and Ames 2005). However, only a few animal studies have investigated the effect of additional n-3 long-chain PUFA supplementation. Supplementation resulted in higher brain levels of DHA at the cost of AA concentration with mixed results on performance in cognitive or behavioral tests (Hadders-Algra 2008; McCann and Ames 2005).

In humans, studies in preterm[1] infants have shown that nutritional insufficiencies adversely affect neuronal development. Due to a shortened gestational period, the accumulation of DHA during the brain growth spurt is partially denied, resulting in significantly lower DHA concentrations in the brain and AT. An infant of 35 wk of gestation has accumulated about 42 % LA, 56 % AA and 50 % of DHA compared to their term peers (Kuipers et al. 2012). Thus, premature babies are at a higher risk of neurodevelopmental disabilities, including for example

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[1] Preterm is defined as born alive before 37 completed wk of gestation (Moon et al. 2016)

impaired executive functioning (Aarnoudse-Moens et al. 2012), attention deficits (Bhutta et al. 2002), or poorer cognitive and language performance (Ionio et al. 2016).

However, in term-born infants the benefit of additional n-3 long-chain PUFAs on neurological development in the offspring is inconclusive. Observational cohort studies point to a positive association between maternal dietary DHA intake and better cognitive test performance (e.g. social, fine motor, language development, etc.) in the offspring (Daniels et al. 2004; Hibbeln et al. 2007; Oken et al. 2008). Likewise, higher concentrations of maternal DHA at the end of pregnancy has been shown to be positively associated with neurocognition in the offspring, such as improved attention in 18-month-old toddlers (Kannass et al. 2009), higher intelligence quotient at 8 y of life (Steer et al. 2013) or better sleep patterns in newborns, pointing towards greater central nervous system maturity (Cheruku et al. 2002). Due to several reasons (in particular causality and exclusion of confounders) (Gould et al. 2013), there exists a further need to investigate the role of DHA in child's neurological development and its therapeutic value in RCTs, known as the most precise way of determining cause-effect relationships (Sibbald and Roland 1998).

RCTs of long-chain PUFA supplementation during pregnancy or pregnancy and lactation on the neurological development of full-term infants have produced conflicting results so far (Janssen and Kiliaan 2014). A meta-analysis from 2013, evaluating the impact of maternal n-3 FA supplementation during pregnancy on early childhood cognitive development, could not make a definitive conclusion at this point of time. Investigators highlighted that most of the included studies had methodological limitations, such as small sample sizes and low statistical power, high attrition rate or incomplete information about the methodology and outcome data, which might have contributed to inconsistent findings. Thus, further clarification of the role of DHA in child's neurodevelopment is warranted (Gould et al. 2013).

1.4   Aim of the thesis

Data for this thesis are based on the Impact of Nutritional Fatty acids during pregnancy and lactation on early human Adipose Tissue development (INFAT) study. The primary aim of the study was to explore short- and long-term effects of a reduction of the n-6/n-3 FA ratio in the maternal diet during pregnancy and lactation on offsprings' AT growth from birth up to 5 y of age. For the assessment of body composition, a range of indirect and direct methods was applied including SFT measurements as the primary outcome, sonographic assessment of abdominal

fat distribution, growth patterns as well as MRI measurements at specific time points. The follow-up study (2–5 y) also included the assessment of child neurodevelopment.

The three main aims of the work presented in this thesis were

1) Chapter I – To verify sonography as a method to measure abdominal AT distribution in early infancy (≤ 1 y of age). Further, to describe how the sonographic measures of fat develop at 6 wk, 4 mo, 1, 2, 3, 4, and 5 y of life

2) Chapter II – To assess the long-term effects of an intervention to reduce the n-6/n-3 fatty acid ratio during pregnancy and lactation on children's AT growth and body composition from 2 to 5 y of life

3) Chapter III – To assess the long-term effects of an intervention to reduce the n-6/n-3 fatty acid ratio during pregnancy and lactation on children's neurodevelopment at 4 and 5 y of life

With regard to 1), the sonographic method of Holzhauer et al. (2009) for the assessment of abdominal subcutaneous and preperitoneal fat areas was adapted for a pediatric population ≤ 1 y of age. The data obtained using this method were discussed regarding feasibility and reproducibility in early infancy as well as to the effect of respiration, age, and sex. Further, the association of the ultrasound (US) measurements with anthropometry and SFT measurements was considered. Further, follow-up data from 2–5 y are presented to investigate long-term trajectories of abdominal fat development and the effect of sex and its correlation with other anthropometric measures.

With regard to 2), we continued the follow-up of the INFAT study that had investigated the impact of a reduced n-6/n-3 fatty acid ratio during pregnancy and lactation on offsprings' AT development from birth up to 1 y of life (Hauner et al. 2012). A follow-up until 5 y of age was performed to explore long-term effects of the intervention. To account for relevant confounding factors, nutritional behavior, as well as physical activity (PA) from the study groups, were considered.

With regard to 3), we investigated the effect of the intervention on neurodevelopmental outcomes of preschool children aged 4 and 5 y. Neurodevelopment was assessed by using a parents' questionnaire and a hand movement test to determine mirror activity. Additionally, associations between cord blood long-chain PUFAs and these outcomes were explored.

2 Study design and methods

2.1 Design of the INFAT study

The INFAT study is an open-label, monocenter, randomized controlled trial, conceived as a proof-of-concept study to test the impact of a reduced n-6/n-3 FA ratio during pregnancy and lactation on early human AT development up to 1 y of age. Between July 2006 and May 2009, 208 healthy pregnant women were recruited before their 15th wk of gestation and were randomly assigned (1:1 block randomization) to an intervention group or a control group. Women in the intervention group received fish oil capsules providing 1.2 g n-3 long-chain PUFAs/day (1020 mg DHA, 180 mg EPA plus 9 mg Vitamin E as an antioxidant) from the 15th wk of gestation until 4 mo postpartum. Besides, they received dietary counseling to reduce their intake of AA to a recommended range of ~90 mg/day (a dietary n-6/n-3 FA ratio of about 3–3.5:1 was planned to achieve). In contrast, the control group received general recommendations for a healthy diet during pregnancy and lactation according to the German Society of Nutrition. Written informed consent was obtained from the mother at the beginning of the study. The study protocol was registered at clinicaltrials.gov as NCT00362089 and was approved by the ethical committee of the Technical University of Munich (1479/06/2006/2/21).

In the first year of life, child body composition was assessed at four defined time points, namely at birth, 6 wk, 4 mo and 1 y of age. The primary endpoint was the sum of 4 SFT measurements, from which fat mass and lean body mass were estimated. The primary endpoint was complemented by anthropometric measurements (weight, length/height, head, arm, and waist circumference), abdominal US and MRI measurements, as well as blood collections. Maternal measurements included dietary records before (15th wk of gestation) and within the intervention (32nd wk of gestation), as well as the collection of biosamples, such as maternal blood, umbilical cord blood, placental tissue and breast milk (Hauner et al. 2009).

First-year-results were published by Hauner et al. (2012). In brief, the women included in the study were on average 32 y old and had a mean prepregnancy body mass index (BMI) of 22 kg/m^2. No significant differences in maternal baseline clinical characteristics, diet, lifestyle factors, and sociodemographic variables were detected between the randomized groups. Compliance in the intervention group was confirmed by FA composition in maternal red blood cells (RBCs) during pregnancy and dietary records, assessing maternal diet at 15th and 32nd wk of gestation and capsule intake. Newborns in the intervention group showed significantly higher values for weight (unadjusted mean difference, 178 g; 95% CI: 31, 324; $P < 0.05$), BMI

(unadjusted mean difference, 0.5 kg/m^2; 95% CI: 0.2, 0.9; $P < 0.01$), weight for length (unadjusted mean difference, 3.1 g/cm; 95% CI: 0.8, 5.3; $P < 0.01$), and ponderal index (unadjusted mean difference, 0.8 kg/m^3; 95% CI: 0.1, 1.5; $P < 0.05$) at birth. Group differences were caused by a prolonged gestation of 4.8 days (95% CI: 1.19, 7.67; $P = 0.001$) in the intervention group and were no longer detectable in the adjusted model (adjusted for sex and pregnancy duration), and at later time points in the unadjusted or adjusted model.

Subcutaneous fat distribution and AT growth were assessed by SFT measurements. Neither analysis of the individual skinfolds, nor the sum of 4 SFTs provided evidence of a difference between the two study groups in the unadjusted or the adjusted model (e.g., at 4 mo: sum of 4 SFTs: mean unadjusted difference, 0.1 mm; 95% CI: −1.2, 1.2). Similarly, non-significant findings were observed for fat mass (g), the percentage of fat mass, subscapular:triceps SFT ratio and trunk-to-total SFTs (%) from birth to 1 y of life. Consistent results were obtained with the method of sonography to assess abdominal subcutaneous and preperitoneal fat areas at 6 wk, 4 mo, and 1 y of life, with no evidence of differences between study groups (e.g., at 4 mo: subcutaneous area$_{sagittal}$: unadjusted mean difference, 0.7 mm^2; 95% CI: −4.1, 5.5; preperitoneal area$_{sagittal}$: unadjusted mean difference, −0.2 mm^2; 95% CI: −1.6, 1.1; both P values > 0.05) (Hauner et al. 2012).

To detect long-term effects of the intervention, a follow-up was conducted between February 2008 and November 2014. Child growth and fat mass development were assessed, using the same measures from the initial study. Children were examined at the study center or during a home visit biannually up to 2 y of age, and after that annually up to 5 y of age. Further secondary endpoints included the assessment of children's diet, active and sedentary behavior in the form of a PA questionnaire as well as neurodevelopment at selected time points. For the follow-up study, written informed consent was obtained from both parents. The study protocol was approved by the ethical committee of the Technical University of Munich (1479/06/2009/10/26).

Figure 1 provides an overview of the study including the design, time schedule, and all examinations from the onset up to 5 y of age (inclusion and exclusion criteria for study enrollment are also stated).
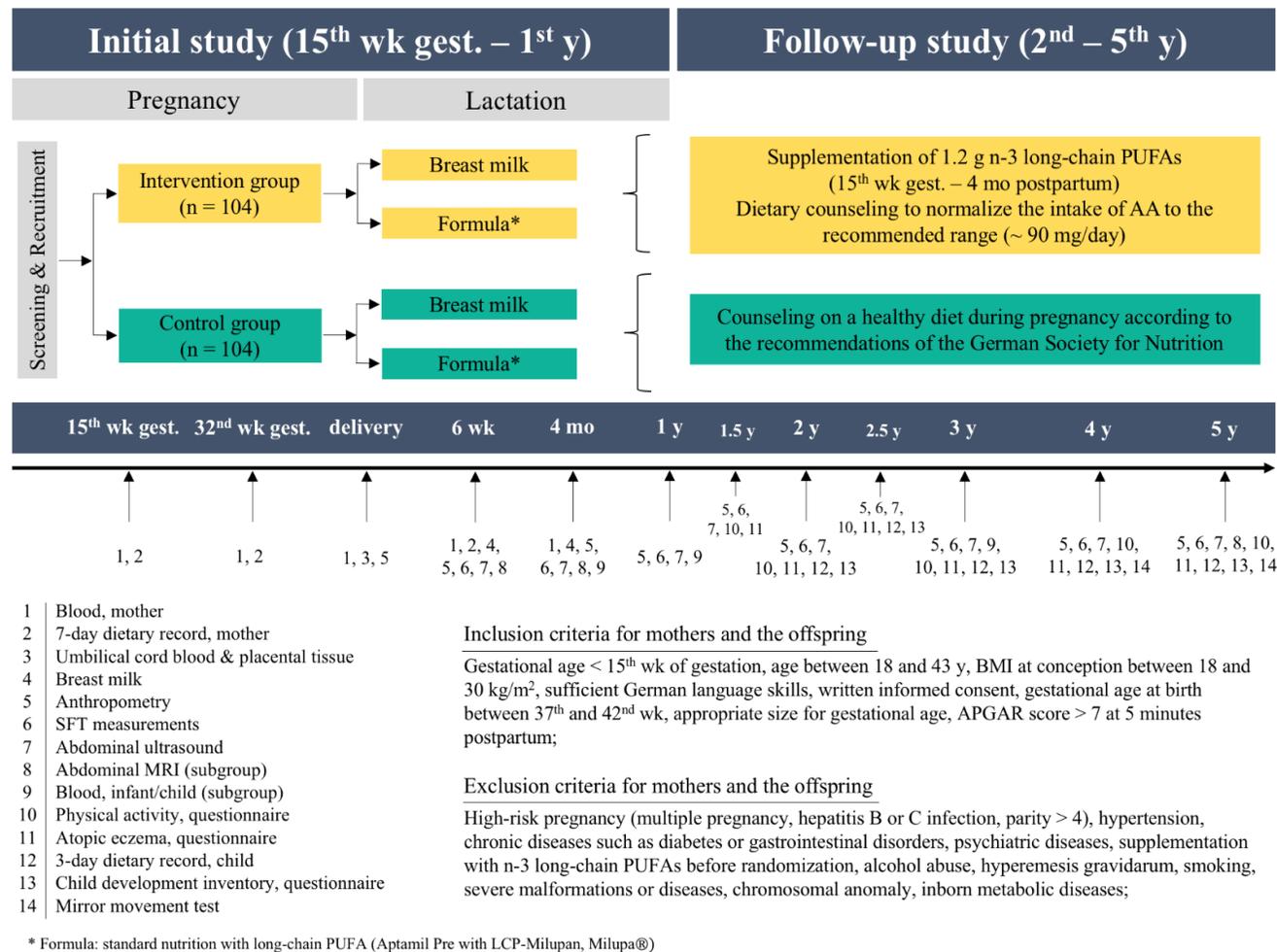
Figure 1 Design of the INFAT study

AA, arachidonic acid; APGAR, appearance, pulse, grimace, activity, respiration; BMI, body mass index; PUFA, polyunsaturated fatty acid; mo, month; MRI, magnetic resonance imaging; SFT, skinfold thickness; wk, week; y, year.
Data source: own contribution.

2.2   Measurements of body composition

2.2.1   Growth parameters

The weight, length, and head circumference of the offspring at birth were obtained from obstetric records in the maternity clinics. At later time points, all examinations were carried out under standardized conditions. From the 6th wk up to the end of the second year of life, infants' weight was measured with the use of a standard infant scale (Babywaage Ultra MBSC-55; myweight) to the nearest of 10 g. The length was obtained by using a measuring stick (Säuglingsmessstab seca 207; seca) to the nearest of 0.5 cm in a supine position with stretched legs. Later examinations were determined in standing position. For weight measurements, a standard flat scale (Seca Clara 803; seca) to the nearest of 100 g was used, and a stadiometer (Stadiometer seca 214; seca) to assess children's height to the nearest of 0.5 cm. Ponderal index (kg/m^3), BMI (kg/m^2), and BMI percentiles were calculated from these measured variables. For the latter, a German reference group according to Kromeyer-Hauschild et al. (2001) was used (https://www.pedz.de/de/rechner.html). Further, head, arm and waist circumferences were measured at study visits.

2.2.2   Skinfold thickness measurements

Skinfold thickness measurements were assessed 3–5 days postpartum in the obstetric clinic or at the family's home and from then on at each study visit. Measurements were performed with a Holtain caliper (Holtain Ltd.) in triplicate under standard conditions at the left body axis at four body sites (biceps, triceps subscapular, and suprailiac). For any given site, the mean from the three measurements was calculated, and the sum of the four respective sites (sum of 4 SFT measurements) was formed. With predictive SFT equations according to Weststrate and Deurenberg (1989), the percentage of body fat was calculated, from which values for percentage of lean body mass, body fat, and lean body mass (kg) could be extrapolated. Additionally, two indexes of fat patterning were calculated: the subscapular-to-triceps SFT ratio (index of central to peripheral fat distribution) according to Haffner et al. (1987) and the central-to-total SFT ratio (percentage of trunk-to-total SFTs[1]) according to Weststrate et al. (1989).

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[1] Percentage of trunk-to-total SFTs = [(subscapular + suprailiac) ÷ (sum of 4 SFTs)] x 100

2.2.3   Abdominal subcutaneous and preperitoneal fat areas by US

Sonographic measurements complemented SFT measurements and were performed in the study center. A high-resolution ultrasonographic system (Siemens Acuson X150 Premium; Siemens) with a 10 MHz linear probe (VFX 13-5, Siemens Medical Solutions, Erlangen, Germany) in b-picture-mode was used. The originally described method from Holzhauer and colleagues (2009) was slightly adapted for our purpose (Brei et al. 2015). Infants/children were located in supine position with both hands resting aside the thighs. Subcutaneous and preperitoneal fat were measured in two defined abdominal regions. For the first measurement, the probe was placed in the sagittal plane in the middle of the xiphoid process to assess subcutaneous and preperitoneal fat areas. The second measurement was performed in axial plane between the middle of the xiphoid process and the navel directly above the linea alba to measure subcutaneous fat area. Measurements were taken at the end of a gentle expiration (Liem et al. 2009; De Lucia Rolfe et al. 2010). For reasons of feasibility in the young study cohort, the cine-loop-function was used to determine pictures at the end of expiration.

The evaluation process was performed at an off-line working station, using the OsiriX software (http://www.osirix-viewer.com/, Geneva, Switzerland). For the evaluation of fat areas as layers of 1-cm length, defined measurement points/distances were set, starting from a reference structure (i.e. in the sagittal plane, xiphoid process; in the axial plane, linea alba) (see Chapter Ia). For each area, three pictures were evaluated. The means of the measured distances were calculated and used to estimate the area of preperitoneal fat in the sagittal plane (preperitoneal area$_{sagittal}$, mm^2), the area of subcutaneous fat in the sagittal plane (subcutaneous area$_{sagittal}$, mm^2) and the area of subcutaneous fat in the axial plane (subcutaneous area$_{axial}$, mm^2). Further, the ratio of preperitoneal to subcutaneous fat tissue from sagittal plane was calculated[1].

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[1] Ratio = preperitoneal area$_{sagittal}$ ÷ subcutaneous area$_{sagittal}$

2.2.4   Abdominal subcutaneous and visceral fat volumes by MRI

In a subgroup of children, abdominal MRI measurements were performed at 5 y of age to determine volumes of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). The examinations were performed at the Klinikum rechts der Isar, on a clinical whole body scanner at 1.5 Tesla without any sedation.

For the MRI measurement, children were positioned supine with feet first, and arms next to their bodies. The imaging protocol was kept as short as possible and lasted approximately 10 minutes. After quantitative scans, planned on localizer images under free-breathing conditions, a 4-point (echo time: 2.38, 4.76, 7.15, and 9.53 ms) Dixon technique according to Glover (1991) was used to obtain water and fat separated images while the children were holding their breath (3.9 s).

In a postprocessing step, acquired MRI data were exported to a remote workstation, and data analysis commenced off-line, using a customized MATLAB program (R2014b; MathWorks). With this procedure, the water and fat images could be calculated, which were further used for SAT and VAT segmentation.

The segmentation of SAT and VAT required a manual identification of measurement limits. Abdominal AT was defined as image slices bounded by the head of the liver to the iliac crest. By a self-written segmentation algorithm in MATLAB (R2014b; MathWorks) developed by Cordes et al. (2015), selected slice images were automatically analyzed regarding fractions of SAT, VAT, and nonadipose tissue (NAT) (mostly water). The segmentation required a subsequent manual adjustment, as shown in **Figure 2**. Total abdominal volumes were then calculated by adding single slice volumes. Besides, ratios of SAT, VAT, and NAT to total volumes were generated[1].

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[1] SAT ratio: [SAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100
VAT ratio: [VAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100
NAT ratio: [NAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100
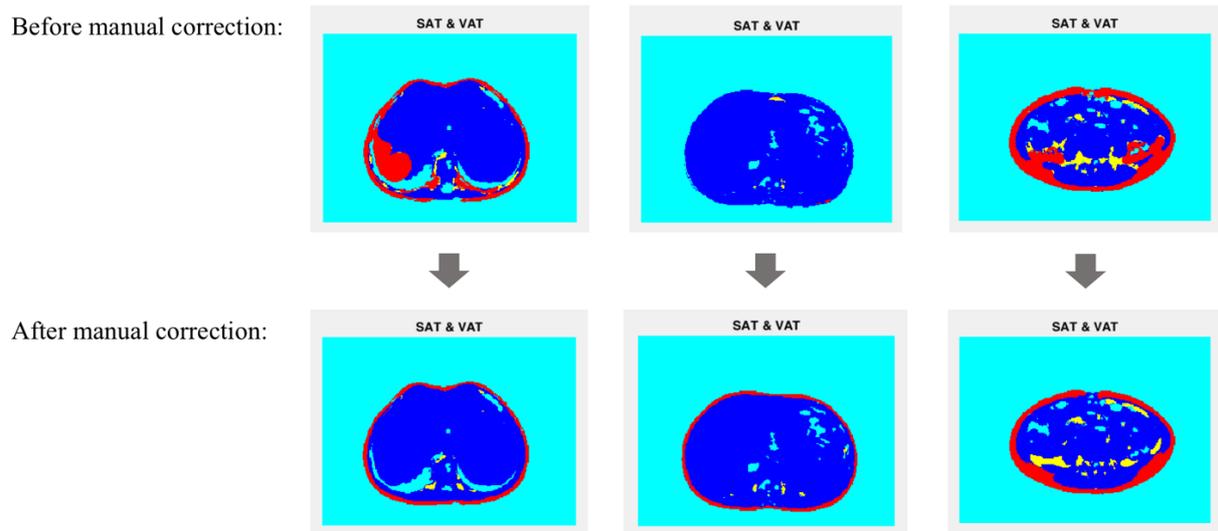
Figure 2 MATLAB segmentation before and after manual correction

Classification of the following compartments: red ≙ SAT; yellow ≙ VAT; dark blue ≙ NAT; turquois ≙ air. NAT, nonadipose tissue; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. Data source: own contribution.

2.3 Neurodevelopment

2.3.1 Child development inventory

The child development inventory (CDI) questionnaire was used to assess children's neurodevelopment. It comprises 270 yes/no answers covering several subject areas. The scales in the questionnaire included the following: social (40 items), self help (40 items), gross motor (30 items), fine motor (50 items), language comprehension (50 items) letters (15 items), numbers (15 items). From these areas, an overall score of general development from the 70 most age-discriminating items was calculated. The questionnaire was originally developed in 1992 (Ireton 1992) to identify children with developmental problems or delay in a clinical setting. The validated method (Doig et al. 1999) was translated into German[1] and normed for the age groups 36 to 67 mo within a total of 758 children (Brandstetter et al. 2002).

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[1] Elternfragebogen zur kindlichen Entwicklung, EFkE

Parents were asked to fill out the questionnaire at specific time points. The form was analyzed by counting the yes-answers for each area. The resulted scores were then compared with age and gender reference scores for German children (Brandstetter et al. 2003) and categorized as either normal development, borderline development (-1.5 SD) or developmentally delayed (-2.0 SD).

2.3.2 Hand movement test

Children's motor development was assessed by evaluating hand mirror movements (MMs) with a PC-supported system (Hermsdörfer et al. 1992) following the procedure of Uttner et al. (2005; 2007). In general, MMs are considered as unintended movements in the homologous muscles of the corresponding limb, in this case, the hand, when the opposite side/hand performs intended movements. MMs are usually observed during childhood and are decreased by its level of intensity along with motor development (Koerte et al. 2010).

For the measurement, children were in a seated position with a force transducer in each hand (diameter: 20 mm, length: 20 mm, weight: 20 g) between the index finger and the thumb, recording grip forces between 0–100 N. A monitor in front of them provided visual feedback about the grip force changes, shown as vertical bars within a box. In a pretest, the maximum grip force strength of both hands was quantified. Children were instructed to press each transducer three times as tight as possible with each hand. For the assessment of MMs, the participants were instructed to increase and decrease their grip force of the right hand (active hand) for 15 s at a low frequency (1/sec) and then at maximum frequency (as fast as possible), and not to squeeze the left hand (mirror hand). The proximate target on the monitor was defined as 40 % of the maximum force (assessed in the pretest). Every measurement was repeated once, with visual feedback for the active hand, only. Subsequently, the roles of active and mirror hand were reversed. **Figure 3** gives an example of the test procedure with the right hand as active hand and left hand as mirror hand at a low frequency (1/sec).
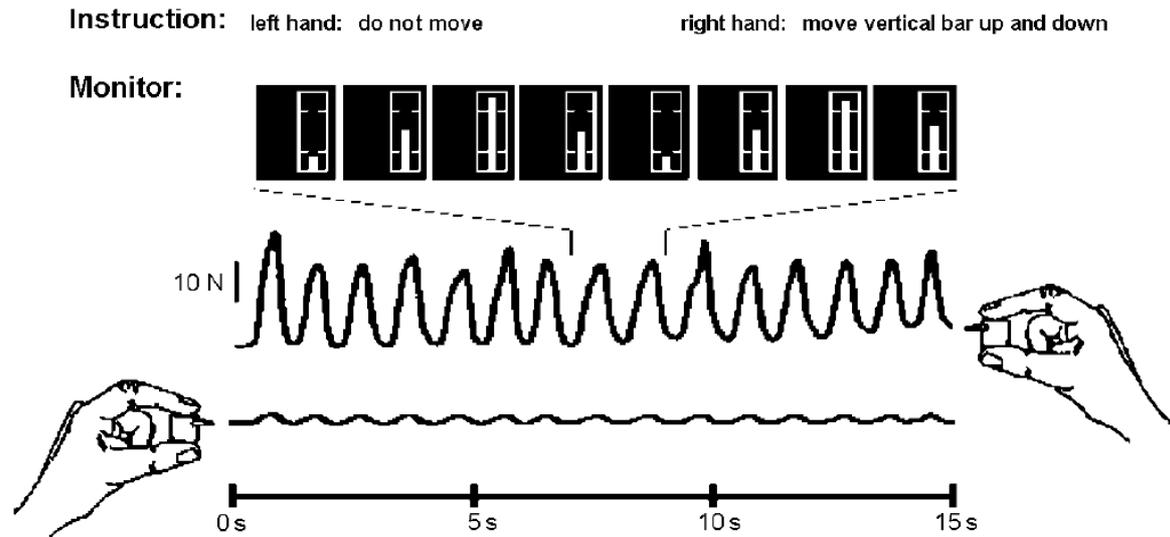
Figure 3 Performance of grip force changes at a low frequency (1/sec)

Data source: Uttner et al. 2005.

Acquired data were saved and stored at an off-line working station. A self-written MATLAB program (R2014b; MathWorks) was used to reject the first 3 seconds of each measurement manually and to remove parts of the examination if children did not follow the technical instructions (e.g. problems of comprehension, concentration problems or lack of motivation). Subsequently, MM ratios, as well as Pearson coefficients of correlation, were computed.

An example of the calculation of the MM ratio is given in **Figure 4**. The program detected maxima and minima of both hands and calculated the amplitudes as peaks of the highest and the lowest values of one unit (active hand: B−A; mirror hand: B'−A'). The ratio of the mean mirror force amplitude to the mean active force amplitude was formed and was corrected for the maximum grip force strength of both hands[1].

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[1] MM ratio (%) = [(mirror amplitude ÷ active amplitude) × (max. grip force active hand ÷ max. grip force mirror hand)] × 100
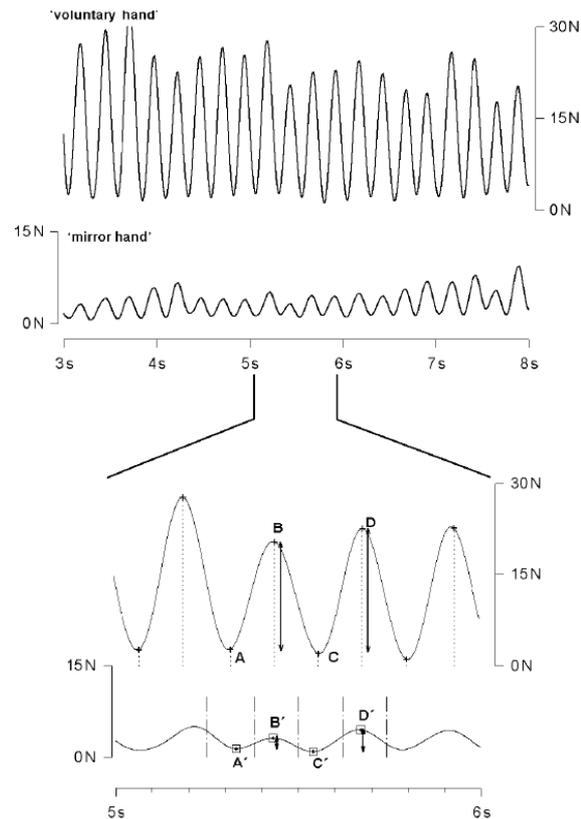
Figure 4 Approach for the calculation of MM ratios

Data source: Uttner et al. 2005.

2.4 Dietary record

Dietary intake was assessed during the follow-up of the INFAT study with 3-day dietary records (see **Appendix A-1**) at specific time points. Parents or daily caregivers were asked to record the consumed amount of food and beverages, ideally by weighing food items with a scale using standard units, or by measuring volumes using household measures (e.g. cups, tablespoons, etc.) to estimate portion sizes. Moreover, they were asked to provide information about the time and location of food intake, the fat content (e.g. in dairy products), and if they had used specific brands. To obtain a representative survey of the week, they were requested to record two weekdays and one weekend day.

A first screening phase revealed that dietary records were not adequately completed, making a standardized estimation based on portion sizes for all food items not feasible, particularly for lunchtime meals. Thus, when no detailed information was given in the protocol, child portion sizes were calculated by a proportional approach developed in the framework of a master thesis (Karla 2015).

Based on a PA level of 1.6 for adult women and children, the reference values for energy intake per day and meal (i.e. one quarter of total daily energy intake) was calculated. Child portion size was adjusted age- and gender-specifically (Alexy et al. 2002). The process was based on a preexisting standardized list of defined portion sizes for middle-aged adult women.

> For example, the reference value for a middle-aged woman (25–51 y) with a PA level of 1.6 is considered as 2100 kcal/day, for a 5-year-old girl 1500 kcal. According to the quarter approach, it was assumed that mean energy intake per main meal is 525 kcal and 375 kcal, respectively. Expressed as a percentage, a child portion of 71.4 % of the adult's portion resulted. Based on the standardized portion of a vegetable lasagna of an adult woman (350 g/portion), a standardized portion of 249.9 g for the girl was defined.

Subsequently, plausibility checks were carried out to determine feasible portion sizes, ranging between portions given by randomly chosen catering companies in Munich and surrounding area and standard portion sizes released by the Research Institute of Child Nutrition[1] (Forschungsinstitut für Kinderernährung Dortmund 2012).

Data entry and analysis was performed with the software OptiDiet (version 5.1.2.065; GOE mbH), which is based on a German nutrient database (Bundeslebensmittelschlüssel).

2.5 Physical activity questionnaire

A basic questionnaire from the German Health Interview and Examination Survey for children and adolescents was used to assess children's physical activity and inactivity (Lampert et al. 2007; Manz et al. 2014). The form contained five questions, of which three were on play and exercise (PA) and two on television viewing and computer consumption (physical inactivity). For questions on physical inactivity, distinctions were made between weekdays and weekend days (see **Appendix A-2**).

Before the examination took place, the documents (including the dietary record, CDI- and PA questionnaire) were sent to the families and were filled out by one of the parents, usually the mother. The forms were returned at the study visit.

2.6 Collection of cord blood and RBC fatty acid analysis

At delivery, cord blood samples were collected in EDTA tubes from the umbilical vein. Samples were centrifuged at 2000 x g for 10 minutes, and RBCs and plasma were separated.

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[1] Forschungsinstitut für Kinderernährung, FKE

The plasma was removed and stored until analysis at –86 °C, while erythrocytes were washed with 0.9 % NaCl solution and aliquoted before they were stored at –86 °C. Coded samples were sent to the Laboratory of Lipid Research, Danone Research-Center for Specialised Nutrition, Friedrichsdorf, Germany, where fatty acid analysis was performed.

The analysis of fatty acid methyl esters (FAME) was used to assess fatty acid values (% of FAs of total FAs) in RBCs. Therefore, RBC fatty acids were transesterified according to a described method (Lepage and Roy 1984). For derivatization, frozen samples were thawed at room temperature and were dissolved in 2 ml methanol/hexane (4:1, vol/vol) and 0.5 % pyrogallol. For methylation, 200 µl acetyl chloride was added at 100 °C. After 1 hour, 5 ml 6 % K_2CO_2 was added, and the solution was centrifuged at 3200 rpm for 10 minutes. The upper phase, containing the FAME, was used for further analysis.

The analysis was performed on a 6890N gas chromatograph (Agilent Technologies, Waldbronn, Germany) with a cold-on-column injector. For separation of FAs, a DB23 column was used (60 m, I.D. 0.25 mm, film 0.25 µm, JW Scientific, Agent Technologies, US). The chromatographic conditions are summarized in **Table 1**. FAs were identified in duplicate according to their retention times relative to standards (GLC 85 standard mix, NuChekPrep, Inc. Elysian, Minnesota, US).

Table 1 Chromatographic conditions for FAME analysis

Injector	60 °C to 270 °C
Carrier gas	Hydrogen at a flow of 1.8 ml/min
Flame ionization detector	280 °C
Oven temperature	60 °C for 0.1 min; from 60 °C to 160 °C at 40 °C/min; 160 °C for 2 min; from 160 °C to 190 °C at 3 °C/min; 190 °C to 220 °C at 4.5 °C/min, 220 °C for 5 min; from 220 °C to 240 °C at 5 °C/min; 240 °C for 25 min

2.7   Statistical analyses

All analyses were performed using the statistical program R (version R 3.1.3; R Foundation for Statistical Computing) or the software package SPSS (version 21.0; SPSS Inc.). A 2-sided *P* value < 0.05 was considered significant. No corrections were made for multiple comparisons. Detailed information about the applied statistics is given in Chapter Ia, II and III, respectively.

Concerning Chapter Ib, statistical analyses are equivalent to Chapter Ia, including all measured time points (6 wk, 4 mo, 1, 2, 3, 4, and 5 y) for the mixed linear models. A paired t-test was used to assess changes in the samples at the specific time points.

3 Publications and additional results

Chapter Ia – Sonographic fat assessment in infants (6 wk–1 y)

"Sonographic assessment of abdominal fat distribution during the first year of infancy"

Christina Brei*, Daniela Much*, Ellen Heimberg, Verena Schulte, Stefanie Brunner, Lynne Stecher, Christiane Vollhardt, Jan S. Bauer, Ulrike Amann-Gassner, and Hans Hauner

*both authors contributed equally to this work

Pediatric Research 78(3): 342–50, 2015[1]

Personal contribution: Christina Brei performed statistical analyses, prepared tables and figures, and wrote and revised the manuscript.

Chapter Ib – Sonographic fat assessment in children (2–5 y)

Unpublished data.

Chapter II – Long-chain PUFAs and offspring body composition

"Reduction of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring body composition: follow-up results from a randomized controlled trial up to 5 y of age"

Christina Brei, Lynne Stecher, Daniela Much, Marie-Theres Karla, Ulrike Amann-Gassner, Jun Shen, Carl Ganter, Dimitrios C. Karampinos, Stefanie Brunner, and Hans Hauner

American Journal of Clinical Nutrition 103(6): 1472–81, 2016[2]

Personal contribution: Christina Brei was responsible for data collection and trial management, analyzed the MRI data, performed statistical analyses, supervised the analysis of dietary records and PA questionnaires, prepared tables and the figure, and wrote and revised the manuscript.

Chapter III – Long-chain PUFAs and offspring neurodevelopment

"Impact of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring neurodevelopment: 5-year follow-up of a randomized controlled trial"

Christina Brei, Lynne Stecher, Stefanie Brunner, Regina Ensenauer, Florian Heinen, Patrick D. Wagner, Joachim Hermsdörfer, and Hans Hauner

European Journal of Clinical Nutrition, 2017[3] [epub ahead of print]

Personal contribution: Christina Brei was responsible for data collection and trial management, analyzed the data, performed statistical analyses, prepared tables and the figure, and wrote and revised the manuscript.

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[1] Approval letter, see **Appendix A-4**
[2] Approval letter, see **Appendix A-5**
[3] Approval letter, see **Appendix A-6**

Chapter Ia – Sonographic fat assessment in infants (6 wk–1 y)

Sonographic assessment of abdominal fat distribution during the first year of infancy

Christina Brei[1], Daniela Much[1], Ellen Heimberg[2], Verena Schulte[1], Stefanie Brunner[1], Lynne Stecher[1], Christiane Vollhardt[1], Jan S. Bauer[3], Ulrike Amann-Gassner[1] and Hans Hauner[1,4]

The first two authors contributed equally to this work.

[1]Else Kröner-Fresenius-Center for Nutritional Medicine, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [2]Department of Pediatrics, Universitätsklinikum Tübingen, Tübingen, Germany; [3]Department of Neuroradiology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [4]ZIEL – Research Center for Nutrition and Food Science, Nutritional Medicine Unit, Technische Universität and München, Freising, Germany.

Correspondence: Hans Hauner (hans.hauner@tum.de)

ABSTRACT

BACKGROUND: Longitudinal data regarding the fat distribution in the early postnatal period is sparse.

METHODS: We performed ultrasonography (US) as a noninvasive approach to investigate the development of abdominal subcutaneous (SC) and preperitoneal (PP) fat depots in infants ≤1 y and compared longitudinal US data with skinfold thickness (SFT) measurements and anthropometry in 162 healthy children at 6 wk, 4 mo, and 1 y postpartum.

RESULTS: US was found to be a reproducible method for the quantification of abdominal SC and PP adipose tissue (AT) in this age group. Thickness of SC fat layers significantly increased from 6 wk to 4 mo and decreased at 1 y postpartum, whereas PP fat layers continuously increased. Girls had a significantly higher SC fat mass compared to boys, while there was no sex-specific difference in PP fat thickness. SC fat layer was strongly correlated with SFT measurements, while PP fat tissue was only weakly correlated with anthropometric measures.

CONCLUSION: US is a feasible and reproducible method for the quantification of abdominal fat mass in infants ≤1 y of age. PP and SC fat depots develop differentially during the first year of life.

INTRODUCTION

Childhood obesity has become a global epidemic (1) and there is growing evidence that the first year of life, a phase of rapid growth, constitutes a critical period for the onset of obesity in later life (2). Many studies among adults suggest that potential health risks of obesity such as cardiovascular disease or type 2 diabetes do not only depend on the amount of body fat, but also on the type of fat distribution. Abdominal fat, especially, has been identified to play a central role for the development of complications because of its close relationship to insulin resistance and metabolic cardiovascular risk factors (3,4). Also in children and adolescents, disturbances of insulin and glucose metabolism as well as signs of an unfavorable lipid profile have been described in relation to abdominal fat (5–7). Thus, the role of abdominal fat distribution during infancy and childhood is gaining recognition.

General growth parameters, such as BMI, skinfold thickness (SFT) measurements, and waist circumference or waist-to-hip ratio are widely used as measures of body fatness in infants and children, but they do not directly quantify fat compartments. Measurement of waist circumference offers an indirect and crude alternative for the assessment of body fat distribution, but cannot distinguish between subcutaneous (SC) and visceral fat, respectively. However, this parameter is not as accurate as direct measures like ultrasonography (US), computer tomography (CT), or magnetic resonance imaging (MRI) (8–10). Techniques like MRI or CT are expensive and represent a burden for the child, because these examinations are uncomfortable and time-consuming or expose the infants to radiation (11).

US is an easily accessible, inexpensive, radiation-free, and noninvasive approach to measure abdominal adipose tissue (AT). Holzhauer *et al*. (12) proposed ultrasound as an adapted technique to measure SC and preperitoneal (PP) fat depots in a cross-sectional study of 212 1-y and 227 2-y olds, respectively, a method which was described by Suzuki *et al*. (13) in 1993. It could be shown that US provides a reliable and reproducible estimate of SC and PP fat depots when compared to CT in which PP fat mass was found to be related to abdominal visceral fat mass. Therefore, US is a suitable method for epidemiological and clinical approaches (13,14). To date, the technique of Holzhauer *et al*. has not been applied to generate longitudinal sonographic data on AT growth in a younger pediatric population <1 y of age. However, a different protocol for estimating visceral and SC fat in the first year of infancy has been applied by a different study (15). Aim of the present study was to generate longitudinal sonographic data on AT growth during early infancy. US measurements were performed in parallel with SFT measurements and anthropometry to assess how these measures are correlated. For this

purpose, the US technique described originally by Holzhauer *et al.* (12) was adapted slightly for use in very young infants (≤ 1 y of age).

RESULTS

Participant Characteristics

The analyses included 162 infants (n = 77 girls, n = 85 boys) aged 6 wk (median 6.33 wk) and 160 infants (n = 79 girls, n = 81 boys) aged 4 mo (median 3.64 mo) who underwent sonographic examinations as well as anthropometric and SFT measurements during regular study visits. Overall 160 children (n = 84 girls, n = 76 boys) completed the investigations at 1 y of age (median 1.04 y). The anthropometric and ultrasound data are presented in **Tables 1** and **2**. All infants were born full-term between the 37th and 42nd week of gestation, except n = 4 preterm infants and one post-term baby. Sixteen infants were born to mothers suffering from gestational diabetes during pregnancy, which was controlled by diet in 12 cases and treated with insulin in 4 cases.

Reproducibility

The intra- and interclass-correlation coefficients (ICC), in **Table 3**, for the observers' estimated distances (mean out of three measurements) and the calculated areas for all measurements suggest strong inter-observer agreement. Additionally, the Bland-Altman plots did not show any relevant differences between observer 1 and 2. On average, the measurements of observer 1 were slightly higher than for observer 2 with a mean difference of 0.38 mm^2 for the area of SC fat in sagittal plane (area sag sc) (**Figure 1a**), 0.81 mm^2 for the area of SC fat in axial plane (area ax sc) (**Figure 1b**) and 0.20 mm^2 for the area of PP fat in sagittal plane (area sag pp) (**Figure 1c**), respectively.

Intra-observer agreement showed comparable results for all measures of the distances and areas of PP and SC fat with ICC ranging from 0.87 (sag caudal pp) to 0.99 (ax r).

When stratified by age group, there was no evidence of a trend in increasing ICC with age (data not shown).

Table 1. Anthropometric variables and skinfold thickness measurements at age 6 weeks, 4 months and 1 year

Age	Parameter	All		Female		Male		Estimated mean difference (95% CI)	P value
		Mean (SD)	n	Mean (SD)	n	Mean (SD)	n		
6 wk	Weight (g)	4,781.4 (625.0)	162	4,638.8 (557.4)	77	4,910.5 (657.3)	85	269.5 (85.8, 453.1)	0.004
	Length (cm)	55.8 (2.4)	162	55.3 (2.2)	77	56.3 (2.5)	85	1.1 (0.4, 1.8)	0.002
	BMI (kg/m^2)	15.3 (1.3)	162	15.2 (1.3)	77	15.4 (1.2)	85	0.2 (–0.1, 0.6)	0.208
	Biceps SFT (mm)	4.4 (0.9)	162	4.2 (0.8)	77	4.5 (1.0)	85	0.3 (0.0, 0.6)	0.034
	Triceps SFT (mm)	6.6 (1.4)	162	6.5 (1.3)	77	6.8 (1.4)	85	0.2 (–0.2, 0.7)	0.251
	Subscapular SFT (mm)	6.2 (1.3)	162	6.3 (1.2)	77	6.1 (1.3)	85	–0.3 (–0.6, 0.1)	0.186
	Suprailiacal SFT (mm)	4.8 (1.1)	162	5.0 (1.1)	77	4.6 (0.9)	85	–0.5 (–0.8, –0.2)	0.003
	Sum 4 SFT (mm)	22.0 (3.8)	162	22.0 (3.6)	77	22.0 (4.0)	85	–0.2 (–1.4, 0.9)	0.684
	Body fat (%)	19.0 (3.0)	162	19.0 (2.8)	77	19.0 (3.2)	85	–0.2 (–1.2, 0.7)	0.599
	Fat mass (g)	918.8 (232.4)	162	891.4 (216.3)	77	943.6 (244.7)	85	41.7 (–28.0, 111.4)	0.241
	Lean body mass (g)	3,862.5 (437.9)	162	3,747.4 (380.7)	77	3,966.9 (461.8)	85	233.7 (105.7, 361.7)	<0.001
	Subscapular-Triceps ratio	1.0 (0.17)	162	0.99 (0.18)	77	0.92 (0.16)	85	–0.07 (–0.12, –0.02)	0.007
	Central-to-total-SFT	0.5 (0.04)	162	0.51 (0.03)	77	0.49 (0.03)	85	–0.03 (–0.04, –0.02)	<0.001
4 mo	Weight (g)	6,394.7 (714.9)	160	6,153.6 (643.0)	79	6,629.8 (706.5)	81	482.9 (278.0, 687.9)	<0.001
	Length (cm)	62.5 (2.1)	160	61.8 (2.0)	79	63.1 (2.0)	81	1.3 (0.7, 1.9)	<0.001
	BMI (kg/m^2)	16.3 (1.4)	160	16.1 (1.3)	79	16.6 (1.3)	81	0.6 (0.1, 1.0)	0.008
	Biceps SFT (mm)	5.1 (1.0)	160	5.1 (1.1)	79	5.2 (0.9)	81	0.1 (–0.2, 0.4)	0.654
	Triceps SFT (mm)	7.8 (1.5)	160	7.8 (1.6)	79	7.8 (1.4)	81	–0.0 (–0.5, 0.5)	0.966
	Subscapular SFT (mm)	6.5 (1.3)	160	6.7 (1.5)	79	6.3 (1.2)	81	–0.3 (–0.7, 0.1)	0.105
	Suprailiacal SFT (mm)	6.0 (1.4)	160	6.3 (1.6)	79	5.7 (1.2)	81	–0.6 (–1.1, –0.2)	0.003
	Sum 4 SFT (mm)	25.4 (4.2)	160	25.9 (4.6)	79	24.9 (3.7)	81	–0.9 (–2.2, 0.3)	0.155
	Body fat (%)	21.2 (2.8)	160	21.5 (3.0)	79	20.9 (2.5)	81	–0.5 (–1.4, 0.3)	0.204
	Fat mass (g)	1,364.0 (278.3)	160	1,331.2 (280.0)	79	1,396.0 (274.6)	81	67.4 (–16.1, 150.8)	0.113

Table 1. Continued

Age	Parameter	All		Female		Male		Estimated mean difference (95% CI)	P value
		Mean (SD)	n	Mean (SD)	n	Mean (SD)	n		
4 mo	Lean body mass (g)	5,030.7 (517.5)	160	4,822.3 (456.0)	79	5,233.8 (494.8)	81	415.1 (269.8, 560.4)	<0.001
	Subscapular-Triceps ratio	0.85 (0.18)	160	0.88 (0.21)	79	0.83 (0.15)	81	−0.05 (−0.11, 0.01)	0.076
	Central-to-total-SFT	0.49 (0.04)	160	0.50 (0.04)	79	0.48 (0.03)	81	−0.02 (−0.03, −0.01)	<0.001
1 y	Weight (g)	9,493.9 (1047.2)	160	9,219.6 (950.1)	84	9,797.1 (1071.6)	76	578.1 (269.5, 886.6)	<0.001
	Length (cm)	75.2 (2.7)	160	74.6 (2.7)	84	75.9 (2.5)	76	1.2 (0.4, 2.0)	0.003
	BMI (kg/m^2)	16.8 (1.4)	160	16.6 (1.5)	84	17.0 (1.4)	76	0.4 (0.0, 0.9)	0.050
	Biceps SFT (mm)	5.2 (1.3)	159	5.3 (1.4)	83	5.0 (1.1)	76	−0.4 (−0.7, 0.0)	0.071
	Triceps SFT (mm)	7.9 (1.7)	159	7.8 (1.4)	83	8.0 (1.9)	76	0.2 (−0.3, 0.7)	0.362
	Subscapular SFT (mm)	6.3 (1.3)	160	6.5 (1.4)	84	6.2 (1.3)	76	−0.4 (−0.8, 0.0)	0.071
	Suprailiacal SFT (mm)	4.5 (1.0)	155	4.7 (1.1)	80	4.3 (0.9)	75	−0.4 (−0.7, −0.1)	0.010
	Sum 4 SFT (mm)	24.0 (4.3)	155	24.4 (4.4)	80	23.5 (4.0)	75	−1.0 (−2.3, 0.4)	0.152
	Body fat (%)	19.7 (2.9)	155	20.0 (3.0)	80	19.3 (2.8)	75	−0.6 (−1.5, 0.3)	0.159
	Fat mass (g)	1,884.2 (431.9)	155	1,857.2 (414.7)	80	1,913.0 (450.5)	75	54.8 (−78.4, 188.0)	0.419
	Lean body mass (g)	7,624.4 (727.0)	155	7,385.1 (656.4)	80	7,879.6 (715.5)	75	498.5 (285.6, 711.5)	<0.001
	Subscapular-Triceps ratio	0.82 (0.16)	159	0.84 (0.15)	83	0.79 (0.17)	76	−0.05 (−0.11, −0.00)	0.035
	Central-to-total-SFT	0.45 (0.04)	155	0.46 (0.03)	80	0.45 (0.04)	75	−0.01 (−0.03, −0.00)	<0.001

Data are presented as mean ± SD (n) along with the nonadjusted mean difference (95% confidence interval) from mixed models containing time, sex and an interaction between sex and time.
SFT, skinfold thickness.

Table 2. SC and PP fat measurements assessed by US at age 6 wk, 4 mo, and 1 y

Age	Parameter	All Mean (SD)	n	Female Mean (SD)	n	Male Mean (SD)	n	Estimated mean difference (95% CI)	*P* value
6 wk	Area ax sc (mm^2)	30.6 (12.4)	162	32.1 (11.6)	77	29.2 (12.9)	85	−3.2 (−7.0, 0.5)	0.088
	Area sag sc (mm^2)	30.8 (12.2)	160	33.0 (11.9)	76	28.8 (12.2)	84	−4.9 (−8.6, 1.2)	0.009
	Area sag pp (mm^2)	10.7 (3.5)	152	10.9 (3.9)	72	10.6 (3.2)	80	−0.4 (−1.5, 0.7)	0.497
	Ratio PP/SC	0.43 (0.36)	152	0.41 (0.43)	72	0.46 (0.27)	80	0.04 (−0.07, 0.15)	0.480
4 mo	Area ax sc (mm^2)	44.8 (16.6)	160	48.1 (17.2)	79	41.6 (15.4)	81	−6.8 (−11.7, −1.9)	0.007
	Area sag sc (mm^2)	41.5 (12.0)	157	44.2 (14.8)	77	38.8 (14.9)	80	−5.2 (−9.7, −0.7)	0.023
	Area sag pp (mm^2)	13.0 (4.0)	150	13.3 (3.9)	74	12.7 (4.2)	76	−0.4 (−1.6, 0.9)	0.583
	Ratio PP/SC	0.34 (0.15)	150	0.33 (0.14)	74	0.36 (0.16)	76	0.03 (−0.01, 0.07)	0.143
1 y	Area ax sc (mm^2)	31.6 (15.4)	158	34.1 (17.3)	82	28.8 (12.6)	76	−5.5 (−10.1, −0.8)	0.021
	Area sag sc (mm^2)	28.4 (13.3)	156	30.4 (14.4)	80	26.4 (11.9)	76	−4.1 (−8.2, −0.1)	0.046
	Area sag pp (mm^2)	17.8 (5.9)	155	18.0 (5.8)	79	17.5 (5.9)	76	−0.6 (−2.4, 1.3)	0.546
	Ratio PP/SC	0.76 (0.48)	155	0.71 (0.36)	79	0.81 (0.57)	76	0.11 (−0.03, 0.25)	0.124

Data are presented as mean ± SD (n) along with the nonadjusted mean difference (95% confidence interval) from mixed models containing time, sex, and an interaction between sex and time.
PP, preperitoneal; SC, subcutaneous; US, ultrasonography.

Table 3. Inter- and intraclass-correlation-coefficients (ICC) of the precision measurements

		Inter-observer agreement ICC (CI 95%)[a]	Intra-observer agreement ICC (CI 95%)[b]
Mean out of 3 measurements	sag cranial pp	0.95 (0.91, 0.97)	0.98 (0.94, 1.00)
	sag caudal pp	0.94 (0.89, 0.97)	0.87 (0.63, 0.96)
	sag cranial sc	0.98 (0.97, 0.99)	0.98 (0.93, 0.99)
	sag caudal sc	0.98 (0.97, 0.99)	0.98 (0.95, 1.00)
	ax r	0.99 (0.97, 0.99)	0.99 (0.98, 1.00)
	ax m	0.98 (0.97, 0.99)	0.97 (0.92, 0.99)
	ax l	0.99 (0.97, 0.99)	0.99 (0.97, 1.00)
Area	Area sag pp	0.97 (0.94, 0.98)	0.97 (0.90, 0.99)
	Area sag sc	0.99 (0.97, 0.99)	0.98 (0.95, 1.00)
	Area ax sc	0.99 (0.98, 0.99)	0.99 (0.97, 1.00)

Data are presented as correlation coefficients (ICC) and corresponding 95% confidence intervals (CI 95%);
[a]Inter-observer agreement n = 45; [b]Intra-observer agreement n = 12.
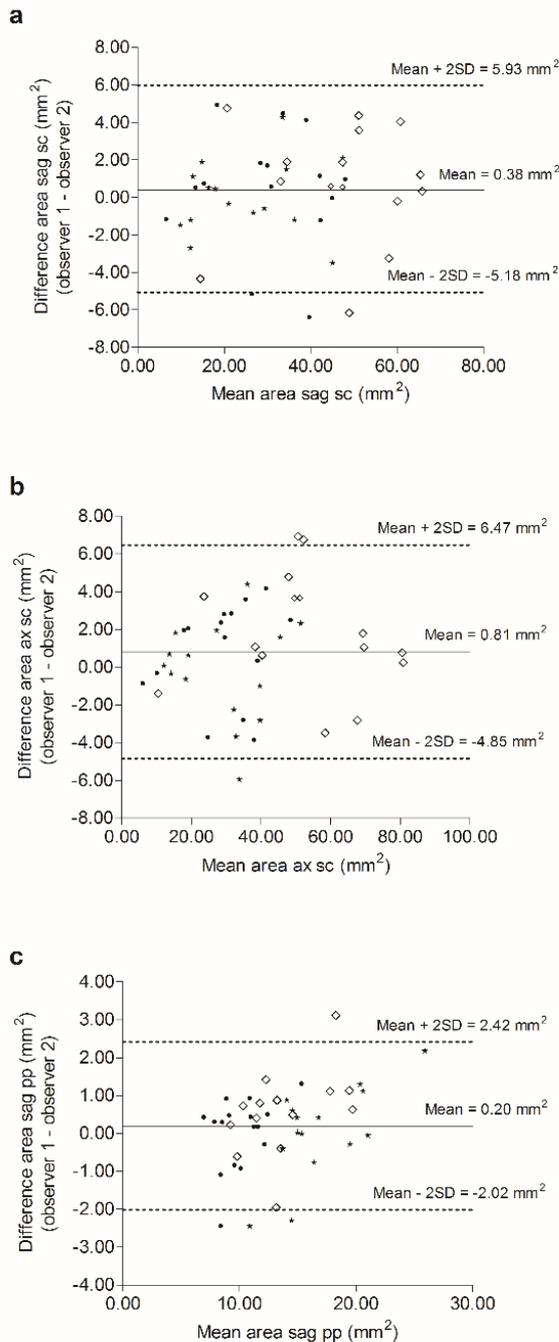
Figure 1. Bland-Altman plots of the area sag sc (**a**), area ax sc (**b**) and area sag pp (**c**), at 6 wk (filled circle), 4 mo (open diamond) and 1 y postpartum (asterisk) of the two observers (observer 1 = E.H., observer 2 = D.M.). The difference between the two observers were plotted against their averages. Average difference and the average difference ± 2 SD, termed as limits of agreements, are plotted.

Effect of Respiration

Breathing phases affect the thicknesses of the fat layers with the greatest thickness at the end of the expiration phase: During inspiration, the liver shifts toward distal direction, reducing the PP fat layer. With increasing expiration, the liver is shifted below the sternum and the layer becomes thicker (data not shown). To consider this effect, measurements were made at the end of expiration using the cine-loop-function.

Effect of Age

The ultrasound investigations showed pronounced differences in the physiological growth of SC and PP fat depots over the first year of life (**Table 2** and **Figure 2a**): Areas of SC fat layers significantly increased from 6 wk (area ax sc = 30.6 mm^2, area sag sc = 30.8 mm^2) to 4 mo postpartum (area ax sc = 44.8 mm^2, area sag sc = 41.5 mm^2, $P < 0.001$) and then significantly decreased toward the first year of life (area ax sc = 31.6 mm^2, area sag sc = 28.4 mm^2, $P < 0.001$).

In contrast, the PP fat layer significantly increased over all measured time points with an estimated mean area sag pp of 10.7 mm^2 in the 6-wk olds, 13.0 mm^2 in the 4-mo and 17.8 mm^2 in the 1-y olds (all $P < 0.001$). Although, some sex differences in the ultrasound measures were estimated, as discussed in the following section, the same general trend with age was observed (**Figure 2b–d**).

The ratio PP/SC of the two AT compartments first decreased slightly from 0.43 to 0.34 due to the greater increase in SC fat. Then, the ratio increased sharply to 0.76 reflecting a shift in the abdominal fat tissue-ratio.

There was large variation in the fat layers between the infants for all measuring positions at each time point of investigation. For example, areas ranged from 3.41 to 40.10 mm^2 in PP fat and ranged from 4.63 to 71.80 mm^2 in SC fat in sagittal plane at 1 y of age.

Effect of Sex

As shown in **Table 2** and **Figure 2b,c**, there is some evidence that females have greater SC fat layers compared to males, particularly at 4 mo and 1 y. There is no evidence that PP fat thickness differs between the sexes (**Table 2** and **Figure 2d**). Consistent with the results measured with US, girls were estimated to have significantly thicker SC fat mass, assessed by higher suprailiac SFT at 6 wk, 4 mo, and 1 y postpartum without consistently significant sex-specific differences in subscapular, biceps, and triceps SFT measurements. The resulting calculated percentage of fat mass was slightly higher at 4 and 12 mo postpartum in girls, but these differences were not statistically significant. Fat distribution was shifted toward a more centralized pattern in the girls compared to the boys: The subscapular-to-triceps SFT ratio was significantly higher in the girls at 6 wk and 12 mo postpartum. In addition, a higher central-to-total SFT ratio was estimated in girls at 6 wk, 4 mo, and 12 mo postpartum (**Table 1**). However, boys had on average a significantly higher body weight and length at each time point of investigation, attributable to a significantly higher lean mass (all $P < 0.001$).
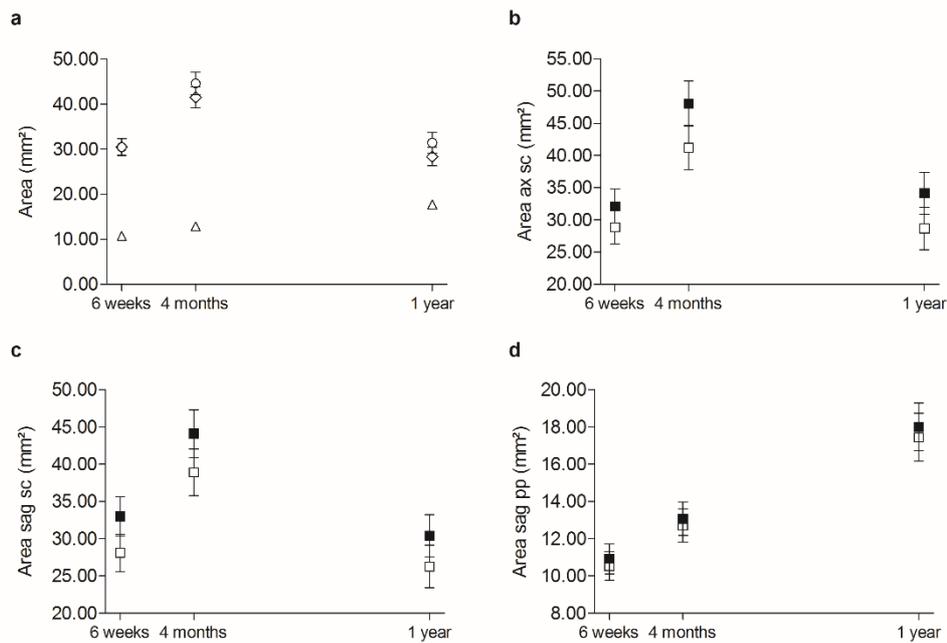
Figure 2. Effect of age and sex on SC and PP fat tissue compartments, stratified by the time point of investigation. (**a**) Comparison of area ax sc (circle), area sag sc (diamond) and area sag pp (triangle) in 6 wk, 4 mo, and 1-y-old infants. Estimated means and 95% confidence intervals are from a mixed linear model with time as a fixed effect. (**b-d**) Effect of sex on area ax sc (**b**), area sag sc (**c**) and area sag pp (**d**) in 6 wk, 4 mo and 1-y-old females (filled squares) and males (open squares). Estimated means and 95% confidence intervals are from a mixed linear model with time, sex and an interaction between sex and time as fixed effects.

Correlation Coefficients of the Different Fat Measures Among Each Other and With Anthropometric Measures

There was strong correlation between the two measures of SC fat at each time point (r > 0.9) (**Figure 3a**). However, the areas calculated in the axial plane were significantly higher at 4 mo and 1 y postpartum (both $P < 0.001$).

The correlations between the PP AT area and the SC AT areas in sagittal and axial plane were weak (Spearman's rho area sag pp/area sag sc r = 0.24, area sag pp/area ax sc r = 0.25) at 6 wk, but increased with increasing age (Spearman's rho at 1 y of age: area sag pp/area sag sc r = 0.47, area sag pp/area ax sc r = 0.47). A scatterplot showing the association between SC and PP in sagittal plane is presented in **Figure 3b**.

PP fat showed weak correlations with the anthropometric measurements at the time points 6 wk and 4 mo. The correlation increased slightly at 1 y for PP fat tissue and subscapular (r = 0.41, $P < 0.001$), suprailiacal (r = 0.31, $P < 0.001$), and sum 4 SFT (r = 0.38, $P < 0.001$). The association between PP fat layers and sum 4 SFT and BMI is shown in **Figure 3c,d**, respectively.

In contrast, the sonographic measures of SC fat tissue were moderately correlated with body weight, length, and BMI at all three time points. They were highly correlated with the sum 4 SFT, especially at 1 y postpartum (area sag sc $r = 0.72$, area ax sc $r = 0.71$, **Table 4**), subscapular (area sag sc $r = 0.70$, area ax sc $r = 0.68$), and suprailiac SFT (area sag sc $r = 0.69$, area ax sc $r = 0.70$), whereas biceps and triceps SFT measurements showed weak to moderate correlations during the assessment period. Associations at each time point between SC fat layers and sum 4 SFT and BMI are shown in **Figure 3e,f**, respectively.
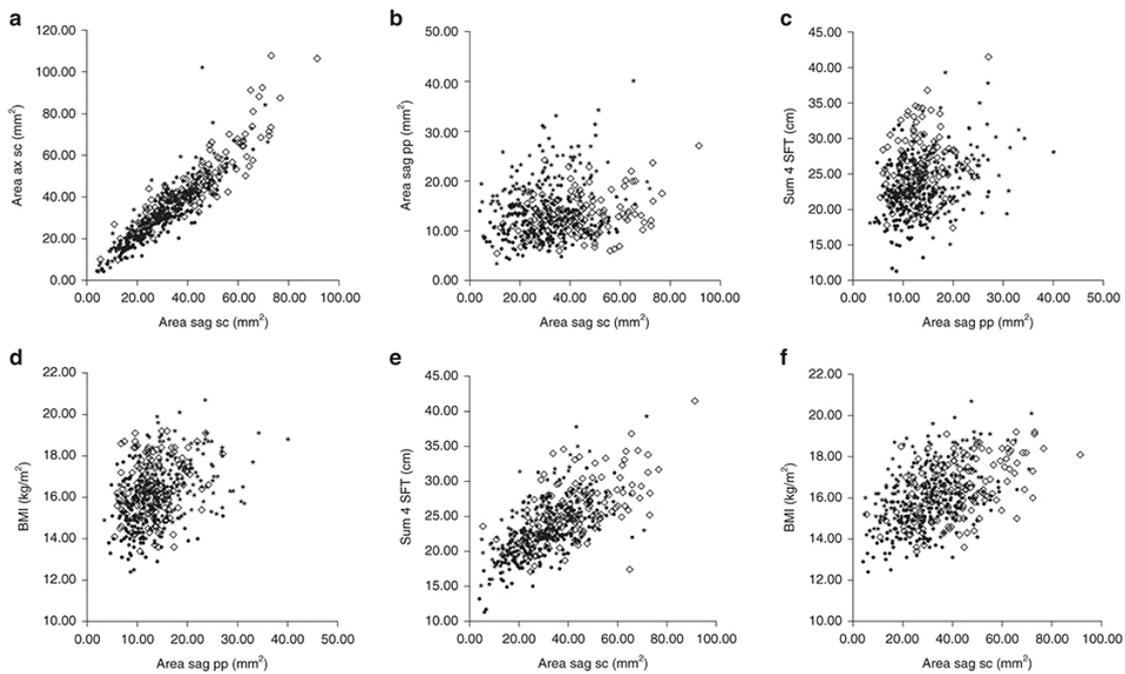
Figure 3. Scatterplots showing the association between subcutaneous (SC) adipose tissue (AT) in sagittal and axial plane (**a**), preperitoneal (PP) and SC AT in sagittal plane (**b**) and between area sag pp (**c, d**) area sag sc (**e, f**) and anthropometric measures at 6 wk (filled circle), 4 mo (open diamond) and 1 y postpartum (asterisk).

Table 4. Spearman-correlation-coefficients between ultrasound and anthropometric measures

6 wk	Area sag pp	Area sag sc	Area ax sc	Ratio PP/SC
Weight	0.17*	0.54†	0.56†	−0.41†
Length	0.14	0.27†	0.25†	−0.22**
BMI	0.10[a]	0.57†[a]	0.60†	−0.43†[a]
Biceps	0.19*	0.39†	0.34†	−0.25**
Triceps	0.18*	0.51†	0.50†	−0.33†
Subscapular	0.11	0.60†	0.64†	−0.48†
Suprailiacal	0.21**	0.65†	0.63†	−0.47†
Sum 4 SFT	0.20*[a]	0.66†[a]	0.65†	−0.46†[a]
4 mo	**Area sag pp**	**Area sag sc**	**Area ax sc**	**Ratio PP/SC**
Weight	0.14	0.40†	0.43†	−0.28†
Length	−0.05	0.02	0.04	−0.12
BMI	0.19*[a]	0.49†[a]	0.52†	−0.28†[a]
Biceps	0.09	0.30†	0.34†	−0.19*
Triceps	0.06	0.48†	0.48†	−0.36†
Subscapular	0.22**	0.53†	0.53†	−0.29†
Suprailiacal	0.25**	0.66†	0.68†	−0.40†
Sum 4 SFT	0.21*[a]	0.63†[a]	0.65†	−0.39†[a]
1 y	**Area sag pp**	**Area sag sc**	**Area ax sc**	**Ratio PP/SC**
Weight	0.25**	0.46†	0.41†	−0.31†
Length	0.10	0.02	−0.04	0.06
BMI	0.26[a]	0.58[a]	0.56	−0.44†[a]
Biceps	0.22**	0.48†	0.46†	−0.33†
Triceps	0.25**	0.45†	0.44†	−0.30†
Subscapular	0.41†	0.70†	0.68†	−0.45†
Suprailiacal	0.31†	0.69†	0.70†	−0.50†
Sum 4 SFT	0.38†[a]	0.72†[a]	0.71†	−0.48†[a]

Values marked with stars show significant correlations at different levels (*P <0.05; ** P <0.01; †
P <0.001); [a]shown in Figure 3.
PP, preperitoneal; SC, subcutaneous; SFT, skinfold thickness.

DISCUSSION

The aim of the present study was to characterize how abdominal PP and SC fat depots, assessed
with US, change over the first year of infancy and to compare these measures with
anthropometric measurements. Therefore, we adapted slightly the technique of sonographic
assessment of PP and SC fat, previously described by Holzhauer et al., (12) for our cohort of
infants ≤1 y of age.

Regarding AT growth, we observed a significant increase of SC fat layers from 6 wk to 4 mo
and a decrease until 1 y postpartum. Our results suggest a different pattern for the development
of PP fat, which increased from 6 wk postpartum up to the age of 1 y (all P < 0.001). There is
some evidence that girls have higher SC fat mass compared to boys, but there was no evidence
of a sex-specific difference in PP fat thickness. While the SC fat layer assessed by US was

strongly correlated with SFT, PP fat tissue was only weakly correlated with conventional anthropometric measures. In addition, we could show for the first time that US is a reproducible method for the quantification of abdominal SC and PP AT in this age group.

A major strength of our study is the longitudinal study design and the relatively large and consistent number of subjects over the survey period. Despite the very young study sample, the results from intra- and inter-observer-analyses and the Bland-Altman plots showed very good agreement and were comparable with the findings of the study of Holzhauer et al. (12), although we have followed a slightly different approach. While the reproducibility in Holzhauer´s paper refers to taking the ultrasound images, we have examined it by analyzing the ultrasound images. However, both approaches showed very good results, indicating a good reproducibility of the method.

Healthy infants gain body fat during their first months of life and SC AT composes the main part of total body fat in the first year postpartum, varying between 89.0 and 92.8% (16). The percentage of total body fat reaches a maximum between 3 and 6 mo postpartum and then slowly decreases during the second half of the first year of life (17,18). This is reflected in our data combining direct and indirect methods and might be due to an increased physical activity at about 6 mo when the crawling phase begins. Holzhauer et al. (12) found a pronounced increase of 45% in the thickness of the PP fat layer during the second year of life, whereas the SC AT showed no increase. Consequently, this resulted in a shift in the abdominal tissue-ratio toward an increase in PP fat mass. We could show that the shift in abdominal fat distribution toward more PP fat already occurs before the age of 1 y. Assuming that PP fat is an approximation of intra-abdominal fat in children (14), our results are consistent with another study which reported a 20% increase in visceral fat between the third and twelfth month postpartum, assessed sonographically (15). Olhager et al. (19) also showed a significant increase in nonsubcutaneous fat layers within the first 4 mo of infancy with MRI scans. Our observations of differential changes in abdominal fat suggest that the two different fat layers develop independently.

Sex differences in the pattern of fat distribution are well known in adults, with women having greater SC and less visceral AT than men (10,20,21). Regarding SC fat, some authors have shown that differences between sexes already occur in childhood from the first year of life on (12,22–25), which could also be demonstrated by our findings. These observations differ from studies that provided no evidence of gender-specific differences under the age of ten (22,25). Regarding PP fat, sex differences in visceral fat seem to be closely linked to age- and puberty-related changes in fat distribution (8,22). However, the question remains open when such

differences in fat patterning first emerge because data in early childhood show conflicting results (9,12,15,22,23,25,26). Further follow-up investigations in children, especially for internal fat, are needed.

Within the first year of life, we found SC fat thickness by US highly correlated with the sum 4 SFT and abdominal SFT, particularly at 1 y of age. The associations with BMI and weight were less pronounced. In contrast, PP fat was rather found to be weakly correlated with these anthropometric measurements. As SFT refers to the measurement of SC fat, this explains why stronger correlation was observed with SC fat than PP fat. Our results are in line with the observations from Liem et al. (27), who showed in 6- to 7-y-old healthy children that the sum of suprailiac and abdominal skinfolds was most strongly associated with SC abdominal AT, assessed by CT, followed by abdominal skinfolds, BMI, suprailiac skinfold, hip, and waist circumferences.

Also in the study of Holzhauer et al., where skinfolds have not been considered, BMI showed only a moderate association with SC fat layers. However, in a meta-analysis of the pediatric literature with 497 children aged 7–16 y, Brambilla et al. (8) identified waist circumference as the best predictor for intraabdominal fat mass and BMI as the best single predictor of SC fat although skinfolds as a predictor were not considered. However, our SFT measurements, especially the sum of 4 SFT and abdominal SFT measurements show stronger correlations with SC fat areas directly measured by US than BMI.

There are some limitations of our study. Although the adaptation to the technique of Holzhauer et al. (15) was minor, our study is lacking a gold standard for comparison such as MRI in this specific age group. To definitively establish this US technique for the assessment of fat distribution in early infancy, an age-specific validation with other direct methods is needed. Furthermore, the study population consisted mostly of German children with a BMI in the normal range. Therefore, the results cannot be generalized to other ethnic groups or to over- or underweight children. A clear differentiation of AT layers with the stated anatomical reference structures is not always possible; there remained some technical difficulties, mostly due to the young age of the participants. For example, the restlessness of the infants made the procedure challenging. Another problem was caused by the high breathing-intensity of the infants, with frequencies of 25–30/min, compared to older children or adults and the influence of liver movements on PP fat thickness. However, it was still possible to obtain high-quality images, which was also reflected by high intra-and interclass-correlation coefficients. Although direct methods, such as CT and MRI represent the gold standard for the assessment of SC and PP AT, they have only a limited application for scientific research in infants. Reasons for that (i.e., apart

from the cost- and time-consuming certainty), include a high sensitivity to breathing motions (i.e., breath-holding techniques are not feasible in this age group), the need for expensive measurement equipment, handled by suitably trained personnel and the exposure to radiation (CT only) (12,15). Mook-Kanamori and colleagues compared in a group of 34 nonobese children with a median age of 9.5 y (95% range 0.3–17.0 y) SC and PP fat thickness and areas by CT and US. Correlation coefficients ranged from 0.75–0.97 (all $P < 0.001$). Two other studies used a different sonographic approach to assess SC and intra-abdominal AT and performed validation studies with children (n = 31, range 6.0–7.9 y) (27) and newborns (n = 22; range 6–19 d) (15). They validated US against MRI measurements and showed moderate to strong positive correlations. However, to definitively establish US for the assessment of fat distribution in children, an additional age-specific validation with a larger sample size for each age group is required.

In summary, our data suggest that US is a feasible method with good reproducibility for the quantification of abdominal SC and PP AT in early infancy. Especially, the latter was described as a discretely developing fat depot. Our results clearly indicate a differential growth of both fat depots towards an increase in PP fat mass during the first year of life. Further studies of longitudinal design, with different assessments over the first year of life and beyond are warranted, to characterize the temporal pattern of AT development at the specific anatomical locations. By associating this data with metabolic parameters, this information may allow a better prediction and prevention of disease risk early in life.

METHODS

Study Population

This analysis was embedded in the INFAT-study, a randomized, controlled trial primarily designed to investigate the effect of fatty acids in maternal nutrition during pregnancy and lactation on infant AT development within the first year of life. Rationale, study design, and the clinical results up to 1 y of age have been described in detail elsewhere (28,29). The study population consisted of 208 healthy pregnant women of Caucasian origin (99.5%) and their newborns, living in the area of Munich, Bavaria, Germany and recruited between July 2006 and May 2009. As there were no significant differences between the study groups with respect to infant body composition (28), study groups were pooled for the following analysis. For the present analysis, only 162 infants with available ultrasound data at 6 wk postpartum were included. The ethical committee of the Technische Universitat München (No. 1479/06/2006/2/21) approved the study protocol. Written informed consent was obtained from all participating mothers.

Data Collection and Anthropometric Measurements

Anthropometric data and SFT were obtained by trained research assistants at 6 wk, 4 mo, and 1 y postpartum as previously described (28). In brief, the infants' weight and length were measured and BMI (kg/m^2) was calculated. SFT measurements were performed in triplicate under standard conditions with a Holtain caliper (Holtain, Croswell, Crymych, UK) at the left body axis at four sites (triceps, biceps, subscapular, suprailiac). The mean of the triplicate measurements was used for analysis. The calculation of body fat (%) was done via predictive skinfold equations according to the method of Weststrate et al. (30). Additionally, we calculated the sum of the four skinfolds and two indices of fat patterning: the subscapular-to-triceps skinfold ratio as an index of central to peripheral fat distribution (31) and the central-to-total skinfolds ratio (trunk-to-total skinfolds %) using the equation (subscapular + suprailiac)/(sum 4 SFT)*100 (32).

Sonographic Assessment of Abdominal Subcutaneous and Preperitoneal Fat

The ultrasound investigations were performed using a high-resolution ultrasonographic system (Siemens Acuson Premium, Munich, Germany). Measurements were performed by two trained research pediatricians (E.H., V.S.). Abdominal SC and PP fat thickness, the latter considered to be an approximation of visceral/intraabdominal fat (12), were measured with a 10 MHz linear probe (VFX 13-5, Siemens Medical Solutions, Erlangen, Germany) in b-picturemode. The infants were located in supine position. Care was taken to minimize movements of the infant. The probe was placed on the skin surface of the upper abdomen of the infant without compression of the tissue layers. We defined two areas of measurement: To determine PP and SC fat, the first measurement was performed in sagittal plane in the middle of the xiphoid process. The second measurement was performed in axial plane, in between the xiphoid process and the umbilicus to determine the SC fat layer. In previous studies in children or adults (27,33), the measurements were performed at the end of a gentle expiration, however, this procedure is not applicable in a young pediatric population. To get a standardized breathing-phase the cine-loop-function was used. By this function, it is possible to save the last 63 pictures taken and after "defreezing", all individual pictures can be displayed. Thereby, it was possible to identify retrospectively single pictures taken at the end of expiration with tissue layers as much as possible in parallel. The images were stored at an off-line working station for evaluation (Apple Power PC G4, Apple, Cupertino, CA).

Evaluation of the Ultrasound Pictures

The size of each individual fat layer was determined with the OsiriX software (http://www.osirix-viewer.com, Genf, Schweiz) in both planes. The evaluation process was performed off-line by two examiners (E.H., D.M.) in a blinded fashion after selecting the three most appropriate pictures in sagittal and axial plane for each case.

Preperitoneal fat. Preperitoneal fat was defined as distance between the linea alba as the upper border until the peritoneum located at the upper margin of the liver as the lower border. The first measurement point was set 0.5 cm caudal from the xiphoid process (sag cranial pp), appearing as a hypoechoic cartilaginous structure, while the second measurement point was set 1.0 cm caudal from the first reference point (sag caudal pp) (**Figure 4**). In each patient, three pictures were evaluated. Means of the measured distances were calculated and used to estimate the area of PP fat by following the formula for trapezoid areas:

$$\text{Area sag pp} \; = \; \frac{\text{sag cranial pp (cm)} + \text{sag caudal pp (cm)}}{2} \; \text{x 1 (cm)}$$

Subcutaneous fat. The SC fat layer was determined in sagittal and axial plane. Fat layers were defined as the echo-poor space between the echo-rich cutis and the echo-rich linea alba or the M. rectus abdominis, respectively. In sagittal plane, the first reference point was set 1.0 cm caudal the xyphoid process, the lower margin of the sternum (sag cranial sc) and the second reference point 1.0 cm caudal of the first reference point (sag caudal sc), with highest parallelism of the layers (**Figure 4**). In axial plane, the first measurement point was set directly above the linea alba (ax m) as well as 1.0 cm on the right (ax r) and left (ax l) of the linea alba between the cutis and the M. rectus abdominis (**Figure 5**).
In sagittal and axial plane, three pictures were evaluated.
Means of the measured distances were calculated and used to estimate the area of SC fat by using the formula:

$$\text{Area sag sc} \; = \; \frac{\text{sag cranial sc (cm)} + \text{sag caudal sc (cm)}}{2} \; \text{x 1 (cm)}$$

$$\text{Area ax sc} \; = \; \left(\frac{\text{ax r (cm)} + \text{ax m (cm)}}{2} \; \text{x 1 (cm)} + \frac{\text{ax l (cm)} + \text{ax m (cm)}}{2} \; \text{x 1 (cm)} \right) / \, 2$$

Moreover, the ratio of PP and SC fat tissue from the sagittal plane was calculated:

$$\text{Ratio PP / SC} \; = \; \frac{\text{Area sag pp}}{\text{Area sag sc}}$$

The technique used was a method originally described by Holzhauer and colleagues (12) with small modifications to assess abdominal fat distribution in children under the age of one. Holzhauer et al. (12) suggest calculating the area with the length of 2.0 cm in sagittal plane. However, this evaluation was not deemed appropriate for the age group <1 y of age. Therefore, we chose a length of 1.0 cm for the calculation of the fat areas. In addition, SC fat layer was determined in sagittal and axial plane, respectively.
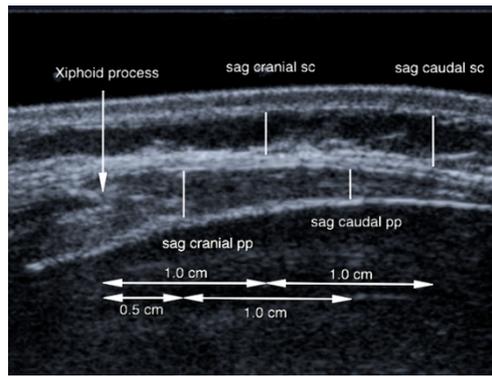
Figure 4. Example for measurements in the sagittal plane with labelling of the xiphoid process (reference structure) and the measurement points sag cranial sc and sag caudal sc (were set 1.0 cm and 2.0 cm on the right of the reference structure, respectively) as well as sag cranial pp and sag caudal pp (were set 0.5 cm and 1.5 cm on the right of the reference structure, respectively); areas are calculated by the formula of trapezoid.
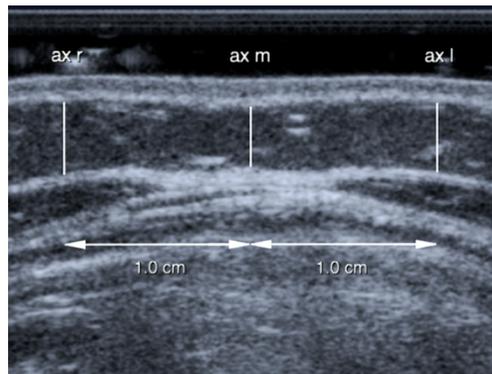
Figure 5. Example for measurements in the axial plane. The measurement is performed directly above the linea alba (ax m) as well as 1.0 cm on the right (ax r) and 1.0 left (ax l) midway between the xiphoid process and the umbilicus; areas are calculated by the formula of trapezoid.

Reproducibility

To calculate intra-observer agreement, the investigations of 12 infants (n = 4 from each time point), were used and analyzed with OsiriX software by one examiner (E.H.) twice. For the assessment of inter-observer variation, 45 randomly chosen ultrasound measurements were independently evaluated with the software by two observers (E.H. and D.M.). For intra- and inter-observer variation, the examiners independently evaluated three pictures and the fat areas were calculated.

Statistical Analysis

Summary ultrasound data, anthropometric data and SFT are presented for infants at 6 wk, 4 mo, and 1 y. Mixed linear models (using an unstructured covariance matrix) were fitted to these repeated measures with time as a fixed effect. To explore how changes over time differ according to sex, sex was added as a fixed effect in the model together with an interaction between sex and time. Estimated mean differences in sex are presented for each measure at each time point, together with 95% confidence intervals. Associations between anthropometric and ultrasound variables were assessed using Spearman-Rho correlation coefficient. Intra- and inter-observer agreements were examined using ICC and their 95% confidence intervals. An ICC of 1 indicates that all of the observed variation is caused by between subject variations. Additionally, we performed Bland-Altman plots of the areas sag pp, sag sc and ax sc. Statistical analyses were performed using PASW software (version 21, SPSS, Chicago, IL). A two-sided *P* value <0.05 was considered statistically significant, and no correction was made for multiple comparisons.

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Chapter Ib – Sonographic fat assessment in children (2–5 y)

Following the method described in Brei et al. (2015), ultrasonography was performed to investigate the development of abdominal subcutaneous and preperitoneal fat depots in children in a follow-up study. In the following, the effect of age, sex and the correlation with anthropometric measures from 2–5 y of age are presented. Data were available for 111 children (n = 48 girls, n = 63 boys) aged 2 y (median 23.3 mo), 103 children (n = 50 girls, n = 53 boys) aged 3 y (median 36.3 mo), 95 children (n = 44 girls, n = 51 boys) aged 4 y (median 48.2 mo) and 98 children (n = 48 girls, n = 50 boys) aged 5 y (median 60.3 mo), respectively.

3.1.1   Effect of age and sex

Differential physiological growth of subcutaneous and preperitoneal fat continued to be observed between 2 and 5 y of age with a large variation at all ages. Results are given in **Table 2**. Areas of preperitoneal fat layers increased significantly every year from 2 to 5 y of age (all P values < 0.001). In comparison, subcutaneous fat layers declined up to 2 y of life, remained relatively stable for one year and showed a significant increase in both planes from 3–4 y (both P values = 0.047). From 4–5 y, changes in fat development were not significant (area$_{axial}$, P = 0.520; area$_{sagittal}$, P = 0.507) (data not shown). Subcutaneous fat measurements were significantly higher in females at each time point investigated. While there was no evidence of a difference between sexes in regard to preperitoneal fat areas up to the second year of life, data showed significant differences from 3 y onwards, with greater areas in females. At 3 y of age, the estimated mean difference was −4.8 mm^2 (95% CI: −8.6, −0.9; P = 0.016).

3.1.2   Correlation coefficients of AT compartments with anthropometric measures

In **Table 3**, Spearman correlation coefficients between AT compartments and anthropometric measurements from 2–5 y of life are given. Waist circumference was amended for follow-up analysis. The observed weak positive correlations between the preperitoneal area and suprailiac SFT and sum of 4 SFTs persisted up to 5 y of age, while suprailiac SFT showed weak to moderate correlations from 2 y onwards. Similar correlations were found for waist circumference, highest at 5 y of age (r_s = 0.326, P = 0.001). The estimates for the correlation coefficients between all other anthropometric measurements and the preperitoneal area did not notably differ from the first-year results except for triceps at 3 y of age (r_s = 0.315, P = 0.002) and biceps at 4 y of age (r_s = 0.349, P = 0.001).

Moderate to strong correlations between subcutaneous fat tissue from both planes and weight, BMI/BMI percentiles and skinfolds were observed with strongest correlations between suprailiac SFT and subcutaneous fat in the axial plane (at 5 y: $r_s = 0.809$, $P < 0.001$). In addition, waist circumference showed relatively strong correlations for subcutaneous fat areas (subcutaneous area$_{sagittal}$ at 5 y: $r_s = 0.593$; subcutaneous area$_{axial}$ at 5 y: $r_s = 0.577$, both P values < 0.001). Consistently negative correlations between the ratio of preperitoneal to subcutaneous fat and anthropometric measures were observed.

Table 2 Subcutaneous and preperitoneal fat measurements by age and gender (2–5 y)

Age	Parameter	All		Female		Male		Estimated mean difference (95% CI)	P
		Mean (SD)	n	Mean (SD)	n	Mean (SD)	n		
2 y	Area ax sc (mm^2)	24.4 (12.3)[1]	111	28.7 (14.3)	48	21.2 (9.4)	63	−7.5 (−11.6, −3.3)	<0.001
	Area sag sc (mm^2)	18.7 (10.9)	111	21.5 (12.6)	48	16.6 (9.0)	63	−5.2 (−8.8, −1.5)	0.006
	Area sag pp (mm^2)	23.7 (7.5)	111	24.4 (6.8)	48	23.1 (8.0)	63	−1.2 (−3.8, 1.3)	0.345
	Ratio PP/SC	1.6 (1.1)	111	1.5 (0.9)	48	1.8 (1.2)	63	0.3 (−0.1, 0.7)	0.097
3 y	Area ax sc (mm^2)	27.2 (17.8)	102	33.3 (20.8)	50	21.4 (11.9)	52	−11.8 (−17.4, −6.2)	<0.001
	Area sag sc (mm^2)	19.6 (12.0)	103	23.8 (13.7)	50	15.7 (8.6)	53	−8.2 (−12.0, −4.5)	<0.001
	Area sag pp (mm^2)	32.6 (11.2)	102	34.6 (11.7)	49	30.8 (10.5)	53	−4.8 (−8.6, −0.9)	0.016
	Ratio PP/SC	2.2 (1.5)	102	1.9 (1.0)	49	2.6 (1.7)	53	0.7 (0.2, 1.2)	0.004
4 y	Area ax sc (mm^2)	28.0 (19.4)	95	34.3 (22.4)	44	22.5 (14.5)	51	−14.8 (−21.6, −8.0)	<0.001
	Area sag sc (mm^2)	19.9 (12.2)	93	24.9 (13.1)	42	15.9 (9.8)	51	−10.4 (−14.6, −6.3)	<0.001
	Area sag pp (mm^2)	40.6 (13.9)	94	44.3 (13.8)	43	37.5 (13.4)	51	−7.4 (−12.2, −2.7)	0.002
	Ratio PP/SC	2.8 (1.8)	93	2.2 (1.3)	42	3.2 (2.0)	51	1.1 (0.4, 1.7)	<0.001
5 y	Area ax sc (mm^2)	29.3 (20.0)	98	35.1 (22.6)	48	23.6 (15.3)	50	−13.8 (−20.9, −6.7)	<0.001
	Area sag sc (mm^2)	20.7 (12.9)	97	24.5 (14.4)	47	17.1 (10.2)	50	−8.7 (−13.2, −4.2)	<0.001
	Area sag pp (mm^2)	48.4 (14.2)	96	51.7 (14.2)	47	45.2 (13.6)	49	−7.7 (−12.6, −2.9)	0.002
	Ratio PP/SC	3.2 (2.1)	96	2.8 (1.6)	47	3.6 (2.5)	49	0.9 (0.2, 1.7)	0.012

[1] Data are presented as mean ± SD (n) along with the nonadjusted mean difference (95% confidence interval) from mixed models containing time, sex, and an interaction between sex and time.

Area ax sc, area of subcutaneous fat in sagittal plane; Area sag pp, area of preperitoneal fat in sagittal plane; Area sag sc, area of subcutaneous fat in sagittal plane; Ratio PP/SC, ratio of preperitoneal/subcutaneous fat areas (from sagittal plane only); US, ultrasonography.

Table 3 Correlation-coefficients between US and anthropometric measures (2–5 y)

Age	Parameter	Area sag pp		Area sag sc		Area ax sc		Ratio PP/SC	
		r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
2 y	Weight (kg)	0.254 [111][1]	0.001	0.457 [111]	0.000	0.508 [111]	0.000	−0.311 [111]	0.001
	Height (cm)	0.129 [111]	0.178	0.166 [111]	0.081	0.222 [111]	0.019	−0.112 [111]	0.243
	BMI (kg/m^2)	0.233 [111]	0.014	0.490 [111]	0.000	0.517 [111]	0.000	−0.345 [111]	0.000
	BMI percentiles	0.254 [111]	0.007	0.510 [111]	0.000	0.540 [111]	0.000	−0.357 [111]	0.000
	Biceps (mm)	0.165 [109]	0.086	0.465 [109]	0.000	0.457 [111]	0.000	−0.397 [109]	0.000
	Triceps (mm)	0.115 [106]	0.239	0.372 [106]	0.000	0.384 [106]	0.000	−0.335 [106]	0.000
	Subscapular (mm)	0.318 [111]	0.001	0.572 [111]	0.000	0.579 [111]	0.000	−0.409 [111]	0.000
	Suprailiac (mm)	0.315 [108]	0.001	0.638 [108]	0.000	0.652 [108]	0.000	−0.496 [108]	0.000
	Sum 4 SFTs (mm)	0.323 [105]	0.001	0.664 [105]	0.000	0.661 [105]	0.000	−0.527 [105]	0.000
	Waist circumference (cm)	0.280 [94]	0.006	0.498 [94]	0.006	0.555 [94]	0.000	−0.353 [94]	0.000
3 y	Weight (kg)	0.270 [102]	0.006	0.383 [103]	0.000	0.395 [102]	0.000	−0.226 [102]	0.023
	Height (cm)	0.287 [102]	0.003	0.182 [103]	0.066	0.154 [102]	0.123	−0.021 [102]	0.837
	BMI (kg/m^2)	0.159 [102]	0.111	0.396 [103]	0.000	0.443 [102]	0.000	−0.296 [102]	0.002
	BMI percentile	0.169 [102]	0.089	0.408 [103]	0.000	0.459 [102]	0.000	−0.303 [102]	0.002
	Biceps (mm)	0.247 [97]	0.015	0.558 [98]	0.000	0.568 [97]	0.000	−0.413 [97]	0.000
	Triceps (mm)	0.315 [97]	0.002	0.337 [98]	0.001	0.374 [97]	0.000	−0.166 [97]	0.104
	Subscapular (mm)	0.188 [97]	0.065	0.591 [98]	0.000	0.591 [97]	0.000	−0.488 [97]	0.000
	Suprailiac (mm)	0.333 [96]	0.001	0.789 [97]	0.000	0.839 [96]	0.000	−0.618 [96]	0.000
	Sum 4 SFTs (mm)	0.342 [96]	0.001	0.655 [97]	0.000	0.696 [96]	0.000	−0.476 [96]	0.000
	Waist circumference (cm)	0.209 [102]	0.035	0.405 [103]	0.000	0.472 [102]	0.000	−0.284 [102]	0.004
4 y	Weight (kg)	0.260 [94]	0.012	0.370 [93]	0.000	0.350 [95]	0.001	−0.249 [93]	0.016
	Height (cm)	0.219 [94]	0.034	0.149 [93]	0.153	0.098 [95]	0.345	−0.051 [93]	0.626
	BMI (kg/m^2)	0.233 [94]	0.024	0.406 [93]	0.000	0.426 [95]	0.000	−0.288 [93]	0.005
	BMI percentile	0.253 [94]	0.014	0.435 [93]	0.000	0.454 [95]	0.000	−0.317 [93]	0.002
	Biceps (mm)	0.349 [92]	0.001	0.490 [91]	0.000	0.438 [93]	0.000	−0.384 [91]	0.000
	Triceps (mm)	0.165 [92]	0.116	0.500 [91]	0.000	0.397 [93]	0.000	−0.478 [91]	0.000
	Subscapular (mm)	0.234 [91]	0.026	0.587 [90]	0.000	0.560 [92]	0.000	−0.531 [90]	0.000
	Suprailiac (mm)	0.339 [91]	0.001	0.738 [90]	0.000	0.751 [92]	0.000	−0.585 [90]	0.000
	Sum 4 SFTs (mm)	0.326 [91]	0.002	0.702 [90]	0.000	0.654 [92]	0.000	−0.601 [90]	0.000
	Waist circumference (cm)	0.266 [94]	0.010	0.501 [93]	0.000	0.492 [95]	0.000	−0.369 [93]	0.000
5 y	Weight (kg)	0.271 [96]	0.008	0.516 [97]	0.000	0.416 [98]	0.000	−0.391 [96]	0.000
	Height (cm)	0.271 [96]	0.008	0.219 [97]	0.004	0.197 [98]	0.052	−0.193 [96]	0.059
	BMI (kg/m^2)	0.153 [96]	0.137	0.485 [97]	0.000	0.453 [98]	0.000	−0.409 [96]	0.000
	BMI percentile	0.164 [96]	0.110	0.503 [97]	0.000	0.474 [98]	0.000	−0.426 [96]	0.000
	Biceps (mm)	0.177 [95]	0.087	0.599 [96]	0.000	0.573 [97]	0.000	−0.569 [95]	0.000
	Triceps (mm)	0.259 [96]	0.011	0.433 [97]	0.000	0.465 [98]	0.000	−0.371 [96]	0.000
	Subscapular (mm)	0.219 [95]	0.033	0.616 [96]	0.000	0.624 [97]	0.000	−0.560 [95]	0.000
	Suprailiac (mm)	0.414 [94]	0.000	0.788 [95]	0.000	0.809 [96]	0.000	−0.632 [94]	0.000
	Sum 4 SFTs (mm)	0.322 [94]	0.002	0.723 [95]	0.000	0.744 [96]	0.000	−0.628 [94]	0.000
	Waist circumference (cm)	0.326 [96]	0.001	0.593 [97]	0.000	0.577 [98]	0.000	−0.452 [96]	0.000

[1]Spearman correlation coefficients; *n* in brackets (all such values).
Area ax sc, area of subcutaneous fat in sagittal plane; Area sag pp, area of preperitoneal fat in sagittal plane; Area sag sc, area of subcutaneous fat in sagittal plane; BMI, body mass index; Ratio PP/SC, ratio of preperitoneal/subcutaneous fat areas (from sagittal plane only); SFT, skinfold thickness.

Chapter II – Long-chain PUFAs and offspring body composition

Reduction of the n–6:n–3 long-chain PUFA ratio during pregnancy and lactation on offspring body composition: follow-up results from a randomized controlled trial up to 5 y of age[1–3]

Christina Brei,[4] Lynne Stecher,[4] Daniela Much,[5,7] Marie-Theres Karla,[4] Ulrike Amann-Gassner,[4] Jun Shen,[6] Carl Ganter,[6] Dimitrios C Karampinos,[6] Stefanie Brunner,[4] and Hans Hauner[4,8]*

[4]Else Kröner-Fresenius-Center for Nutritional Medicine, [5]Forschergruppe Diabetes, and [6]Department of Diagnostic and Interventional Radiology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [7]Institute of Diabetes Research, Helmholtz Zentrum München, Munich, Germany; and [8]ZIEL–Institute for Food and Health, Nutritional Medicine Unit, Technische Universität München, Freising, Germany

ABSTRACT

Background: It has been hypothesized that the n–6:n–3 (ω-6:ω-3) long-chain polyunsaturated fatty acid (LCPUFA) ratio in the maternal diet during the prenatal and early postnatal phase positively affects the body composition of the offspring. However, only limited data from prospective human intervention studies with long-term follow-up are available.

Objective: We assessed the long-term effects of a reduced n–6:n–3 LCPUFA ratio in the diets of pregnant and lactating women [1020 mg docosahexaenoic acid (DHA) plus 180 mg eicosapentaenoic acid (EPA)/d together with an arachidonic acid–balanced diet compared with a control diet] on the body weights and compositions of their offspring from 2 to 5 y of age with a focus on the 5-y results.

Design: Participants in the randomized controlled trial received follow-up assessments with annual body-composition measurements including skinfold thickness (SFT) measurements (primary outcome), a sonographic assessment of abdominal subcutaneous and preperitoneal fat, and child growth. In addition, abdominal MRI was performed in a subgroup of 5-y-old children. For the statistical analysis, mixed models for repeated measures (MMRMs) were fit with the use of data from each visit since birth (except for MRI).

Results: Maternal LCPUFA supplementation did not significantly influence the children's sum of 4 SFTs [means ± SDs at 5 y of age: intervention, 23.9 ± 4.7 mm ($n = 57$); control, 24.5 ± 5.0 mm ($n = 55$); adjusted mean difference, −0.5 (95% CI: −2.2, 1.2)], growth, or ultrasonography measures at any time point in the adjusted MMRM model (all P values < 0.05). Results were consistent with abdominal MRI measurements ($n = 44$) at 5 y of age, which showed no significant differences in subcutaneous and visceral adipose tissue volumes and ratios.

Conclusion: The current study provides no evidence that a dietary reduction of the n–6:n–3 LCPUFA ratio in the maternal diet during pregnancy and lactation is a useful early preventive strategy against obesity at preschool age. This trial was registered at clinicaltrials. gov as NCT00362089. *Am J Clin Nutr* doi: 10.3945/ajcn.115. 128520.

Keywords: body composition, LCPUFA, obesity, preschool age, prevention

INTRODUCTION

The increasing prevalence of overweight and obesity, particularly in early life stages, has negative implications for individuals and society as a whole (1). To prevent and overcome this problem, different approaches are being pursued on a global level (2). These methods include approaches that are based on the concept of fetal programming, which hypothesizes that the intrauterine environment during the prenatal period is associated with lifelong, adverse health-related outcomes in the offspring. One such outcome is risk of obesity and its associated diseases (3). Specific emphasis has been placed on intake and the ratio of essential n–3 and n–6 long-chain PUFAs (LCPUFAs)[9] because they may be involved in early adipocyte differentiation (4). On the basis of in vitro and animal studies (5), it was hypothesized that the ratio of n–6:n–3 LCPUFA intake during the prenatal and early postnatal period influences the development of fat mass in offspring, with a lower ratio preventing excess adipose tissue development (6).

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On the basis of this consideration, several prospective human cohort studies and randomized controlled trials (RCTs) have been performed that investigated the impact observed on altering the n–6:n–3 fatty acid ratio. These studies showed conflicting results (7–12). However, they were mostly post hoc analyses in trials that were primarily designed to investigate other outcomes such as infant neurodevelopment. To our knowledge, the INFAT (Impact of Nutritional Fatty Acids during Pregnancy and Lactation on Early Human Adipose Tissue Development) study was the first human RCT to focus on the impact of a dietary change or modification of the n–6:n–3 LCPUFA ratio in pregnant and lactating women on infant adipose tissue growth as the primary outcome. Supplementation with fish-oil capsules (1020 mg DHA plus 180 mg EPA/d) together with an arachidonic acid (AA)–balanced diet provided no evidence that this dietary intervention could prevent excess adipose tissue growth in infants ≤1 y of age (13, 14). According to a recent meta-analysis by Stratakis et al. (15), there is currently no conclusive evidence to support a favorable programming effect of n–3 LCPUFA supplementation during pregnancy or lactation on BMI of preschool children. However, because of between-study heterogeneity and methodologic limitations, such as small sample sizes or selective attrition rates, the authors claimed that additional high-quality studies are required (15). Furthermore, the need to assess the long-term effects has also been highlighted (15–18). To assess the long-term effects of the intervention, the infants in the INFAT study were followed up until they reached preschool age. In the current article, we present the findings of the follow-up study with a primary focus on the 5-y results.

METHODS

The INFAT study was conducted as an open-label, mono-center, randomized, controlled dietary intervention trial with 2 parallel groups, with each group consisting of 104 pregnant women. Originally, the study was designed to investigate the effect of a reduction in the n–6:n–3 LCPUFA ratio in the diets of pregnant women and breastfeeding mothers on adipose tissue growth in their infants aged ≤1 y. To investigate the long-term effects, the infants were followed up until the age of 5 y. Details of the rationale, study design (including the sample-size determination, eligibility criteria, and process of random assignment), participant characteristics, and clinical results on the fat mass of infants ≤1 y old together with maternal and fetal fatty acid profiles have been previously described (13, 14, 19).

Subjects

In total, 208 healthy, pregnant women with a mean age of 32 y and a mean prepregnancy BMI (in kg/m^2) of 22 were recruited before the 15th wk of gestation between July 2006 and May 2009. From enrollment until 4 mo postpartum, women in the intervention group received 1200 mg LCPUFAs (1020 mg DHA plus 180 mg EPA plus 9 mg vitamin E) as fish-oil capsules daily. In addition, the dietary n–6:n–3 LCPUFA ratio was further reduced through specific, individualized dietary counseling aimed at lowering AA intake. The control group received general recommendations regarding healthy nutrition during pregnancy. Baseline maternal clinical characteristics, dietary habits, lifestyle factors, and sociodemographic variables did not differ significantly

between the 2 study groups. Within the study, 188 women gave birth to healthy infants (n = 90 girls; n = 98 boys) with a mean difference in pregnancy duration of 4.8 d (95% CI: 1.19, 7.67) between study groups. For the initial study, infants were assessed at ≤1 y of age (13, 14). Follow-up assessments were performed between February 2008 and November 2014 to detect the possible long-term effects of the intervention in the offspring at 2, 3, 4, and 5 y of age. All follow-up assessments included the same measurements of infant growth and fat mass that were performed during the first year of life with skinfold thickness (SFT) measurements remaining as the primary outcome measurement. Participant data were collected at the study center or were assessed by a study team member during a home visit. Furthermore, an abdominal MRI at 5 y of age (Department of Radiology, Klinikum rechts der Isar, Munich) and the assessment of the children's diet and physical activity (PA) at 3 respective time points (3, 4, and 5 y of age) were investigated. The ethical committee of the Technische Universität München approved the study protocol (1479/06/2009/ 10/26). Written informed consent for follow-up was obtained from both parents of each child.

Child growth and development

For infants ≤2 y of age, weight was measured to the nearest 10 g with the use of a standard infant scale (Babywaage Ultra MBSC-55; myweight), and length was measured with the use of a measuring stick (Säuglingsmessstab seca 207; seca) to the nearest 0.5 cm while the infant was supine with stretched legs. At later time points, a standard flat scale (Seca Clara 803; seca) was used to determine weight to the nearest 100 g. In addition, a stadiometer (Stadiometer seca 214; seca) was used to measure the child height to the nearest 0.5 cm with both measures performed with the child in a standing position. BMI percentiles were determined with the use of the German reference group according to Kromeyer-Hauschild et al. (20).

Fat mass and fat distribution of the children

SFT

The infant's SFTs as a primary outcome was measured in triplicate with the use of a Holtain caliper (Holtain Ltd.) at 4 different body sites on the left body axis (triceps, biceps, subscapular, and suprailiac). Measurements were performed at 2, 3, 4, and 5 y of age at the study center or at the family's home. For each site, the mean of the 3 measurements was used for the SFT value, and the sum of the 4 SFTs was calculated. Fat mass and the percentage of body fat were calculated with the use of predictive skinfold regression equations according to Weststrate and Deurenberg (21).

Ultrasound

In addition, ultrasonography was performed to determine abdominal subcutaneous and preperitoneal fat areas with the use of a high-resolution ultrasonographic system (Siemens Acuson ×150 Premium; Siemens) at 2, 3, 4, and 5 y of age. The originally described method from Holzhauer et al. (22) was slightly modified for our purposes and has been described in detail (23). Three trained research assistants (CB, SB, and K Pusch) collected data on infant growth, SFT measurements, and ultrasounds.

MRI measurement

In addition, abdominal MRI in a subgroup of 5-y old children was performed to quantify abdominal adipose tissue. Although all participating families were approached, data for the analysis were only available from 44 children. Before the examination, additional written informed consent was obtained from the accompanying parent. An MRI examination was performed without sedation on a clinical whole body scanner at 1.5 T (Magnetom Avanto; Siemens Medical Solutions). The children were positioned supine (feet first) with arms next to their bodies and with spine array receive coils included in the patient table. In addition, a body matrix coil was applied ventrally, which covered the entire abdominal region. To mitigate the possible degradation of image quality because of the limited ability to comply with the MRI scan, the imaging protocol had to be kept as short as possible. The quantitative scans were planned on localizer images, the latter of which were obtained under free-breathing conditions (field of view: 500 mm; 3 orientations; duration: 13 s). Water and fat separation was based on a 4-point (echo time: 2.38, 4.76, 7.15, and 9.53 ms) Dixon technique as described by Glover (24). The total measurement consisted of a series of blocks of 4 transverse spoiled gradient echo images, which were acquired in inspiration (breath hold of 3.9 s). The resolution was 2.34×2.34 mm^2 in plane and 10 mm in the head-feet direction (80 mm slice thickness and an additional gap of 2 mm). Additional imaging variables included repetition time of 50 ms, flip angle of 55°, and a monopolar readout gradient to avoid phase shifts between in-phase and opposed-phase images.

Postprocessing of MRI data

The acquired data were exported to a remote workstation and analyzed with self-written software in the MatLab program (R2014b; MathWorks) according to the procedure outlined by Glover (24). On the basis of the phase images, a (smoothed) B0 map was calculated that included a local off resonance and a hardware-related offset (coil phase) with the exclusion of the chemical shift between water and fat. Subsequently, the complex images were B0 corrected and transformed into real-value images. The ratio of the in-phase and opposed-phase magnitude images (evaluated independently and subsequently averaged) allowed for an estimate of the T2* decay, which further improved the water and fat separation (24). Finally, the water and fat images were calculated with the use of the Dixon approach. Images were further used for subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) segmentation.

MRI analysis: SAT and VAT segmentation

For image analyses, single slices that were bounded by the head of the liver to the iliac crest were manually identified from 2 individuals (C Cordes and CB). Selected slice images were automatically analyzed with a segmentation algorithm written in MatLab software (R2014b) according to the method of Cordes et al. (25). This algorithm was used to obtain an initial fat classification of the following 3 compartments: *1*) SAT, which is the fraction between the dermis and external fascia of the abdominal muscle wall; *2*) VAT, which is within the inner contour of the SAT compartment; and *3*) nonadipose tissue (NAT), which is the remaining fraction (mostly water). Image processing required manual correction and was con-

ducted by a single person (CB). Volumes of SAT, VAT, and NAT were quantified by summing the individual slices. Ratios of SAT, VAT, and NAT volumes to total volume were also generated.

Dietary intake

The children's diets were assessed with the use of 3-d estimated food records at 3, 4, and 5 y of age and were completed by their parents or the daycare personnel. Data were entered in a standardized manner by one person (M-TK) with the use of OptiDiet Plus software (version 5.1.2.065; GOE mbH), which is a program that is based on a German nutrient database (Bundeslebensmittelschlüssel). Energy and macronutrient intake were analyzed.

PA questionnaires

We assessed the PA and inactivity of the children with the use of a basic questionnaire from the German Health Interview and Examination Survey for children and adolescents at 3 respective time points (3, 4, and 5 y of age) (26, 27). One of the parents of each child filled out the questionnaire during the follow-up visits or at home. The protocol contained questions on play, exercise, television viewing, and computer consumption and was evaluated by a single person (M-TK).

Statistical analysis

All analyses were based on children who actively participated at the follow-up. For each group, means ± SDs of anthropometric data, SFT, and ultrasound data are presented for children at 2, 3, 4, and 5 y of age; dietary values are presented for children at 3, 4, and 5 y of age; and MRI analyses are presented for children at 5 y of age. For outcomes measured more than once (all except MRI), likelihood-based mixed models for repeated measures (MMRMs) according to Bell et al. (28) were fit with the use of data from each visit (birth and 6 wk, 4 mo, and 1, 2, 3, 4, and 5 y of age). The independent variables included were the visit number as a factor variable and indicator variables for the group assignment at each visit. In adjusted analyses, sex and pregnancy duration were also included as independent variables. Unstructured covariance matrices were used to model the within-subject error. Although the difference in groups at 5 y of age was the focus of this study, estimated mean differences between the groups are presented for each measure at each time point together with 95% CIs. For the MRI data, differences between groups were analyzed with the use of simple and multiple linear regression models that were adjusted for sex and pregnancy duration. The binary outcomes from the PA questionnaire were compared between groups, at each time point separately, with the use of chi-square tests. All statistical analyses were performed with the use of the statistical program R (version R 3.1.3; R Foundation for Statistical Computing); in particular, the mixed models were fit with the use of the gls function within the nlme library and with PASW software (version 21.0; SPSS Inc.). A 2-sided *P* value <0.05 was considered statistically significant, and no correction was made for multiple comparisons.

RESULTS

Participants

Of the 208 women included at the beginning of the study, data for the body composition of 170 children (81.7%) were available at 1 y of age (13). For the follow-up study, we analyzed the data from 118 children at 2 y of age (56.7%), 120 children at 3 y of age (57.7%), 107 children at 4 y of age (51.4%), and 114 children at 5 y of age (54.8%) with similar numbers between study groups (at 5 y: intervention group, $n = 58$; control group, $n = 56$). Data from all children who participated at the follow-up are presented in **Figure 1**. The most common reasons for dropout were a lack of time or relocation.

Child growth and development

Growth patterns, including weight, height, and head, arm, and waist circumferences for children aged between 2 and 5 y are summarized in **Table 1**. These outcome variables did not significantly differ between the intervention and control group in the unadjusted and adjusted MMRM analyses at any time point except for weight and BMI at 4 y of life. These 2 variables showed significantly higher values for the intervention group in the unadjusted analysis but not in the adjusted analysis. BMI percentiles, which were estimated with the use of German reference data (20), showed values in the recommended range for most but not all children. For example, 51 of 58 children aged
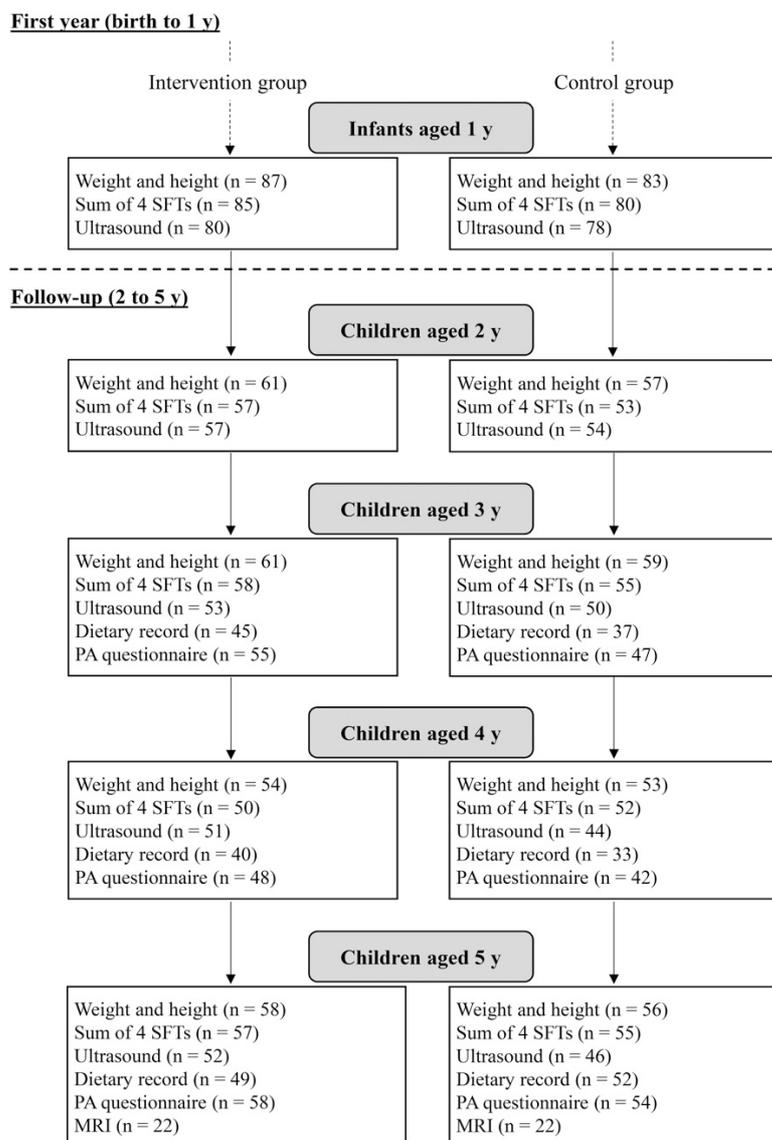
FIGURE 1   Flowchart of follow-up data of participants in the INFAT study. The flowchart of the first year of life has been published elsewhere (13). As indicated in parentheses, data for the sum of 4 SFTs, ultrasounds, dietary records, and PA questionnaires were not available for all children. INFAT, Impact of Nutritional Fatty Acids during Pregnancy and Lactation on Early Human Adipose Tissue Development; PA, physical activity; SFT, skinfold thickness.

TABLE 1
Growth patterns from 2 to 5 y of life

Variable and age	Intervention group	Control group	Unadjusted difference[1]	P	Adjusted difference[2]	P
Weight, kg						
2 y	12.5 ± 1.4 [61][3]	12.3 ± 1.3 [57]	0.4 (−0.1, 0.9)[4]	0.082	0.2 (−0.2, 0.6)	0.372
3 y	14.8 ± 1.9 [61]	14.3 ± 1.5 [59]	0.5 (−0.1, 1.1)	0.122	0.2 (−0.3, 0.8)	0.452
4 y	17.0 ± 2.2 [54]	16.2 ± 1.7 [53]	0.8 (0.1, 1.5)	0.032	0.5 (−0.2, 1.1)	0.146
5 y	19.2 ± 3.0 [58]	18.4 ± 3.2 [56]	0.7 (−0.3, 1.6)	0.174	0.3 (−0.6, 1.1)	0.548
Height, cm						
2 y	87.1 ± 2.9 [61]	87.0 ± 2.7 [57]	0.3 (−0.6, 1.3)	0.492	−0.2 (−1.1, 0.8)	0.758
3 y	96.4 ± 3.8 [61]	96.0 ± 3.5 [59]	0.3 (−0.8, 1.5)	0.580	−0.2 (−1.3, 1.0)	0.804
4 y	104.0 ± 4.3 [54]	103.3 ± 3.6 [53]	0.7 (−0.6, 1.9)	0.312	0.1 (−1.2, 1.4)	0.829
5 y	112.2 ± 4.8 [58]	110.7 ± 4.0 [56]	0.5 (−0.9, 2.0)	0.465	−0.0 (−1.5, 1.4)	0.975
BMI percentile[5]						
2 y	56.7 ± 27.5 [61]	52.2 ± 27.9 [57]	4.6 (−4.5, 13.7)	0.325	2.3 (−6.9, 11.6)	0.621
3 y	53.9 ± 27.6 [61]	45.9 ± 23.1 [59]	5.7 (−2.7, 14.1)	0.186	3.4 (−5.2, 12.0)	0.437
4 y	54.3 ± 24.6 [54]	41.3 ± 27.1 [53]	10.0 (1.2, 18.8)	0.026	7.8 (−1.0, 16.6)	0.081
5 y	49.8 ± 27.4 [58]	45.0 ± 24.3 [56]	3.8 (−4.9, 12.5)	0.394	1.6 (−7.2, 10.3)	0.722
Head circumference, cm						
2 y	48.7 ± 1.3 [61]	48.6 ± 1.3 [57]	0.2 (−0.2, 0.6)	0.305	−0.0 (−0.4, 0.4)	0.983
3 y	49.9 ± 1.4 [61]	49.8 ± 1.3 [59]	0.2 (−0.2, 0.6)	0.326	−0.0 (−0.4, 0.4)	0.985
4 y	50.6 ± 1.3 [54]	50.6 ± 1.2 [53]	0.1 (−0.3, 0.5)	0.486	−0.1 (−0.5, 0.3)	0.715
5 y	51.2 ± 1.3 [58]	51.2 ± 1.3 [56]	0.1 (−0.3, 0.6)	0.549	−0.1 (−0.5, 0.3)	0.679
Arm circumference, cm						
2 y	15.7 ± 1.1 [61]	15.5 ± 1.1 [57]	0.2 (−0.1, 0.6)	0.222	0.2 (−0.2, 0.6)	0.323
3 y	16.1 ± 1.3 [61]	15.9 ± 1.1 [59]	0.2 (−0.2, 0.6)	0.417	0.1 (−0.3, 0.5)	0.545
4 y	16.5 ± 1.3 [53]	16.3 ± 1.1 [52]	0.1 (−0.3, 0.5)	0.557	0.1 (−0.3, 0.5)	0.710
5 y	16.9 ± 1.4 [58]	16.8 ± 1.0 [56]	0.0 (−0.4, 0.5)	0.837	0.0 (−0.4, 0.4)	0.987
Waist circumference, cm						
2 y	47.8 ± 2.9 [50]	48.0 ± 2.7 [51]	0.2 (−0.9, 1.2)	0.767	0.1 (−1.0, 1.1)	0.877
3 y	50.0 ± 3.0 [61]	49.4 ± 2.2 [59]	0.5 (−0.4, 1.4)	0.316	0.4 (−0.6, 1.3)	0.417
4 y	52.0 ± 2.9 [54]	51.3 ± 2.9 [52]	0.4 (−0.6, 1.5)	0.410	0.4 (−0.7, 1.4)	0.502
5 y	53.4 ± 5.2 [58]	52.8 ± 2.9 [56]	0.5 (−1.0, 2.0)	0.501	0.4 (−1.1, 1.9)	0.568

[1]From mixed models for repeated measures with the use of data from each visit since birth.

[2]From mixed models for repeated measures with the use of data from each visit since birth and controlled for sex and pregnancy duration for all variables except BMI percentiles (controlled for pregnancy duration).

[3]Mean ± SD; *n* in brackets (all such values). Values were calculated from the observed data.

[4]Mean; 95% CI in parentheses (all such values).

[5]Calculated according to Kromeyer-Hauschild et al. (20).

5 y (87.9%) from the intervention group and 49 of 56 children aged 5 y (87.5%) from the control group were within the recommended range. There was no significant evidence of a difference in BMI percentiles between groups at 5 y of age (adjusted mean difference at 5 y: 1.6; 95% CI: −7.2, 10.3). At 5 y of age, 5.2% of the children in the intervention group were defined as overweight (including obesity), and 1.7% of the children in the intervention group were defined as obese compared with 3.6% of children who were overweight and 0% of children who were obese in the control group.

Fat mass and fat distribution of the children

SFT

Results for the sums of the 4 SFTs from 2, 3, 4, and 5 y are provided in **Table 2**. At 5 y of age, the sum of 4 SFTs (sum of the 4 individual SFTs) was 23.9 ± 4.7 mm in the intervention group (*n* = 57) and 24.5 ± 5.0 mm in the control group (*n* = 55), with no significant evidence of a difference between groups provided by the unadjusted or adjusted analyses. Similar results were observed when the 4 individual SFTs were analyzed sep-

arately (**Supplemental Table 1**). Likewise, the percentage of body fat and body fat mass (kg) as estimated by the SFT equations (21) did not significantly differ between groups during the follow-up period. The same results applied to lean body mass (kg) and the percentage of lean body mass (Table 2).

Ultrasound

Table 3 presents results from the adipose tissue growth and abdominal fat distribution at 2, 3, 4, and 5 y of age as assessed with the use of ultrasonography. Consistent with the SFT measurements, the unadjusted and adjusted analyses showed comparable abdominal subcutaneous and preperitoneal fat areas with increasing mean values observed over time. At 5 y of age, the adjusted mean difference between the groups was 0.28 mm^2 (95% CI: −4.75, 5.31 mm^2) in the preperitoneal area, −2.29 mm^2 (95% CI: −6.91, 2.34 mm^2) in the subcutaneous area$_{sagittal}$ and −3.88 mm^2 (95% CI: −11.22, 3.46 mm^2) in the subcutaneous area$_{axial}$. There was also no evidence of a difference in the fat distribution between groups on the basis of the preperitoneal: subcutaneous ratio in the sagittal plane (adjusted mean difference at 5 y of age: 0.49; 95% CI: −0.25, 1.24).

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TABLE 2
Adipose tissue development, subcutaneous fat, and lean body mass distribution from 2 to 5 y of life assessed with the use of SFT measurements[1]

Variable and age	Intervention group	Control group	Unadjusted difference[2]	P	Adjusted difference[3]	P
Sum of 4 SFTs,[4] mm						
2 y	23.8 ± 3.3 [57][5]	23.5 ± 3.5 [53]	0.5 (−0.7, 1.7)[6]	0.398	0.6 (−0.6, 1.8)	0.307
3 y	23.4 ± 3.7 [58]	23.3 ± 3.6 [55]	0.4 (−0.9, 1.6)	0.543	0.5 (−0.7, 1.7)	0.455
4 y	23.6 ± 3.5 [50]	23.4 ± 3.8 [52]	0.2 (−1.1, 1.6)	0.762	0.3 (−1.0, 1.6)	0.648
5 y	23.9 ± 4.7 [57]	24.5 ± 5.0 [55]	−0.6 (−2.3, 1.1)	0.493	−0.5 (−2.2, 1.2)	0.549
Fat mass,[7] kg						
2 y	2.4 ± 0.5 [57]	2.3 ± 0.5 [53]	0.1 (−0.1, 0.3)	0.211	0.1 (−0.1, 0.3)	0.246
3 y	2.7 ± 0.7 [58]	2.6 ± 0.5 [55]	0.1 (−0.1, 0.3)	0.286	0.1 (−0.1, 0.3)	0.321
4 y	3.1 ± 0.7 [50]	2.9 ± 0.6 [52]	0.2 (−0.1, 0.4)	0.183	0.1 (−0.1, 0.4)	0.211
5 y	3.5 ± 1.1 [57]	3.4 ± 0.8 [55]	0.1 (−0.3, 0.4)	0.734	0.1 (−0.3, 0.4)	0.770
Lean body mass,[8] kg						
2 y	10.1 ± 1.0 [57]	9.9 ± 0.9 [53]	0.2 (−0.1, 0.5)	0.252	0.1 (−0.2, 0.4)	0.439
3 y	12.0 ± 1.3 [58]	11.7 ± 1.2 [55]	0.1 (−0.3, 0.5)	0.480	0.1 (−0.3, 0.5)	0.702
4 y	13.9 ± 1.8 [50]	13.3 ± 1.4 [52]	0.4 (−0.1, 0.9)	0.095	0.3 (−0.1, 0.8)	0.165
5 y	15.8 ± 2.1 [57]	15.3 ± 1.7 [55]	0.3 (−0.3, 0.9)	0.375	0.2 (−0.4, 0.8)	0.527
Fat mass,[7] %						
2 y	19.2 ± 2.3 [57]	19.0 ± 2.4 [53]	0.4 (−0.5, 1.2)	0.400	0.4 (−0.4, 1.3)	0.274
3 y	18.4 ± 2.6 [58]	18.3 ± 2.6 [55]	0.3 (−0.6, 1.2)	0.501	0.4 (−0.5, 1.2)	0.378
4 y	18.2 ± 2.6 [50]	17.9 ± 3.0 [52]	0.3 (−0.8, 1.3)	0.627	0.3 (−0.6, 1.3)	0.495
5 y	17.9 ± 3.4 [57]	18.1 ± 3.6 [55]	−0.2 (−1.4, 1.0)	0.766	−0.1 (−1.3, 1.0)	0.840
Lean body mass,[9] %						
2 y	80.8 ± 2.3 [57]	81.0 ± 2.4 [53]	−0.4 (−1.2, 0.5)	0.402	−0.5 (−1.3, 0.4)	0.276
3 y	81.6 ± 2.6 [58]	81.7 ± 2.6 [55]	−0.3 (−1.2, 0.6)	0.502	−0.4 (−1.2, 0.5)	0.380
4 y	81.8 ± 2.6 [50]	82.1 ± 3.0 [52]	−0.3 (−1.3, 0.8)	0.627	−0.3 (−1.3, 0.6)	0.496
5 y	82.1 ± 3.4 [57]	81.9 ± 3.6 [55]	0.2 (−1.0, 1.4)	0.766	0.1 (−1.1, 1.3)	0.840

[1]SFT, skinfold thickness.

[2]From mixed models for repeated measures with the use of data from each visit since birth.

[3]From mixed models for repeated measures with the use of data from each visit since birth and controlled for sex and pregnancy duration.

[4]Sum of 4 SFTs was calculated as biceps + triceps + subscapular + suprailiac SFTs.

[5]Mean ± SD; n in brackets (all such values). Values were calculated from the observed data.

[6]Mean; 95% CI in parentheses (all such values).

[7]Calculated according to Weststrate and Deurenberg (21).

[8]Lean body mass (kg) was calculated as body weight (kg) − fat mass (kg).

[9]Percentage of lean body mass was calculated as 100 − the percentage of fat mass.

MRI

In a subgroup of 44 children, an additional abdominal MRI was performed at 5 y of age (**Table 4**). The mean number of analyzed slices was 16.6 ± 1.2 in the intervention group (n = 22) compared with 16.7 ± 1.3 in the control group (n = 22). Mean SAT, VAT, and NAT volumes did not differ between control and intervention groups [SAT-volume adjusted mean difference: −8.84 cm^3 (95% CI: −105.51, 87.83 cm^3); VAT-volume adjusted mean difference: −7.18 cm^3 (95% CI: −28.65, 14.29 cm^3); NAT-volume adjusted mean difference: 160.44 cm^3 (95% CI: −62.48, 383.37 cm^3)]. Similarly, the calculated percentages (SAT, VAT, and NAT ratio) were not significantly different between groups after adjustment for sex and pregnancy duration. Therefore, these findings were consistent with the results of the other methods.

Dietary intake

Mean energy in kcal and MJ and macronutrient intakes in grams and percentages of energy are provided in **Table 5**. There was a gradual increase in the mean energy intake in both groups. However, the mean proportion of caloric intake from carbohydrates, fat, and protein did not change notably over time. In the investigated period, the analysis of the mean daily energy and macronutrient intakes showed no significant evidence of a group difference in daily energy and macronutrient intakes at any time point.

PA

The results of the PA questionnaires provided no evidence regarding differences between groups in active or sedentary behaviors (**Supplemental Table 2**). At 5 y of age, 53 of 58 children (91%) in the intervention group and 50 of 54 children (93%) in the control group regularly participated in a sport activity (≥1 time/wk). Furthermore, all children in both groups played outside ≥3 times/wk. As regards sedentary behavior, 19 of 54 children (35%) in the control group and 16 of 58 children (28%) in the intervention group regularly watched television or played on a computer ≥1 h/d [number of children who played on a computer ≥1 h/d: intervention group: one of 58 (2%); control group: one of 54 (2%)].

DISCUSSION

Our previous analysis did not provide any evidence that a dietary intervention with fish-oil capsules (1020 mg DHA

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TABLE 3

Adipose tissue growth and abdominal fat distribution from 2 to 5 y of life assessed by ultrasonography

Variable and age	Intervention group	Control group	Unadjusted difference[1]	P	Adjusted difference[2]	P
Preperitoneal area$_{sagittal}$,[3] mm^2						
2 y	22.91 ± 7.04 [57][4]	24.48 ± 7.97 [54]	−1.71 (−4.30, 0.89)[5]	0.196	−1.61 (−4.22, 0.99)	0.223
3 y	32.87 ± 11.05 [52]	32.34 ± 11.43 [50]	1.07 (−2.89, 5.03)	0.594	1.16 (−2.80, 5.12)	0.564
4 y	41.10 ± 13.26 [51]	39.99 ± 14.83 [43]	1.02 (−3.94, 5.98)	0.683	1.13 (−3.84, 6.09)	0.654
5 y	48.89 ± 12.32 [51]	47.77 ± 16.18 [45]	0.18 (4.85, 5.20)	0.944	0.28 (−4.75, 5.31)	0.913
Subcutaneous area$_{sagittal}$,[3] mm^2						
2 y	16.80 ± 9.59 [57]	20.74 ± 11.93 [54]	−1.93 (−5.70, 1.84)	0.313	−2.41 (−6.18, 1.37)	0.210
3 y	18.86 ± 12.73 [53]	20.43 ± 11.25 [50]	−0.82 (−4.83, 3.18)	0.685	−1.32 (−5.19, 2.54)	0.499
4 y	19.21 ± 11.48 [51]	20.84 ± 13.15 [42]	−1.06 (−5.57, 3.44)	0.641	−1.57 (−5.83, 2.68)	0.466
5 y	20.23 ± 13.76 [52]	21.24 ± 11.92 [45]	−1.78 (−6.58, 3.01)	0.463	−2.29 (−6.91, 2.34)	0.330
Subcutaneous area$_{axial}$,[6] mm^2						
2 y	22.00 ± 9.55 [57]	26.96 ± 14.36 [54]	−3.31 (−7.61, 1.00)	0.131	−3.88 (−8.14, 0.38)	0.074
3 y	26.36 ± 19.35 [52]	28.10 ± 16.28 [50]	−0.29 (−6.25, 5.68)	0.925	−0.75 (−6.59, 5.09)	0.800
4 y	26.44 ± 17.18 [51]	29.77 ± 21.69 [44]	−3.40 (−10.68, 3.88)	0.357	−3.81 (−10.91, 3.29)	0.290
5 y	28.53 ± 20.68 [52]	30.08 ± 19.39 [46]	−3.46 (−10.95, 4.04)	0.363	−3.88 (−11.22, 3.46)	0.298
Preperitoneal:subcutaneous ratio[7]						
2 y	1.79 ± 1.31 [57]	1.49 ± 0.82 [54]	0.20 (−0.17, 0.58)	0.279	0.21 (−0.16, 0.58)	0.270
3 y	2.48 ± 1.77 [52]	1.95 ± 1.03 [50]	0.37 (−0.10, 0.85)	0.124	0.38 (−0.10, 0.86)	0.121
4 y	2.82 ± 1.86 [51]	2.69 ± 1.79 [42]	−0.02 (−0.65, 0.62)	0.958	−0.01 (−0.65, 0.62)	0.969
5 y	3.42 ± 2.42 [51]	2.94 ± 1.73 [45]	0.48 (−0.26, 1.23)	0.201	0.49 (−0.25, 1.24)	0.194

[1]From mixed models for repeated measures with the use of data from each visit from 6 wk onward.

[2]From mixed models for repeated measures with the use of data from each visit from 6 wk onward and controlled for sex and pregnancy duration.

[3]Sagittal subcutaneous and preperitoneal fat were measured as areas of 1-cm length in the middle of the xiphoid process according to an adapted method of Holzhauer et al. (22); adapted method described in detail elsewhere (23).

[4]Mean ± SD; n in brackets (all such values). Values were calculated from the observed data.

[5]Mean; 95% CI in parentheses (all such values).

[6]Axial subcutaneous fat was measured between the middle of the xiphoid process and the navel directly above the linea alba.

[7]Ratio of preperitoneal to subcutaneous fat was calculated as preperitoneal area$_{sagittal}$ ÷ subcutaneous area$_{sagittal}$.

plus 180 mg EPA/d) combined with an AA-balanced diet in pregnant and lactating women had an effect on adipose tissue growth in their offspring for ≤1 y of life (as indicated by SFT measurements and ultrasonography) (13). With the use of the same methods, we followed this cohort carefully up to the fifth year of life. Data from the current study revealed no evidence of any long-term effects of the intervention at 2, 3, 4, and 5 y of age, which was consistent with our previous findings (the observed significant difference in weight and BMI between the 2 groups in the unadjusted MMRM model at 4 y of age should be treated with caution because it may be an artifact of multiple testing). In addition, abdominal MRI measurements at 5 y of age in a subgroup of children showed no significant difference in abdominal fat distribution (SAT and VAT) between intervention and control groups. Furthermore, we showed no differences in dietary energy and macronutrient intakes or PA over the time period between the 2 groups.

TABLE 4

Abdominal subcutaneous and visceral adipose tissue volumes and ratios at 5 y of life assessed by MRI[1]

	Intervention group (n = 22)	Control group (n = 22)	Unadjusted difference[2]	P	Adjusted difference[3]	P
SAT volume, cm^3	563.41 ± 154.00[4]	563.61 ± 160.31	−0.21 (95.85, 95.44)[5]	0.997	−8.84 (−105.51, 87.83)	0.854
VAT volume, cm^3	100.20 ± 35.28	108.17 ± 32.39	−7.97 (−28.57, 12.64)	0.440	−7.18 (−28.65, 14.29)	0.503
NAT volume, cm^3	3136.01 ± 371.91	3056.59 ± 385.81	114.25 (−151.14, 309.99)	0.491	160.44 (−62.48, 383.37)	0.154
SAT ratio,[6] %	14.68 ± 2.52	15.04 ± 3.18	−0.36 (−2.10, 1.39)	0.681	−0.85 (−2.47, 0.77)	0.298
VAT ratio,[7] %	2.59 ± 0.65	2.91 ± 0.84	−0.32 (−0.77, 0.14)	0.170	−0.35 (−0.82, 0.13)	0.152
NAT ratio,[8] %	82.73 ± 2.87	82.05 ± 3.74	0.68 (−1.35, 2.70)	0.505	1.19 (−0.72, 3.11)	0.216

[1]NAT, nonadipose tissue; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

[2]Calculated with the use of Student's t test.

[3]Calculated with the use of a multiple regression analysis (F test; ANCOVA) and controlled for sex and pregnancy duration.

[4]Mean ± SD (all such values). Values were calculated from the observed data.

[5]Mean; 95% CI in parentheses (all such values).

[6]Ratio was calculated as [SAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100.

[7]Ratio was calculated as [VAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100.

[8]Ratio was calculated as [NAT volume ÷ (SAT volume + VAT volume + NAT volume)] × 100.

TABLE 5
Energy and macronutrient intakes from 3 to 5 y of life[1]

Variable and age	Intervention group	Control group	Unadjusted difference[2]	P
Energy, kcal/d				
3 y	1361.56 ± 284.45 [45][3]	1300.51 ± 276.26 [37]	73.74 (−49.05, 196.52)[4]	0.236
4 y	1546.75 ± 227.79 [40]	1492.45 ± 344.74 [33]	47.89 (−78.91, 174.68)	0.455
5 y	1557.24 ± 303.02 [49]	1610.13 ± 328.14 [52]	−53.50 (−176.91, 69.91)	0.392
Energy, MJ/d				
3 y	5.70 ± 1.19 [45]	5.44 ± 1.16 [37]	0.31 (−0.21, 0.82)	0.236
4 y	6.47 ± 0.95 [40]	6.24 ± 1.44 [33]	0.20 (−0.33, 0.73)	0.456
5 y	6.52 ± 1.27 [49]	6.73 ± 1.37 [52]	−0.22 (−0.74, 0.29)	0.391
Protein, g/d				
3 y	47.96 ± 12.61 [45]	45.21 ± 10.75 [37]	3.51 (−1.63, 8.65)	0.178
4 y	54.80 ± 11.88 [40]	49.88 ± 13.54 [33]	4.38 (−1.11, 9.87)	0.117
5 y	53.45 ± 13.79 [49]	54.41 ± 14.19 [52]	−1.15 (−6.58, 4.29)	0.676
Fat, g/d				
3 y	51.55 ± 16.68 [45]	49.26 ± 11.72 [37]	2.92 (−3.53, 9.37)	0.371
4 y	56.93 ± 12.01 [40]	56.21 ± 15.77 [33]	−0.51 (−6.79, 5.78)	0.873
5 y	55.21 ± 12.78 [49]	59.88 ± 15.30 [52]	−4.54 (−10.10, 1.01)	0.108
Carbohydrate, g/d				
3 y	172.53 ± 36.92 [45]	164.95 ± 44.12 [37]	8.07 (−9.24, 25.38)	0.357
4 y	199.13 ± 31.85 [40]	191.85 ± 49.30 [33]	8.96 (−9.36, 27.29)	0.333
5 y	206.80 ± 43.82 [49]	208.15 ± 49.44 [52]	−1.14 (−19.46, 17.18)	0.902
Protein, % of energy				
3 y	14.41 ± 1.98 [45]	14.33 ± 2.47 [37]	0.22 (−0.75, 1.18)	0.654
4 y	14.55 ± 2.56 [40]	13.64 ± 1.74 [33]	0.86 (−0.13, 1.85)	0.086
5 y	14.02 ± 2.10 [49]	13.82 ± 2.07 [52]	0.18 (−0.63, 0.99)	0.661
Fat, % of energy				
3 y	34.86 ± 5.86 [45]	35.44 ± 5.54 [37]	−0.42 (−2.93, 2.08)	0.738
4 y	34.10 ± 4.10 [40]	35.04 ± 5.95 [33]	−1.28 (−3.61, 1.04)	0.275
5 y	32.99 ± 4.01 [49]	34.70 ± 5.47 [52]	−1.74 (−3.64, 0.16)	0.073
Carbohydrate, % of energy				
3 y	52.31 ± 6.33 [45]	51.74 ± 6.34 [37]	0.29 (−2.47, 3.06)	0.833
4 y	52.87 ± 4.60 [40]	52.76 ± 6.20 [33]	0.45 (−2.02, 2.93)	0.716
5 y	54.48 ± 4.71 [49]	52.96 ± 6.25 [52]	1.60 (−0.58, 3.78)	0.148

[1]Dietary records were collected at the ages of 35–39 mo (3 y), 47–52 mo (4 y), and 59–63 mo (5 y).

[2]From mixed models for repeated measures with the use of data from 3, 4, and 5 y of age.

[3]Mean ± SD (all such values). Values were calculated from the observed data.

[4]Mean; 95% CI in parentheses (all such values).

The strengths and uniqueness of the current follow-up study are that it provides one of the largest sets of combined methods for the assessment of body composition and fat distribution. Other RCTs have primarily assessed growth measures, such as weight, height (29), BMI and/or BMI *z* scores (30–33), waist and head circumferences (29, 33), and SFT measurements (33) in preschool-age children. In the current study, we combined several methods (anthropometric measures, SFT measurements, and ultrasound) in a longitudinal approach with annual assessments. With consideration of the importance of MRI measurements as a gold standard, providing one of the most precise estimates of adipose tissue deposition in children (34), this method was used to complement our 5-y results. With the use of several tools of body-composition assessments in a combinational approach (all of which indicated the same conclusion), the current study contributes strong evidence that suggests that reducing the n–6:n–3 LCPUFA ratio in the maternal diet during pregnancy and lactation does not affect adipose tissue growth in preschool-age children.

Another strength of our study was the use of 3-d estimated food records, which are a valid instrument to assess diet in toddlers and children (35). The exploration of preschool child nutrition over time revealed that energy and macronutrient intakes were not significantly different between study groups. Likewise, the concentration of PA was regularly assessed with the use of validated instruments and was also shown to be not significantly different between groups, thereby excluding relevant confounding by lifestyle influences.

Our findings are consistent with the current scientific literature. A number of reviews on this topic have emerged (15–17, 36–38). The latest review, which primarily focused on early fatty acid exposure and obesity risk in later life, concluded that current data from observational studies and RCTs have been inconsistent. However, the data available provide little evidence to support the proposed fatty acid hypothesis (16). Our results are similar to those in the meta-analysis conducted by Stratakis et al. (15) on the effect of an n–3 LCPUFA supplementation during pregnancy or lactation on adiposity status in childhood. Stratakis et al. included 6 RCTs with a total of 2847 participants in the meta-analysis. For the preschool-age category (≤5 y), they examined 4 RCTs and looked at BMI as the primary outcome. They showed no effect of a maternal n–3 LCPUFA

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supplementation during pregnancy and/or lactation on childhood BMI and without an association of the supplemented n–3 LCPUFA dosage or age (15). Preschool-age ($\leq$5-y) and school-age (6–12-y) data of 4 primary and follow-up studies each were included in the meta-analysis. However, to our knowledge, there has only been one study to date with a follow-up period that extended into adolescence ($>$13 y of age) that reported data on body composition at the age of 19 y with no difference in BMI and waist circumference (39).

Some limitations may have weakened our findings such as the small sample size that included only 104 pregnant women/study group. Because the study was initially planned for 1 y, there was a relatively high attrition rate after this particular time with a slight decrease that continued over the follow-up period. These factors resulted in the loss of statistical power. However, considerable efforts were made to obtain the compliance of the families, in particular for the 5-y follow-up. Because of missing outcome data, an MMRM approach was used for the analysis with the use of data from each visit (birth and 6 wk, 4 mo, and 1, 2, 3, 4, and 5 y of age). This approach has been recommended for the analysis of longitudinal data from an RCT, and it is a valid method under the assumption that data are missing at random. We considered this assumption that data were missing at random, which was conditional on the observed values for each outcome, to be reasonable for our study (28). With consideration of the open-label design of this study, both participants and investigators who performed the measurements and analysis were not blinded to the treatment, which may have introduced a potential bias. In addition, our sample was rather lean at the time of study entry (mean prepregnancy BMI: 22) and relatively well educated. These factors may have reflected a more health-conscious behavior in our study group than is present in the general population. This behavior might have resulted in lower prevalence rates in children who were overweight or obese at 5 y of age than the current German prevalence rates from school-enrollment examinations (prevalence of overweight including obesity: 8.4–11.9%; prevalence of obesity: 3.3–5.4%) (40). Furthermore, we determined energy intake and expenditure with the use of 3-d estimated food records and PA questionnaires, which may not have accurately reflected the true values in terms of underreporting and overreporting, respectively. The given information, which was provided by parents or daycare personnel, could be, for example, biased because of social desirability (41).

In conclusion, the current study does not provide evidence that a dietary reduction of the n–6:n–3 LCPUFA ratio would be a useful early preventive strategy against obesity at preschool age. One strong point of the current study is the use of several tools of body-composition assessment in a combinational approach and longitudinal manner with consistent results. However, the impact of LCPUFAs during pregnancy and lactation on offspring adipose tissue development is still a subject of interest. Data for adolescents and adults are limited.

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Supporting material for Chapter II

Supplemental Table 1. Individual SFT measurements (biceps, triceps, subscapular, suprailiac) from 2 to 5 y of life of the INFAT study

	Intervention group	Control group	Unadjusted difference[1]	*P* value	Adjusted difference[2]	*P* value
Biceps (mm)						
2 y	4.8 ± 0.9 [59][3]	4.8 ± 0.8 [55]	0.1 (−0.3, 0.4)[4]	0.729	0.1 (−0.2, 0.4)	0.577
3 y	4.9 ± 1.0 [58]	4.9 ± 0.8 [57]	0.1 (−0.3, 0.4)	0.766	0.1 (−0.3, 0.4)	0.623
4 y	4.8 ± 0.9 [51]	5.0 ± 1.0 [52]	−0.1 (−0.4, 0.3)	0.648	−0.0 (−0.4, 0.3)	0.796
5 y	4.8 ± 1.2 [57]	5.0 ± 1.2 [56]	−0.2 (−0.7, 0.2)	0.289	−0.2 (−0.6, 0.2)	0.363
Triceps (mm)						
2 y	8.9 ± 1.8 [58]	8.5 ± 1.6 [54]	0.4 (−0.3, 1.0)	0.249	0.4 (−0.2, 1.0)	0.198
3 y	8.9 ± 1.6 [58]	8.8 ± 1.7 [56]	0.3 (−0.3, 0.9)	0.389	0.3 (−0.3, 0.9)	0.319
4 y	9.4 ±1.4 [51]	9.0 ± 1.8 [52]	0.5 (−0.1, 1.1)	0.120	0.5 (−0.1, 1.1)	0.090
5 y	9.4 ±1.9 [58]	9.4 ± 1.9 [56]	0.0 (−0.7, 0.7)	0.980	0.0 (−0.6, 0.7)	0.886
Subscapular (mm)						
2 y	6.1 ± 1.1 [61]	6.0 ± 1.1 [57]	0.1 (−0.2, 0.5)	0.449	0.2 (−0.2, 0.5)	0.420
3 y	5.6 ± 0.9 [58]	5.5 ± 1.0 [57]	0.2 (−0.2, 0.5)	0.343	0.2 (−0.2, 0.5)	0.340
4 y	5.3 ± 0.9 [50]	5.2 ± 1.0 [52]	0.1 (−0.2, 0.5)	0.497	0.1 (−0.2, 0.5)	0.465
5 y	5.3 ± 1.0 [57]	5.4 ± 1.8 [56]	0.0 (−0.4, 0.4)	0.981	0.0 (−0.4, 0.4)	0.953
Suprailiac (mm)						
2 y	4.1 ± 0.8 [58]	4.2 ± 0.9 [55]	−0.1 (−0.4, 0.2)	0.481	−0.1 (−0.4, 0.2)	0.510
3 y	4.0 ± 0.9 [58]	4.1 ± 1.0 [55]	−0.1 (−0.4, 0.2)	0.607	−0.1 (−0.4, 0.2)	0.576
4 y	4.0 ± 1.0 [50]	4.3 ± 1.1 [52]	−0.3 (−0.7, 0.1)	0.105	−0.3 (−0.7, 0.1)	0.104
5 y	4.4 ± 1.5 [57]	4.7 ± 1.5 [55]	−0.3 (−0.9, 0.2)	0.191	−0.3 (−0.8, 0.2)	0.184

[1] From mixed models for repeated measures, using data from each visit since birth; SFT, skinfold thickness; INFAT, impact of nutritional fatty acids during pregnancy and lactation on early human adipose tissue development.
[2] From mixed models for repeated measures, using data from each visit since birth and controlled for sex and pregnancy duration.
[3] Mean ± SD calculated from the observed data; n in brackets (all such values).
[4] Mean; 95% CI in parentheses (all such values).

Supplemental Table 2. Physical activity and electronic media use from 3 to 5 y of life assessed by a questionnaire of the INFAT study[1]

	Type of behavior	Time point	Intervention group	Control group	*P* value
Regularly play outside (at least 3 times a week)	active	3 y	54/55 (98%)[2]	47/47 (100%)	>0.999[3]
		4 y	46/48 (96%)	42/42 (100%)	0.535
		5 y	58/58 (100%)	54/54 (100%)	/[4]
Regularly play sport (at least once a week either as part of a club or not)	active	3 y	42/55 (76%)	28/47 (60%)	0.108
		4 y	42/48 (88%)	36/42 (86%)	>0.999
		5 y	53/58 (91%)	50/54 (93%)	>0.999
Regularly watch television (at least 1 hour a day on weekdays or weekend)	sedentary	3 y	4/55 (7%)	9/47 (19%)	0.135
		4 y	10/48 (21%)	9/42 (21%)	>0.999
		5 y	16/58 (28%)	19/54 (35%)	0.507
Regularly play on a computer (at least 1 hour a day on weekdays or weekend)	sedentary	3 y	1/55 (2%)	1/47 (2%)	>0.999
		4 y	0/48 (0%)	0/42 (0%)	/
		5 y	1/58 (2%)	1/54 (2%)	>0.999

[1] Questionnaires were collected at 35–39 mo (3 y), 47–52 mo (4 y), and 59–63 mo (5 y); INFAT, impact of nutritional fatty acids during pregnancy and lactation on early human adipose tissue development.
[2] /n (percentage).
[3] From chi-square tests comparing proportions between the two groups.
[4] Chi-square test could not be performed due to either all or no children undertaking this activity.

Chapter III – Long-chain PUFAs and offspring neurodevelopment

Impact of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring neurodevelopment: 5-year follow-up of a randomized controlled trial

C Brei[1], L Stecher[1], S Brunner[1], R Ensenauer[2,3], F Heinen[4], PD Wagner[5], J Hermsdörfer[5] and H Hauner[1,6]

[1]Else Kröner-Fresenius Center for Nutritional Medicine, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [2]Department of General Pediatrics, Neonatology and Pediatric Cardiology, Experimental Pediatrics and Metabolism, University Children's Hospital, Heinrich Heine University Düsseldorf, Düsseldorf, Germany; [3]Research Center, Dr von Hauner Children's Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; [4]Department of Pediatric Neurology and Developmental Medicine, Dr von Hauner Children's Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; [5]Department of Sport and Health Sciences, Institute of Human Movement Science, Technische Universität München, Munich, Germany and [6]ZIEL – Institute for Food and Health, Nutritional Medicine Unit, Technische Universität München, Freising, Germany.

Correspondence: Professor H Hauner, Else Kröner-Fresenius Center for Nutritional Medicine, Klinikum rechts der Isar, Technische Universität München, Georg-Brauchle-Ring 60/62, Munich D-80992, Germany. E-mail: hans.hauner@tum.de

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

The analysis of fatty acids was performed by the Laboratory of Lipid Research, Danone Research—Center for Specialised Nutrition using coded samples. There was no intervention from any sponsor with any of the research aspects of the study, including study design, intervention, data collection, data analysis and interpretation, as well as writing of the manuscript.

AUTHOR CONTRIBUTIONS

HH designed the research; RE and FH were involved in the methodological design and gave scientific advice; CB and SB were responsible for the data collection and trial management; PDW and JH developed the algorithm for the mirror movement analysis, provided scientific advice regarding the analysis and edited previous versions of the manuscript; CB analyzed the data; CB and LS were responsible for statistical analysis; CB wrote the manuscript; and all authors contributed to the critical revision of the manuscript.

ABSTRACT

BACKGROUND/OBJECTIVES: Evidence regarding the effect of n-3 long-chain polyunsaturated fatty acid (LCPUFA) supplementation during pregnancy on offspring's neurodevelopment is not conclusive.

SUBJECTS/METHODS: In this analysis, the effect of a reduced n-6:n-3 LCPUFA ratio in the diet of pregnant/lactating women (1.2 g n-3 LCPUFA together with an arachidonic acid (AA)-balanced diet between 15th wk of gestation-4 months postpartum vs control diet) on child neurodevelopment at 4 and 5 years of age was assessed. A child development inventory (CDI) questionnaire and a hand movement test measuring mirror movements (MMs) were applied and the association with cord blood LCPUFA concentrations examined.

RESULTS: CDI questionnaire data, which categorizes children as 'normal', 'borderline' or 'delayed' in different areas of development, showed no significant evidence between study groups at 4 (n = 119) and 5 years (n = 130) except for the area 'letters' at 5 years of age ($P = 0.043$). Similarly, the results did not strongly support the hypothesis that the intervention has a beneficial effect on MMs (for example, at 5 years: dominant hand, fast: adjusted mean difference, − 0.08 (− 0.43, 0.26); $P = 0.631$). Children exposed to higher cord blood concentrations of docosahexaenoic acid, eicosapentaenoic acid and AA, as well as a lower ratio of n-6:n-3 fatty acids appeared to show beneficial effects on MMs, but these results were largely not statistically significant.

CONCLUSIONS: Our results do not show clear benefits or harms of a change in the n-6:n-3 LCPUFA ratio during pregnancy on offspring's neurodevelopment at preschool age. Findings on cord blood LCPUFAs point to a potential influence on offspring development.

INTRODUCTION

Pregnancy is considered a window of opportunity for offspring's future health.[1] Increasingly, attention has focused on long-chain polyunsaturated fatty acids (LCPUFAs) in fetal neurological development since they are crucial components of the brain membrane lipids, predominantly accumulated in the neuron-rich cortical gray matter, but also to a lesser extent in white matter.[2] A specific role is thereby played by docosahexaenoic acid (DHA, 22:6n-3), the most abundant omega-3 fatty acid, involved, for example, in signal transduction, neurotransmission and gene expression.[3] The most important period during brain development is the third trimester of pregnancy, when it is estimated that $67 - 75$ mg DHA per day is accumulated in utero.[4] Continuing up to 18 months after birth, rapid brain growth and DHA accumulation takes place, referred to as a brain growth spurt.[2] Although genetic factors are discussed to be involved in the transfer of DHA from the mother to the fetus, a significant determinant is considered to be maternal DHA status.[5] Thus, an adequate supply during pregnancy is recommended.[6]

A large number of studies have investigated the effects of a higher n-3 LCPUFA intake during the prenatal phase on offspring's mental and motor skill development. Results from observational cohort studies suggest that higher maternal dietary intake of DHA from fish and seafood,[7–9] higher concentrations of maternal DHA in late gestation[10] and at delivery,[11] and higher DHA plasma concentrations in infants' cord blood[12–14] are associated with positive developmental outcomes in the offspring. Although results from these cohort studies seem promising, the randomized controlled trials, examining the effect of prenatal supplementation on offspring development from early infancy up to adolescents,[15] have resulted in inconsistent findings so far. Several systematic reviews[16–19] have reviewed the body of literature, but found no clear evidence of an effect of n-3 LCPUFA supplementation during pregnancy on child's neurodevelopment. The authors note that most of the included studies had methodological limitations such as small sample sizes, high attrition rate or incomplete information about the methodology and outcome data. Therefore, there exists a further need to investigate the role of DHA on child's neurological development and its potential benefit.

In this paper, we present secondary analysis results to assess whether a reduced n-6:n-3 fatty acid ratio during pregnancy and lactation supports offsprings' neurodevelopment at 4 and 5 years by using data from the German Impact of Nutritional Fatty acids during pregnancy and lactation on early human Adipose Tissue development (INFAT) study. Neurodevelopment was assessed by using (I) a global questionnaire, covering several domains of neurodevelopment

and (II) a specific hand movement test, measuring unintended/mirror hand movements. Furthermore, cord blood LCPUFAs were associated with these outcomes.

MATERIALS AND METHODS

Study design, subjects and dietary intervention

The INFAT study, an open-label, monocenter, randomized controlled dietary intervention, with 104 pregnant women in each group, was originally designed to investigate the effect of a reduced n-6:n-3 LCPUFA ratio during pregnancy and lactation on infant adipose tissue development up to the first year of life. Follow-up of the infants continued until the fifth year of life enabling long-term effects of the intervention to be explored. Details of the study design, participant characteristics, maternal and fetal fatty acid profiles, and clinical results on fat mass development of infants/ children from birth up to 5 years have been published elsewhere.[20–24] In brief, between 2006 and 2009, healthy pregnant women before their 15th wk of gestation in the Munich area in Germany were randomized to an intervention or control group. In the intervention group a reduced n-6:n-3 fatty acid ratio was achieved by daily fish oil supplements (1020 mg DHA + 180 mg EPA + 9 mg Vitamin E) from the 15th wk until 4 months postpartum as well as dietary counseling aimed at lowering arachidonic acid (AA) intake. Women in the control group received general information about a healthy diet during pregnancy according to the German guidelines. In addition to the primary outcome (skinfold thickness measurements at four body sites), children's neurological development at 4 and 5 years of age was assessed by using two methods: a parents' questionnaire based on the child development inventory (CDI)[25] and a hand movement test, which was administered at the study center. Cord blood fatty acid analysis was performed in the laboratory of Lipid Research, Danone Research— Center for Specialised Nutrition, Friedrichsdorf, Germany.[22] The procedures of this study were approved by the ethical committee of the Technische Universität München (1479/06/2009/10/26). Written informed consent for the follow-up study was obtained from both parents.

Measures

Child development inventory. Children's development was assessed with the use of a German questionnaire (Elternfragebogen zur kindlichen Entwicklung, EFkE), completed by one of the parents, usually by the mothers, at home or at the study center. The questionnaire is based on a translation of the American CDI[25] and has been normed within a sample of 758 German children

for the ages 1–6 years.[26] In the following, the questionnaire is referred to as CDI. It comprises 270 yes/no questions covering different areas of child development: social (40 items), self-help (40 items), gross motor (30 items), fine motor (30 items), expressive language (50 items), language comprehension (50 items), letters (15 items) and numbers (15 items). From this, an overall score of general development was calculated from the 70 most age-discriminating items in these areas. The scores for each area separately and for general development were then compared with the German age and gender reference scores, and categorized as either normal development, borderline development or developmentally delayed. Borderline development was defined as − 1.5 s.d., developmentally delayed as − 2.0 s.d. below age cutoffs.[27]

Assessment of mirror movements. Due to the hypothesis that lower mirror movements (MMs) indicate a more progressed neuronal development, children performed grip force measurements described previously.[28–30] Trained research assistants guided the measurement in the study center.

In brief, children were in a seated position during the examination and had a transducer in each hand (diameter: 20 mm, length: 20 mm, weight: 20 g), between the thumb and the index finger. In a pretest, the maximum grip force (highest force magnitude achieved in one out of three trials) for both hands was determined.

The main tests took place with 40% of the maximum force (indicated as an approximate target on the monitor). Children were instructed to squeeze the transducer with the right hand (active hand) for 15 s, first at a low frequency (1/s), and afterwards at maximum frequency (as fast as possible), without squeezing the transducer in their other hand (mirror hand). A visual feedback on the monitor was only provided for the active hand. Each measurement was performed twice, followed by measurements with a switch of active and mirror hand. Acquired data were saved and data analysis commenced offline, using a customized MATLAB program (R2014b; MathWorks, Natick, MA, USA). Time series of grip forces were smoothed by applying a 10th order low-pass digital Butterworth filter with a cut-off frequency of 10 Hz to the raw data. The algorithm detected maxima and minima of grip forces for the sinusoidal-like grip force profiles of the voluntary active hand. Mirror force changes are obvious as involuntary changes of the grip force of the mirror hand that have a similar sinusoidal time course as the profile of the active hand though typically a smaller amplitude. Accordingly, the MATLAB algorithm searched maxima and minima in the grip force profile of the mirror hand in the vicinity of the pre-determined maxima and minima of the active hand. If the grip force of the non-active hand is largely constant, very-small amplitudes between consecutive maxima and minima will be detected due to signal noise. If there are overt MMs a clearly larger amplitude will be found.

MM ratios (%) were calculated and adjusted by the ratio of the individual maximum grip force of both hands by using the formula:

$$\text{Mirror movement ratio (\%)} = \frac{\text{mirror amplitude}}{\text{active amplitude}} \times \frac{\text{max. grip force (active hand)}}{\text{max. grip force (mirror hand)}} \times 100$$

A MM ratio close to 0% would mean that the force was approximately constant in the non-active hand and there was no mirror activity, while a MM ratio of 100% would mean that force changes are applied in the mirror hand that have the same amplitude as in the active hand. Further, to quantify the similarities of the sinusoidal-like grip forces profiles of the active and the mirror hand, Pearson correlation coefficients between both time series were calculated. A Pearson correlation of 0 would indicate that there is no linear association between the force profiles, the grip force profile in the mirror and the active hand— that is, no mirror activity, while a positive correlation would suggest a similar time course of the grip force changes in both hands—a clear indicator of mirror activity.

Data analysis required manual intervention (CB): the first 3 s of each measurement have been excluded and short parts were cut out, if necessary (for example, transducers dropped down). A measurement duration of at least 4 s was required, otherwise the measurement was rejected. When children did not follow the technical instruction voluntarily or involuntarily, indicated by frequencies ≤ 0.3 (1/s), low-frequency trials with higher or equal frequency as maximum frequency trials, or MM ratios $\geq 50\%$, measurements have been excluded for analysis. Data are provided in relation to handedness (information was requested from the accompanying parent).

Statistical analysis

Continuous baseline variables are presented as median (P25, P75) and compared between intervention and control groups using Mann–Whitney U-test due to some deviations from normality. For qualitative variables, the χ2-test was used. Fisher's exact test was used to assess the association between group (intervention or control) with the CDI questionnaire outcome (normal, borderline or delayed) for each type of development. The association between fatty acid levels and the general development score was assessed using logistic regression models. For this, the categories of borderline and delayed development were pooled. The outcome variables from the hand movement test are defined based upon whether the dominant or non-dominant hand (based on handedness) was the active hand and whether it was a slow or fast test. Therefore, for each age group, for both the MM ratio and Pearson correlation, four outcome variables were analyzed. Due to skewness, the MM ratio variables were log transformed. Mixed

models were fit to the outcome variables to assess differences according to the group. These models included a random effect term for participant due to repeated measurements. Further, the models were adjusted for handedness and frequency. The estimated coefficients for the group effect were presented with 95% confidence intervals. Analogous mixed models were fitted to assess the association between fatty acid levels and MM ratio. Statistical analyses were performed with R software package (version R 3.1.3; R Foundation for Statistical Computing, Vienna, Austria) and with SPSS Statistics software (version 21.0; IBM, Armonk, NY, USA). A two-sided P-value < 0.05 was considered statistically significant and no adjustment has been made for multiple comparisons.

RESULTS

Participants

From 208 women randomized to the study, data were available at birth for 188 children (intervention, n = 92; control, n = 96)[21] and 132 cord blood samples (intervention, n = 67; control, n = 65).[22] For this analysis, data from the CDI questionnaire were available from 119 children (intervention, n = 63; control, n = 56) at 4 years and 130 children (intervention, n = 70; control, n = 60) at 5 years, respectively. Regarding the MM test, data were assessed from 88 children at 4 years (intervention, n = 47; control, n = 41) and 92 children (intervention, n = 48; control, n = 44) at 5 years, respectively. A flowchart showing available data at each time point is given in the supplement (Supplementary Appendix 1). Baseline and perinatal characteristics in children who completed the 5-year CDI questionnaire are given in Table 1. Variables did not differ between treatment groups except for pregnancy duration (P = 0.024) and cord blood red blood cells fatty acids, namely eicosapentaenoic acid (EPA), DHA and both n-6:n-3 ratios (all P < 0.01). Testing for baseline differences between CDI completers and non-completers did not reveal evidence of a difference (all P > 0.05) (data not shown).

Table 1. Baseline and perinatal characteristics in children who completed the 5-year CDI questionnaire

Characteristics	Intervention group	Control group	P-value
Maternal age	33.0 (29.0, 36.0) [70][a]	32.0 (28.3, 35,0) [60]	0.557
Primiparae (n (%))	39 (52.7)	35 (47.3)	0.764
Pregnancy duration (wk)	40.1 (39.4, 40.8) [70]	39.6 (38.6, 40.6) [60]	0.024
Education (≥ 12 years at school) (n (%))	49 (75.0)	45 (70.0)	0.525
Infant, sex (n (%))			
Male	37 (52.9)	32 (53.3)	0.957
Female	33 (47.1)	28 (46.7)	
Mode of infant feeding, 4 mo postpartum (n (%))			
Exclusively breastfed	46 (54.8)	38 (45.2)	
Partially breastfed	9 (45.0)	11 (55.0)	0.666
Formula fed	15 (57.7)	11 (42.3)	
Cord blood RBCs fatty acid profile[b]			
20:4n-6, AA	8.00 (3.40, 12.18) [57]	6.48 (3.52, 11.49) [43]	0.694
20:5n-3, EPA	0.18 (0.07, 0.38) [56]	0.05 (0.02, 0.12) [40]	0.000
22:6n-3, DHA	3.63 (1.24, 6.48) [57]	1.86 (0.80, 4.25) [43]	0.003
n-6:n-3[c]	2.01 (1.76, 2.57) [56]	3.49 (2.74, 4.39) [40]	0.000
n-6:n-3[d]	3.01 (2.49, 3.78) [57]	4.58 (3.95, 5.39) [43]	0.000

Abbreviations: AA, arachidonic acid; CDI, child development inventory; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; RBCs, red blood cells. For quantitative variables, the Mann–Whitney U-test was used ($P < 0.05$); for qualitative variables, the $\chi2$-test was used ($P < 0.05$). [a]Median (P25, P75) [n] (all such values). [b]Values for fatty acids are expressed as percentage of the fatty acid of the total fatty acids (% of total FAs). [c]Ratio: 20:4n-6/(20:5n-3 + 22:6n-3). [d]Ratio: (20:2n-6 + 20:3n-6 + 20:4n-6 + 22:2n-6 + 22:4n-6 + 22:5n-6)/(20:3n-3 + 20:4n-3 + 20:5n-3 + 21:5n-3 + 22:3n-3 + 22:5n-3 + 22:6n-3).

Child development inventory

Table 2 shows the CDI classification scales at 5 years of age categorized as normal development, borderline development and developmentally delayed for both groups. There was no evidence of an impact of group allocation on children's development at 5 years of age in all areas except for the area letters, where 4/60 children in the control group were categorized as borderline/ developmentally delayed vs 0/70 in the intervention group ($P = 0.043$). At 4 years, no influence of the group assignment was observed (Supplementary Appendix 2).

Mirror movements

In Table 3, the results of the analyses comparing outcomes of the hand movement tests at 5 years between the intervention and control groups are presented. Excluding the 'non-dominant hand, fast' outcomes, the point estimates suggest a trend with lower outcome values in the intervention group with a significant difference between groups for the 'dominant hand, slow' (intervention group, 1.3 (0.7, 2.6); control group, 1.8 (1.0, 3.6); adjusted mean difference −0.39 (−0.76, −0.02); $P = 0.039$). For all other outcomes considered, however, there was no statistically significant evidence of a difference between groups. This is consistent with the results at 4 years (Supplementary Appendix 3).

Associations with fatty acids

The results of analyses to assess the association between cord blood red blood cells fatty acids and cognitive outcomes at 5 years are presented in Table 4. The calculated point estimates indicated that a higher concentration of DHA, EPA and AA is mostly associated with improved cognitive outcomes (lower MM ratio and a higher general development score). By calculating n-6:n-3 fatty acid ratios, the results suggest that a lower ratio is beneficial regarding MM ratios but not beneficial for the general development score. However, apart from two exceptions, the results do not provide statistically significant evidence of an association. Similarly, inconclusive evidence at 4 years of age is given in the Supplementary Material (Supplementary Appendix 4).

Table 2. CDI classification scales at 5 years of age

CDI scales	Intervention group n (%)	Control group n (%)	*P*-value[a]
Social			
Normal[b]	69 (98.6)	60 (100.0)	
Borderline[b]	1 (1.4)	0 (0.0)	1.000
Developmentally delayed[b]	0 (0.0)	0 (0.0)	
Self help			
Normal	68 (97.1)	57 (95.0)	
Borderline	2 (2.9)	2 (3.3)	0.797
Developmentally delayed	0 (0.0)	1 (1.7)	
Gross motor			
Normal	67 (95.7)	60 (100.0)	
Borderline	1 (1.4)	0 (0.0)	0.499
Developmentally delayed	2 (2.9)	0 (0.0)	
Fine motor			
Normal	66 (94.3)	60 (100.0)	
Borderline	2 (2.9)	0 (0.0)	0.249
Developmentally delayed	2 (2.9)	0 (0.0)	
Expressive language			
Normal	69 (98.9)	57 (95.0)	
Borderline	0 (0.0)	2 (3.3)	0.460
Developmentally delayed	1 (1.4)	1 (1.7)	
Language comprehension			
Normal	70 (100.0)	56 (94.9)	
Borderline	0 (0.0)	3 (5.1)	0.093
Developmentally delayed	0 (0.0)	0 (0.0)	
Letters			
Normal	70 (100.0)	56 (93.3)	
Borderline	0 (0.0)	2 (3.3)	0.043
Developmentally delayed	0 (0.0)	2 (3.3)	
Numbers			
Normal	69 (98.6)	57 (95.0)	
Borderline development	0 (0.0)	2 (3.3)	0.460
Developmentally delayed	1 (1.4)	1 (1.7)	
General development[c]			
Normal	67 (95.7)	54 (91.5)	
Borderline	0 (0.0)	0 (0.0)	0.468
Developmentally delayed	3 (4.3)	5 (8.5)	

Abbreviation: CDI, child development inventory. Questionnaires were collected at 60.5 ±1.3 months at 5 years with no significant age differences between groups (mean difference −0.06 (−0.51; 0.38), $P = 0.774$). [a]Fisher's exact test. [b]Terms are used to designate development within or below age expectations: borderline development was defined as − 1.5 s.d., developmentally delayed was defined as − 2.0 s.d. [c]General development scale: overall index of development, consisting of the 70 most age-discriminating items from the different scales.

Table 3. Mirror movement ratio and Pearson coefficients of correlation between intervention and control group in relation to speed and handedness at 5 years of age

	Intervention group	**Control group**	**Estimated difference**	*P*-value
Dominant hand, slow				
MM ratio [%]	1.3 (0.7, 2.6) [84, 47][a]	1.8 (1.0, 3.6) [76, 41][a]	−0.39 (−0.76, −0.02)[b,c]	0.039
Correlation coefficient	0.36 ± 0.18 [84, 47][d]	0.41 ± 0.22 [76, 41][d]	−0.05 (−0.12, 0.02)	0.142
Dominant hand, fast				
MM ratio [%]	2.0 (1.3, 3.9) [95, 48]	2.3 (1.3, 5.6) [82, 42]	−0.08 (−0.43, 0.26)	0.631
Correlation coefficient	0.22 ± 0.19 [95, 48]	0.24 ± 0.18 [82, 42]	−0.02 (−0.08, 0.05)	0.627
Non-dominant hand, slow				
MM ratio [%]	1.8 (0.9, 3.1) [77, 43]	1.8 (1.1, 3.8) [68, 37]	−0.15 (−0.53, 0.24)	0.450
Correlation coefficient	0.42 ± 0.21 [77, 43]	0.42 ± 0.21 [68, 37]	−0.01 (−0.09, 0.07)	0.828
Non-dominant hand, fast				
MM ratio [%]	2.5 (1.6, 6.1) [92, 47]	2.9 (1.7, 4.8) [80, 43]	−0.01 (−0.41, 0.39)	0.974
Correlation coefficient	0.33 ± 0.20 [92, 47]	0.31 ± 0.17 [80, 43]	0.02 (−0.05, 0.09)	0.586

Abbreviations: CI, confidence interval; MM ratio, mirror movement ratio. [a]Median (P25, P75) [number of observations, number of participants] (all such values). [b]From mixed models with a participant random effect and adjusted for frequency and handedness. The MM ratio variables were log transformed. [c]Estimated mean difference; 95% CI in parentheses (all such values). [d]Mean ±s.d. [number of observations, number of participants] (all such values).

Table 4. Association between cord blood RBCs fatty acids and cognitive outcomes at 5 years of age

Cord blood RBCs fatty acids	Test and outcome variable				
	MM ratio [%][a]				CDI questionnaire[b]
	Dominant hand, slow (n = 57)	Dominant hand, fast (n = 57)	Non-dominant hand, slow (n = 57)	Non-dominant hand, fast (n = 59)	General development score (n = 87)
20:4n-6, AA	−0.03 (−0.08, 0.02), $P = 0.281$[c]	−0.02 (−0.07, 0.03), $P = 0.372$[c]	−0.05 (−0.10, 0.00), $P = 0.073$[c]	−0.04 (−0.09, 0.01), $P = 0.123$[c]	0.89 (0.72, 1.06), $P = 0.224$[4]
20:5n-3, EPA	−0.27 (−1.75, 1.22), $P = 0.721$	−0.53 (−1.84, 0.78), $P = 0.422$	−0.23 (−1.63, 1.18), $P = 0.747$	−0.57 (−2.03, 0.88), $P = 0.434$	0.00 (0.00, 0.98), $P = 0.158$
22:6n-3, DHA	−0.06 (−0.16, 0.03), $P = 0.194$	−0.04 (−0.13, 0.04), $P = 0.322$	−0.08 (−0.17, 0.01), $P = 0.088$	−0.08 (−0.17, 0.01), $P = 0.068$	0.72 (0.43, 1.02), $P = 0.115$
n-6:n-3[e]	0.31 (0.03, 0.59), $P = 0.033$	0.17 (−0.09, 0.43), $P = 0.185$	0.20 (−0.06, 0.46), $P = 0.121$	0.32 (0.07, 0.58), $P = 0.014$	1.84 (0.96, 3.61), $P = 0.063$
n-6:n-3[f]	0.06 (−0.09, 0.22), $P = 0.397$	−0.01 (−0.15, 0.13), $P = 0.900$	0.12 (−0.02, 0.27), $P = 0.093$	0.10 (−0.05, 0.25), $P = 0.203$	1.34 (0.90, 1.95), $P = 0.118$

Abbreviations: AA, arachidonic acid; CDI, child development inventory; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MM ratio, mirror movement ratio; RBCs, red blood cells. [a]Analyzed using mixed models with a participant random effect and adjusted for frequency and handedness. The MM ratio variables were log transformed. [b]Analyzed using logistic regression with delayed and borderline development categories pooled. [c]Coefficient estimate from mixed model corresponds to the estimated mean change in log MM ratio for a unit increase in fatty acid value; 95% CI in parentheses, P-value (all such values). [d]Estimated odds ratio for a unit increase in fatty acid values; 95% CI in parentheses, P-value (all such values). [e]Ratio: 20:4n-6/(20:5n-3 + 22:6n-3). [f]Ratio: (20:2n-6 + 20:3n-6 + 20:4n-6 + 22:2n- 6 + 22:4n-6 + 22:5n-6)/(20:3n-3 + 20:4n-3 + 20:5n-3 + 21:5n-3 + 22:3n-3 + 22:5n-3 + 22:6n-3).

DISCUSSION

In this study, we explored the neurodevelopment of children, whose mothers received either a dietary intervention with n-3 fatty acids (1020 mg DHA plus 180 mg EPA per day) combined with an AA-balanced diet or a general counseling on a healthy diet without an additional supplementation during pregnancy and lactation. Results of the CDI questionnaire did not significantly differ between preschoolers at 4 and 5 years apart from the area 'letters' at 5 years of age, favoring the intervention group. There was also no evidence of a difference between MM ratios at 4 and 5 years with one exception. Further, there is only weak evidence, largely not significant, of associations between cord blood red blood cells fatty acids and the investigated outcomes.

The American version of the CDI questionnaire is an age-standardized and validated measure of neurodevelopment. It has been compared with the Bayley Scales of Infant Development, Mental Development Index, 2nd Edition: sensitivity 80%, specificity 96%.[31] Initial validation studies have been performed for the German version as well.[32] It was designed as a simple tool to identify children with developmental problems or delay in a clinical setting, indicating the need for a more expanded assessment if children fall within the borderline/delayed range.[25] When applying the CDI in this collective, observed difference between groups was only significant in the area 'letters' at 5 years of age. However, this finding should be treated with caution, as only four children in this category were defined as borderline/ delayed. It may be that standardized questionnaires, such as the CDI or Bayley scales of infant development, are not sensitive enough.[33]

To detect more subtle effects of the intervention, the parents' questionnaire was complemented by a specific test to measure MMs. MMs, also known as motor overflow, are unintended movements, which occur in homologs muscles on the opposite part of the body.[30] In the general healthy population, MMs are present in children up to 10 years of age and can be detected once again in old age. One explanation is that the nervous system is not fully myelinated, leading to impairments in white matter.[34] A study from Barnea-Goraly et al.[35] could show that white matter maturation is age-dependent, taking place from childhood through adolescence. Thereby, DHA is also involved in myelination and transmission of nerve impulses in white matter.[36] Hence, the sensitive method to quantify MMs might be a suitable indicator of neuronal development. Obtained MM ratios are comparable with normative values for neurologically healthy German children,[37] but this study does not provide significant evidence of a difference in MM ratios and Pearson coefficients of correlation between study groups.

Interestingly, albeit lacking statistical significance, analysis between cord blood LCPUFA concentrations and cognitive outcomes suggests that the higher the concentrations of EPA, DHA, but also AA, the lower the MM ratios. Our findings might suggest a potential influence of LCPUFAs on neurodevelopment, along with a balanced n-6:n-3 ratio, as a higher ratio tended to be associated with greater MMs. This observed trend might not have reached statistical significance between study groups due to high variability in the data and the small number of subjects.

A strength of our study is that women in the intervention group received a high dosage of n-3 LCPUFA (1.2 g per day) during pregnancy starting from the 15th week of gestation resulting in a significant difference in n-3 LCPUFA status at birth compared to the control group. Furthermore, the study provides a detailed phenotyping of our participants with regular assessments in preschool age. However, we have to acknowledge some limitations, which could have led to bias. Our study is limited by its sample size (≤ 70 per study group) and missing data. Due to logistical reasons, 132 cord blood samples from initially 188 children at birth (70.2%) were available. Five-year data from the CDI questionnaire were available for 130 children and due to the MM test requiring attendance at the study center, data from only 92 children were available. The high dropout rate is largely because the study was originally planned for 1 year and several families were not interested in participating in the follow-up (2–5 years). However, we went to considerable effort to obtain follow-up data, particularly for the last time point of data collection. An additional study limitation is that the CDI questionnaire is parent and not physician-based.

In addition to insufficient statistical power, other factors including inappropriate test selection, ethnic and sex differences in sample populations, supplement dosage and duration, and compliance measurements have been discussed as possible reasons for the inconsistent findings among randomized controlled trials,[33] suggesting a need for further clarification. Most of the previous studies have focused on child outcomes in children younger than 18 months of age.[38] To our knowledge, there are only eight publications from five randomized controlled trials that have assessed the effects of prenatal n-3 LCPUFA supplementation (DHA dosage ranging between 400 and 2200 mg per day) on child development beyond the age of 3,[15,39-45] with the longest follow-up until 12 years of age.[15] The majority has not obtained differences between study groups, applying different methodologies to test neurodevelopment. By investigating LCPUFA blood levels on neurological performance in the offspring, observational and interventional studies have reported mixed results.[13,41,46,47] In one such study, cord blood DHA levels revealed a significant impact on the neurological optimality score (Touwen examination)

at 5.5 years, concluding that higher DHA levels might result in a better neurological outcome, while no group differences at 5.5 years were observed.[41]

While there is a broad consensus concerning the importance of LCPUFAs in brain and neural development,[38] the current evidence for the impact of n-3 supplementation during pregnancy on child neurodevelopment is not conclusive.[19] The results of our study also did not show clear benefits or harms of a change in the n-6:n-3 fatty acid ratio during pregnancy by supplementing n-3 LCPUFAs along with a dietary reduction of AA on offspring's neurodevelopment in preschool age. Nevertheless, our findings on cord blood red blood cells fatty acids point to a potential influence on offspring neurodevelopment suggesting a need for further elucidation.

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Supporting material for Chapter III
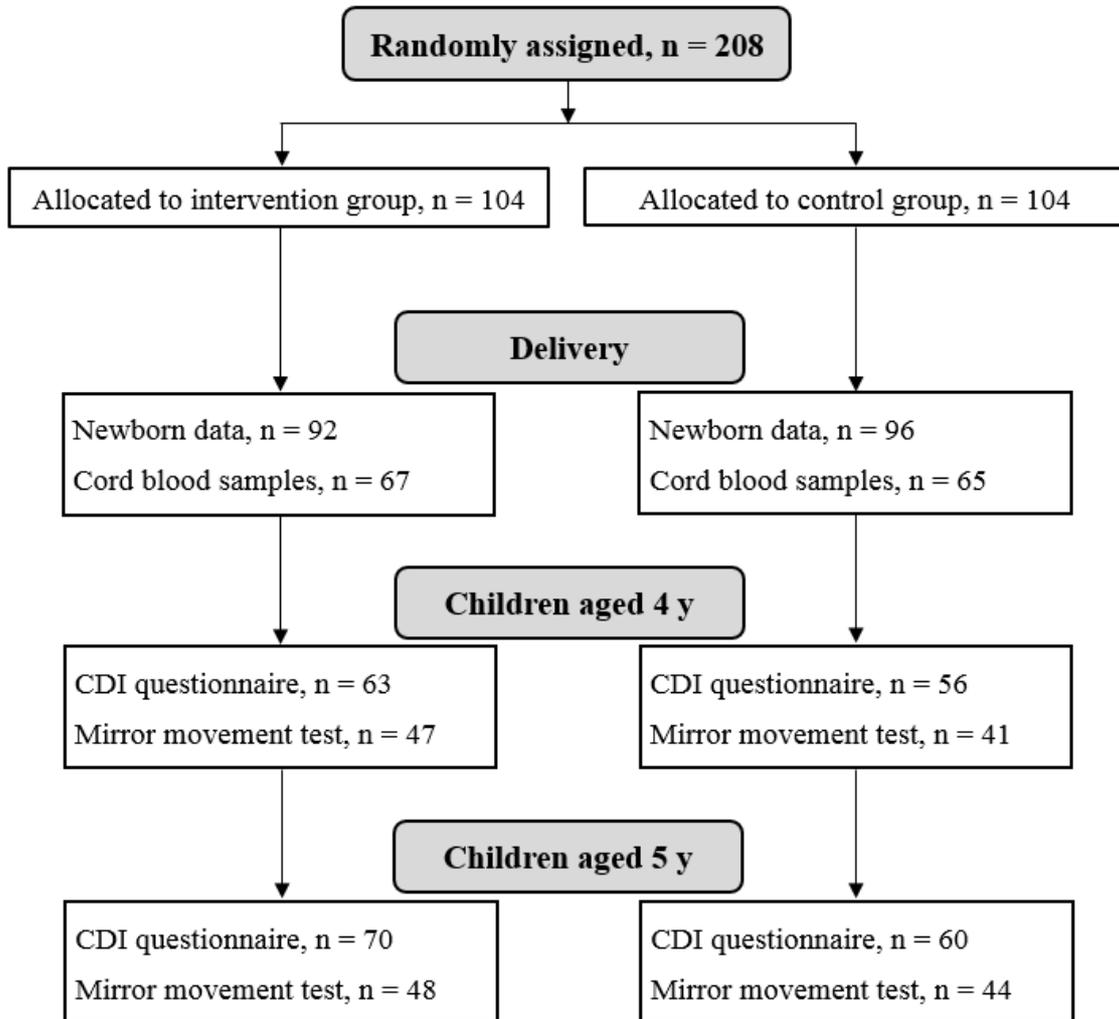
Supplementary Appendix 1. Flow-chart showing available data at each time point.
Abbreviation: CDI, child development inventory.

Supplementary Appendix 2. CDI classification scales at 4 years of age

CDI scales	Intervention group n (%)	Control group n (%)	P-value[a]
Social			
Normal[b]	62 (100.0)	56 (100.0)	
Borderline[b]	0 (0.0)	0 (0.0)	1.000
Developmentally delayed[b]	0 (0.0)	0 (0.0)	
Self help			
Normal	61 (96.8)	54 (96.4)	
Borderline	1 (1.6)	1 (1.8)	1.000
Developmentally delayed	1 (1.6)	1 (1.8)	
Gross motor			
Normal	61 (96.8)	54 (98.2)	
Borderline	0 (0.0)	0 (0.0)	1.000
Developmentally delayed	2 (3.2)	1 (1.8)	
Fine motor			
Normal	60 (95.2)	52 (94.5)	
Borderline	0 (0.0)	1 (1.8)	0.821
Developmentally delayed	3 (4.8)	2 (3.6)	
Expressive language			
Normal	56 (91.8)	54 (96.4)	
Borderline	3 (4.9)	1 (1.8)	0.713
Developmentally delayed	2 (3.3)	1 (1.8)	
Language comprehension			
Normal	57 (91.9)	49 (87.5)	
Borderline	2 (3.2)	1 (1.8)	0.475
Developmentally delayed	3 (4.8)	6 (10.7)	
Letters			
Normal	63 (100.0)	56 (100.0)	
Borderline	0 (0.0)	0 (0.0)	1.000
Developmentally delayed	0 (0.0)	0 (0.0)	
Numbers			
Normal	61 (98.4)	54 (96.4)	
Borderline development	0 (0.0)	1 (1.8)	0.736
Developmentally delayed	1 (1.6)	1 (1.8)	
General development[c]			
Normal	53 (86.9)	44 (81.5)	
Borderline	3 (4.9)	3 (5.6)	0.734
Developmentally delayed	5 (8.2)	7 (13.0)	

Abbreviation: CDI, child development inventory. Questionnaires were collected at 48.4 ± 1.0 months at 4 years with no significant age differences between groups (mean difference 0.12 (−0.26; 0.50), $P = 0.534$). [a]Fisher's exact test. [b]Terms are used to designate development within or below age expectations: borderline development was defined as − 1.5 s.d., developmentally delayed was defined as −2.0 s.d. [c]General development scale: overall index of development, consisting of the 70 most age-discriminating items from the different scales.

Supplementary Appendix 3. Mirror movement ratio and Pearson coefficients of correlation between intervention and control group in relation to speed and handedness at 4 years of age

	Intervention group	Control group	Estimated difference	P-value
Dominant hand, slow				
MM ratio [%]	1.6 (1.0, 2.8) [73, 40][a]	1.3 (0.8, 2.5) [67, 37][a]	−0.05 (−0.43, 0.33)[b,c]	0.784
Correlation coefficient	0.29 ± 0.17 [73, 40][d]	0.36 ± 0.18 [67, 37][d]	−0.06 (−0.13, 0.00)	0.065
Dominant hand, fast				
MM ratio [%]	1.8 (1.0, 4.4) [75, 40]	2.3 (1.2, 4.6) [71, 38]	−0.22 (−0.66, 0.23)	0.331
Correlation coefficient	0.27 ± 0.21 [75, 40]	0.26 ± 0.24 [71, 38]	0.03 (−0.06, 0.11)	0.565
Non-dominant hand, slow				
MM ratio [%]	2.1 (1.3, 4.3) [70, 41]	3.2 (1.5, 4.9) [56, 35]	−0.23 (−0.62, 0.17)	0.259
Correlation coefficient	0.38 ± 0.20 [70, 41]	0.41 ± 0.20 [56, 35]	−0.02 (−0.10, 0.07)	0.667
Non-dominant hand, fast				
MM ratio [%]	2.9 (1.7, 6.8) [76, 42]	3.5 (1.7, 6.1) [63, 38]	−0.02 (−0.43, 0.39)	0.918
Correlation coefficient	0.29 ± 0.20 [76, 42]	0.35 ± 0.20 [63, 38]	−0.04 (−0.13, 0.04)	0.277

Abbreviations: CI, confidence interval; MM ratio, mirror movement ratio. [a]Median (P25, P75) [number of observations, number of participants] (all such values). [b]From mixed models with a participant random effect and adjusted for frequency and handedness. The MM ratio variables were log transformed. [c]Estimated mean difference; 95% CI in parentheses (all such values). [d]Mean ± s.d. [number of observations, number of participants] (all such values).

Supplementary Appendix 4. Association between cord blood RBCs fatty acids and cognitive outcomes at 4 years of age

Cord blood RBCs fatty acids	Test and outcome variable				
	MM ratio [%][a]				CDI questionnaire[b]
	Dominant hand, slow (n = 57)	Dominant hand, fast (n = 57)	Non-dominant hand, slow (n = 57)	Non-dominant hand, fast (n = 59)	General development score (n = 87)
20:4n-6, AA	−0.07 (−0.12, −0.03), $P = 0.003$[c]	−0.03 (−0.08, 0.02), $P = 0.273$[c]	−0.04 (−0.08, 0.01), $P = 0.129$[c]	−0.06 (−0.11, −0.01), $P = 0.025$[c]	0.98 (0.85, 1.11), $P = 0.710$[d]
20:5n-3, EPA	−1.90 (−3.17, −0.63), $P = 0.004$	−0.87 (−2.24, 0.50), $P = 0.210$	−0.43 (−1.64, 0.78), $P = 0.479$	−0.88 (−2.25, 0.50), $P = 0.206$	0.32 (0.00, 12.31), $P = 0.581$
22:6n-3, DHA	−0.13 (−0.21, −0.05), $P = 0.003$	−0.05 (−0.15, 0.04), $P = 0.258$	−0.05 (−0.13, 0.03), $P = 0.200$	−0.07 (−0.16, 0.02), $P = 0.105$	0.96 (0.74, 1.21), $P = 0.713$
n-6:n-3[e]	0.23 (−0.01, 0.46), $P = 0.061$	0.04 (−0.22, 0.29), $P = 0.764$	0.06 (−0.17, 0.28), $P = 0.631$	0.08 (−0.18, 0.34), $P = 0.554$	1.43 (0.82, 2.43), $P = 0.188$
n-6:n-3[f]	0.16 (0.03, 0.30), $P = 0.021$	0.08 (−0.07, 0.23), $P = 0.281$	0.09 (−0.04, 0.21), $P = 0.179$	0.08 (−0.06, 0.23), $P = 0.262$	−0.04 (0.64, 1.35), $P = 0.845$

Abbreviations: AA, arachidonic acid; CDI, child development inventory; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MM ratio, mirror movement ratio; RBCs, red blood cells. [a]Analyzed using mixed models with a participant random effect and adjusted for frequency and handedness. The MM ratio variables were log transformed. [b]Analyzed using logistic regression with delayed and borderline development categories pooled. [c]Coefficient estimate from mixed model corresponds to the estimated mean change in log MM ratio for a unit increase in fatty acid value; 95% CI in parentheses, P-value (all such values). [d]Estimated odds ratio for a unit increase in fatty acid values; 95% CI in parentheses, P-value (all such values). [e]Ratio: 20:4n-6/(20:5n-3 + 22:6n-3). [f]Ratio: (20:2n-6 + 20:3n-6 + 20:4n-6 + 22:2n-6 + 22:4n-6 + 22:5n-6)/(20:3n-3 + 20:4n-3 + 20:5n-3 + 21:5n-3 + 22:3n-3 + 22:5n-3 + 22:6n-3).

Summary of findings

This thesis was based on the INFAT study, where the effects of a reduced n-6/n-3 long-chain PUFA ratio [dietary intervention with fish-oil capsules containing 1020 mg DHA and 180 mg EPA combined with an AA-balanced diet (~ 90 mg/day)] during pregnancy and lactation on primary and secondary outcomes from healthy, predominantly term-born children was assessed. For the assessment of abdominal fat compartments, a previous described sonographic method was adapted for a pediatric population aged ≤ 1 y. The data were also used to investigate long-term trajectories of abdominal fat growth and distribution. The key findings from Chapter Ia, II, and III are as follows:

Chapter Ia – Sonographic fat assessment in infants (6 wk–1 y)

The direct method of US to measure abdominal AT growth and distribution (i.e. subcutaneous and preperitoneal fat areas) was applied at three time points in infants aged ≤ 1 y following slight adaptions to the method described by Holzhauer et al. (2009). This sonographic method was found to be feasible and reproducible in early infancy, as reproducibility of fat areas showed strong inter- and intra-observer agreement with correlation coefficients of 0.97–0.99 at 6 wk–1 y of life. Additionally, Bland-Altman plots did not show any relevant differences between the two observers. However, certain aspects during the phase of measurement and evaluation have to be taken into consideration, including the prevention of moving artifacts resulting from the restlessness of the infants and the consideration of respiration and its effect on fat thickness. To compensate for these effects, the cine-loop-function was applied during measurement. The analysis results suggested age- and sex-dependent development of the fat compartments. A differential growth of subcutaneous and preperitoneal fat depots toward an increase in preperitoneal fat mass was observed, reflected by the shift in the calculated ratio of preperitoneal to subcutaneous fat tissue between 4 mo and 1 y of life. Girls tended to have significantly greater subcutaneous fat areas than boys from 6 wk onwards, while preperitoneal areas were not influenced by gender in the first year of life. Subcutaneous fat areas showed strong positive correlations with central SFTs as well as the sum of 4 SFTs in the first year of life, while preperitoneal fat was only weakly correlated at 6 wk and 4 mo with a slight increase in central and the sum of 4 SFTs at 1 y.

Chapter II – Long-chain PUFAs and offspring body composition

Long-term effects of the intervention on AT growth and fat distribution were assessed in children from 2–5 y. Consistent with the 1-year data (Hauner et al. 2012), the findings do not provide evidence that a dietary reduction of the n-6/n-3 long-chain PUFA ratio during pregnancy and lactation has long-term effects on body composition in preschool children. No evidence of a difference between the intervention and control group was observed in the adjusted models for the sum of 4 SFTs, growth patterns (i.e. weight, height, BMI percentiles, head, arm and waist circumference) and abdominal US (all P values > 0.05). Further, abdominal subcutaneous and visceral MRI measurements in a subgroup of children at 5 y did not reveal evidence of a difference in AT volumes and ratios. Influencing factors (i.e. diet and physical activity) could be excluded as confounding factors, as no differences between study groups in energy- and macronutrient intake as well as in PA within the ages 3 and 5 were observed.

Chapter III – Long-chain PUFAs and offspring neurodevelopment

As a secondary endpoint, the impact of the intervention on child neurological and cognitive functions at the ages 4 and 5 was investigated. The applied methods included the CDI questionnaire and a hand movement test (assessment of mirror activity), which have not been used previously in this field. The findings do not support the hypothesis that a dietary reduction of the n-6/n-3 long-chain PUFA ratio during pregnancy and lactation has long-term effects on neurodevelopment, as analyses of both methods showed mostly non-significant differences between the intervention and control group at the ages 4 and 5. Moreover, the association between the cord blood long-chain PUFA concentrations (for the groups pooled) and the neurodevelopment outcomes was assessed. Children from mothers who had higher DHA, EPA and AA cord blood concentrations as well as lower ratios of n-6/n-3, appeared to show better outcomes on MMs, but these results were largely not significant and not clinically relevant.

4 Discussion

4.1 US, a direct method to assess abdominal AT growth and distribution

The previously described and validated sonographic method from Holzhauer et al. (2009), applied in 1- and 2-year-old children from data coming from the Generation R study, was slightly adapted for our purpose in a young cohort ≤ 1 y. Uebel et al. (2014) applied the method described in Chapter Ia in 44 infants of lean and obese mothers with and without gestational diabetes mellitus at birth, 6 wk, 4 mo and 1 y with consistent growth patterns for subcutaneous and preperitoneal fat compartments.

At later stages, US-data are reported in 5- (Gruszfeld et al. 2016), 6- (Gishti et al. 2014; Santos et al. 2016) and 9.5-year-old children (Mook-Kanamori et al. 2009). In 2016, Vogelezang and colleagues provided US-data from 2 and 6-year olds, to report on the degree of tracking abdominal fat from 2 to 6 years of age (Vogelezang et al. 2016). However, longitudinal data on abdominal AT growth and distribution in preschool age are lacking. Therefore, we followed up our cohort up to 5 y of life, with the use of the sonographic method described in Chapter Ia. We observed a monotonous increase in preperitoneal fat area, while subcutaneous AT declined from 1 to 2 y and slightly increased until the fifth year of life. Data suggest that areas of subcutaneous fat remained significantly greater in girls up to 5 y of age. In contrast, preperitoneal fat areas did not differ significantly between girls and boys up to the second year of life, while there was evidence for greater areas in females from 3 y onwards. Following our cohort up to preschool age, associations with anthropometric measures at 2, 3, 4, and 5 y were largely consistent with the results observed in early infancy (≤ 1 y), with the sum of 4 SFTs and the suprailiac skinfold being the best predictor of subcutaneous fat areas. In contrast, correlations of preperitoneal fat with anthropometric measures were rather weak.

Holzhauer et al. (2009) observed similar growth patterns in preperitoneal fat areas from 1 to 2 y in a group of 210 children with a significant increase of 45 % (Generation R study) compared with 33 % (INFAT study) (both $P < 0.001$). These findings are also in accordance with a review by Samara et al. (2012), who concluded that the accumulation of visceral fat is taking place very early in life. From 1 to 2 y we have seen a decrease of the subcutaneous fat thickness in the sagittal and axial plane by 34.2 % and 22.8 %, respectively, in our cohort (both $P < 0.001$), while Holzhauer et al. observed an increase of 1.0 % ($P = 0.78$). This discrepancy might be due to heterogeneity between studies. Notably, we used a longitudinal approach with smaller sample size, while in Holzhauer's study a cross-sectional design with different children for the

investigated two time points was used. From 2 to 5 y, we observed largely stable subcutaneous fat areas for the sexes combined. In general, a reduction in fat percentage is observed between 2 and 5 y (Toro-Ramos et al. 2015), with a minimum of body fat at 5 to 7 y, before body fatness increases into adulthood, referred to as adiposity rebound (Hughes et al. 2014; Rolland-Cachera and Péneau 2013). When considering the sexes separately, our anthropometric data suggest that the adiposity rebound is already present in girls (indicated by a minimum percentage of fat mass at 4 y while values already increased at 5 y) (see **Appendix A-3**). This nadir in the percentage of body fat was not yet apparent in the boys from our cohort, consistent with other data that the adiposity rebound appears earlier in girls compared to boys (Srdic et al. 2012; Plachta-Danielzik et al. 2013). Following the preschoolers up to the age of 6 or 7 would have been helpful to gain further information on the trajectories in our cohort.

Sex-specific differences in AT are well documented in adults, with adult females having higher rates of subcutaneous fat, while adult males gain higher rates of visceral fat (Taylor et al. 2010). However, findings in children and adolescents are mixed and not consistent across studies (Staiano and Katzmarzyk 2012; Staiano et al. 2013). In contrast to those in adults, several studies have shown significantly higher rates of intraabdominal AT growth in girls compared to boys, yet the age at which point the shift happens differs between studies (Gishti et al. 2014; Holzhauer et al. 2009; Benfield et al. 2008; Gruszfeld et al. 2016; Santos et al. 2016). In a cross-sectional sample of 499 subjects aged 5–88 y, Shen and coworkers aimed to explore visceral and subcutaneous AT distribution by MRI measurements across the lifespan. Their results suggest that females have larger SAT volumes at all ages, while VAT was larger in females up to 12 y with a subsequent change resulting in higher amounts in males, thereafter (Shen et al. 2009). In another analysis from the Generation R study, fat patterning from 199 boys and 194 girls at 2 and 6 y was examined. In regard to subcutaneous fat, differences were already present at 2 y, while preperitoneal fat area was considerably higher in girls at 6 y (girls: median, $0.4 \, cm^2$; 90 % range: 0.2, 0.8; boys: median, $0.3 \, cm^2$; 90 % range: 0.2, 0.6; $P < 0.01$) but not at 2 y (girls and boys: median, $0.3 \, cm^2$; 90 % range: 0.2, 0.5; $P = 0.37$) (Vogelezang et al. 2016). Our data are consistent with these findings, supporting the idea that sex differences are already present in very early infancy and increase with age. In contrast, one study reported greater amounts of US visceral depths in boys compared to girls at 12 mo ($P = 0.04$), but not at 3 mo ($P = 0.9$), while subcutaneous depths did not differ significantly at both time points (De Lucia Rolfe et al. 2013).

Benfield et al. (2008) found higher levels of abdominal intraabdominal and subcutaneous AT for 13-year-old girls compared to their male counterparts. They also observed a significantly higher ratio of preperitoneal to subcutaneous fat areas in males, and postulate that this could indicate the start of sexual dimorphism in fat patterning. However, a significantly higher ratio in boys, which indicates proportionally more AT deposited intra-abdominally, was already observed by us very early in life (3 y of age) and by others between 2 and 7 y of age (Vogelezang et al. 2016; Gruszfeld et al. 2016; Liem et al. 2009).

Existing data on gender differences in early infancy to childhood are limited and have not yet been clearly identified. However, literature points towards the pubertal/early postpubertal period, where the shift in visceral fat emerges. Sexual maturation and hormone secretion are discussed to be a significant determinant. Longitudinal studies during infancy and childhood would provide a better understanding of gender-specific subcutaneous and preperitoneal/intra-abdominal fat development (Staiano et al. 2013).

In respect to the association of abdominal fat areas with other anthropometric parameters, our results from Chapter Ia were confirmed by a very recent study. Breij et al. (2016) showed moderate to good correlations of the subcutaneous fat area with BMI and sum of central[1] and peripheral[2] skinfolds at 3 and 6 mo postpartum in both sexes, while, in contrast, no notable association between visceral fat thickness with these measures was observed. Waist circumference is considered to be the best predictor of intra-abdominal fat mass in adolescents between 7–16 y, accounting for 64.8 % of the variance in visceral AT (Brambilla et al. 2006), but is also used in early childhood (Toro-Ramos et al. 2015). However, a validation study with newborns suggested that waist circumference as a predictor of visceral fat is likely to be ineffective in early stages, showing almost no correlation between waist circumference and VAT volume assessed by MRI (r = 0.08, $P > 0.05$). In contrast, VAT assessed by US was significantly positively correlated with VAT by MRI (r = 0.48, $P < 0.05$) (De Lucia Rolfe et al. 2013). Due to the benefit of the differentiation between SAT and VAT, the method of US is likely to enable a prediction of better accuracy and a better indicator of metabolic risk.

US contributes significant advantages over MRI, CT, and DXA and has been proposed as a promising method for AT growth and fat distribution, especially in the pediatric population (Toro-Ramos et al. 2015; Horan et al. 2015). Here, the applied US method has been characterized as a non-invasive and easy-to-handle approach to assess regional abdominal

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[1] Central skinfolds = sum of subscapular and suprailiac skinfolds
[2] Peripheral skinfolds = sum of biceps and triceps skinfolds

subcutaneous and preperitoneal AT development accurately in very young infants ($\leq$ 1 y). By following our cohort until 5 y of life, the evaluation revealed a differential development of the two fat compartments, depending on children's age and sex. As stated in Chapter Ia, by associating these data with metabolic parameters, this information may allow a better prediction and prevention of disease risk early in life.

4.2   Impact of n-3 long-chain PUFAs on offspring AT growth

To date, there exist 14 publications from eight individual RCTs on the impact of n-3 long-chain PUFA supplementation during pregnancy and/or lactation on offspring AT composition with sample sizes ranging between 69 and 1531. **Figure 5**, an updated version of a figure published in 2013 (Hauner et al.), gives an overview of available human data from these RCTs. Data are available from birth up to 19 y of age, with 57 % of those focusing on the ages between 4–7 y. The majority did not find a significant effect on offspring body composition (Stein et al. 2011; Gonzalez-Casanova et al. 2015; Rytter et al. 2011; Escolano-Margarit et al. 2011; Campoy et al. 2011; Muhlhausler et al. 2016; Asserhoj et al. 2009; Lauritzen et al. 2017; Bergmann et al. 2012; Helland et al. 2008; Hauner et al. 2012; Brei et al. 2016) with the exception of two studies (Lauritzen et al. 2005; Bergmann et al. 2007).

In one such study, the authors reported significantly higher values of BMI, BMI z-scores and a larger waist circumference in the fish oil group (supplementation of 1.5 g n-3 FAs from birth until 4 mo postpartum) compared with those in the olive oil group at 2.5 y of age (Lauritzen et al. 2005). In a German cohort, Bergmann et al. (2007) described significantly lower values of BMI and BMI z-scores at 21 mo postpartum in the offspring of mothers receiving a daily dose of 260 mg n-3 long-chain PUFAs from 21 wk of gestation until 3 mo postpartum, compared to a control group. However, when the cohorts were followed up about 4 y later, the significant differences between study groups were no longer detectable (Asserhoj et al. 2009; Bergmann et al. 2012).
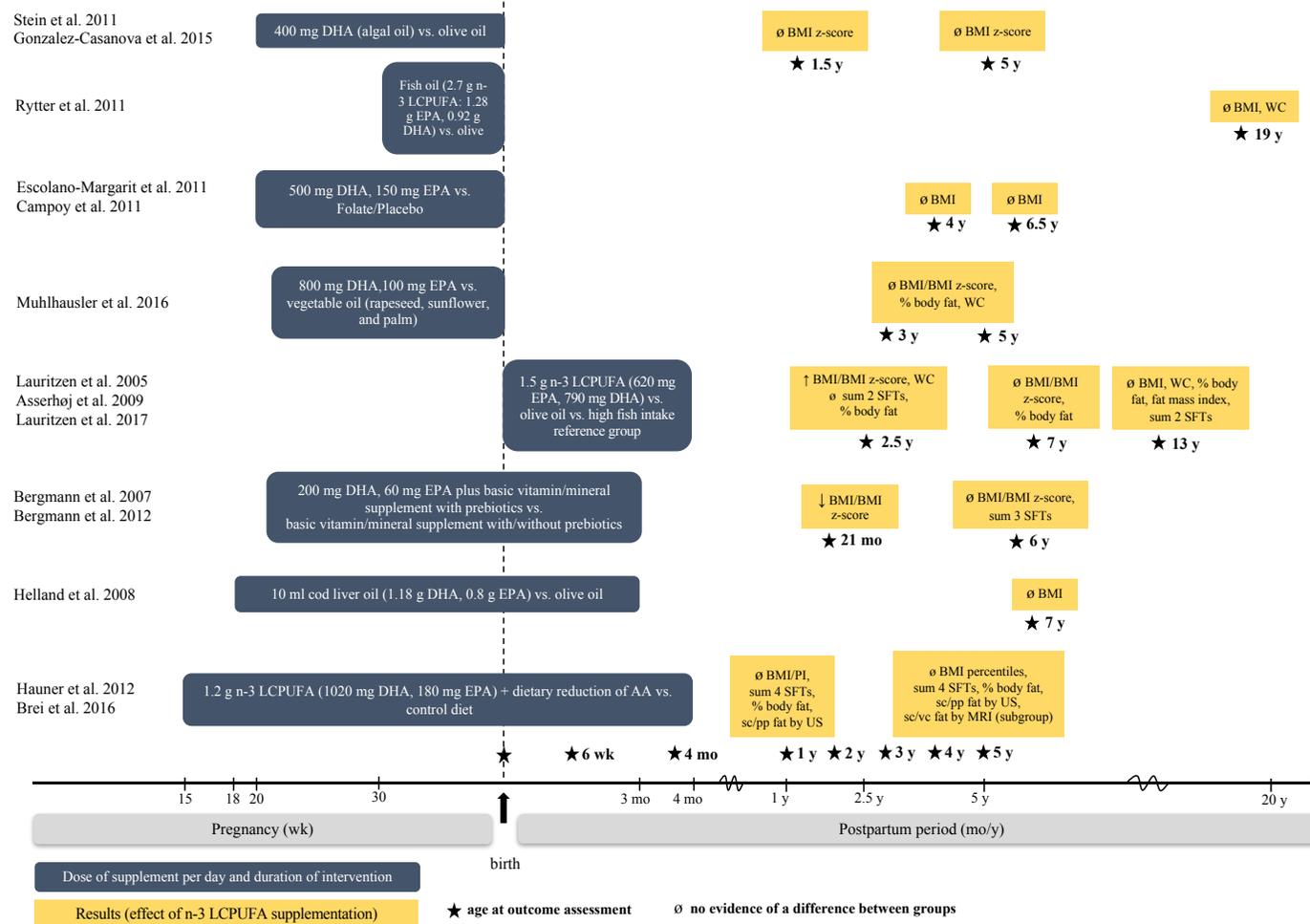
Figure 5 RCTs of n-3 long-chain PUFA supplementation on offspring body composition

AA, arachidonic acid; BMI, body mass index; DHA, docosahexaenoic acid; EPA eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; mo, months; MRI, magnetic resonance imaging; PI, ponderal index; pp, preperitoneal; sc, subcutaneous; US, ultrasound; vc, visceral; WC, waist circumference; wk, week; y, year;
Data source: modified from Hauner et al. 2013.

Of note, approximately half of the studies used rather indirect growth parameters to determine body composition, such as BMI, BMI z-scores, and waist circumference (Stein et al. 2011; Gonzalez-Casanova et al. 2015; Rytter et al. 2011; Escolano-Margarit et al. 2011; Campoy et al. 2011; Helland et al. 2008), while other studies applied a broader range of methods (Muhlhausler et al. 2016; Lauritzen et al. 2005; Asserhoj et al. 2009; Lauritzen et al. 2017). One study enlarged the range of methods by determining skinfolds during follow-up measurements (Bergmann et al. 2012). The INFAT study used the largest set of combined methods for the assessment of direct and indirect methods, including anthropometry, SFT measurements at 4 body sites, abdominal sonography as well as abdominal MRI examination in a subgroup of children at 5 y of age. However, in addition to other limitations discussed in Chapter II, the INFAT study is limited in its power by the low number of participants (104 participants per study group) and the high attrition rate during the follow-up period (~ 55 participants per study group at 5 y).

In the same issue of the American Journal of Clinical Nutrition where the follow-up data of the INFAT study were published, Muhlhausler et al. (2016) presented findings on body composition data at 3 and 5 y of age. Data came from the DOMInO (DHA to Optimize Mother Infant Outcome) trial, where pregnant women took daily supplements containing 900 mg n-3 long-chain PUFAs or placebo from 20th wk of gestation to delivery. At 3 and 5 y of age, no influence of group assignment concerning BMI/BMI z-scores (BMI z-score at 5 y: adjusted mean difference, 0.02; 95% CI: −0.08, 0.12; $P = 0.66$), percentage of body fat mass (at 5 y: adjusted mean difference, 0.11; 95% CI: −0.60, 0.82; $P = 0.75$) or waist circumference (at 5 y: adjusted mean difference, 0.10; 95% CI: −0.31, 0.51; $P = 0.62$) was observed. This study provides most robust data due to its large sample size so far, with a total of 1531 children for both time points and high retention rates (92.2 %). Interim analysis of 7-year data, presented at the 12th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) in 2016, gave results consistent with the earlier time points[1].

The most recent work is a follow-up from the Danish National Birth Cohort. They published 13-year data in January 2017 (Lauritzen et al. 2017). Consistent with their follow-up at 7 y of age (Asserhoj et al. 2009), they found no evidence of a difference between study groups

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[1] Interim analysis of 7-year data from 252 DOMInO-children (n = 133 males, n = 117 females) revealed no evidence of a difference between study groups in regard to percentage of body fat, fat free mass, weight, waist and hip circumference or BMI/BMI z-scores (Wood et al. 2016)

regarding BMI, fat mass index, body fat percentage, the sum of SFTs (triceps and subscapular) and waist circumference (all P values > 0.05).

In conclusion, the INFAT study investigated the original hypothesis of Ailhaud and Guesnet (2004), if a reduced n-6/n-3 long-chain PUFA ratio during pregnancy and lactation has the potential on perinatal programming towards the primary prevention of childhood obesity. Applying several methods to assess body composition, and in accordance with the first-year results (Hauner et al. 2012), no long-term effects between the randomized groups from 2–5 y have been identified. Thus, akin to other reported studies (shown in **Figure 5**) and one meta-analysis (Stratakis et al. 2014), we could not verify the hypothesis that a dietary intervention to reduce the maternal dietary n-6/n-3 FA ratio is a useful strategy to limit AT growth and thereby prevent obesity in preschool age.

Even if available RCTs provide strong evidence for the lack of benefit of n-3 long-chain PUFA supplementation during pregnancy and/or lactation on later obesity risk, various approaches have been discussed for further investigations:

The critical windows for AT development (timing and duration) are still of interest. Even if the first appearance of adipocytes is documented between 14 and 16 wk of gestation (Poissonnet et al. 1983), Blumfield (2016) hypothesized that nutritional modification before this period (i.e. between 5 and 12 wk of gestation) could program offspring physiology. Future research could be designed to determine whether maternal long-chain PUFA supplementation impacts offspring body composition when the intervention is commenced earlier in pregnancy (i.e. the phase of preconception and/or early pregnancy).

The region for the assessment of AT development could also be of interest in subsequent publications. Previous work found a significant positive association between maternal PUFA intake (% of energy/day) during pregnancy and the fetal mid-thigh lean area (%) and a corresponding negative association with the fetal mid-thigh subcutaneous area (%), whereas abdominal visceral/subcutaneous areas tended to be unaffected by the nutrient supply of PUFAs. Data were assessed from 145 pregnant women with US scans from 19th to 36th wk of gestation (Blumfield et al. 2012b). In future research, body composition measurements should include measurements in the gluteo-femoral regions.

An increased focus could be placed on the proadipogenic role of n-6 long-chain PUFAs in human studies. Cohort studies have linked increased maternal n-6 long-chain PUFA concentrations to offspring adiposity (Donahue et al. 2011; de Vries et al. 2014; Moon et al.

2013; Vidakovic et al. 2016). In contrast, only the present RCT from our group focused on a modification of the n-6/n-3 long-chain PUFA ratio in the maternal diet (Hauner et al. 2009; Hauner et al. 2012; Brei et al. 2016). All other RCTs determined the effects of an increased intake of n-3 FAs alone. Additional studies could be designed to investigate this issue further.

4.3   Impact of n-3 long-chain PUFAs on offspring neurodevelopment

The only meta-analysis performed by Gould et al. (2013), did not conclusively support or refute that maternal n-3 long-chain PUFA supplementation during pregnancy affects offspring's cognitive development in early childhood. The authors' literature search (last update August 2012) resulted in 19 published articles and 4 abstracts/conference proceedings from 11 individual trials, which were listed in the review. Studies included in the meta-analysis showed no differences in standardized test scores in regard to cognitive (7 studies), motor (4 studies), or language development (2 studies), except for cognitive scores in 2–5-year-old children (2 studies; mean difference, 3.92; 95% CI: 0.77, 7.08; n = 156; P = 0.01). Both studies were in favor of the supplemented group (Dunstan et al. 2008; Helland et al. 2003) and resulted in significance in the fixed-effect model. As the general development score from the CDI-questionnaire is considered as a general index of cognitive development (Ireton 1992), our results with 4- and 5-year-old children (n = 244) are in contrast to what they have observed.

Subsequent to the above meta-analysis, and to the best of my knowledge, 10 RCTs have been published after August 2012, presenting data with mixed results (Gustafson et al. 2013; Mulder et al. 2014; Makrides et al. 2014; Gould et al. 2014; Hurtado et al. 2015; Meldrum et al. 2015; Ramakrishnan et al. 2016; Colombo et al. 2016; Gould et al. 2017). Even if some studies report on a beneficial effect of prenatal n-3 supplementation on individual areas between birth and 5 y of life, such as objective measures of attention (Ramakrishnan et al. 2016; Colombo et al. 2016), motor and orientation abilities (Gustafson et al. 2013), and improved language development (Mulder et al. 2014), most studies did not provide significant evidence of an effect. Supplementation during pregnancy did not affect mental or psychomotor development at 12 mo (Hurtado et al. 2015), problem-solving and fine/gross motor skills at 14 and 18 mo (Mulder et al. 2014), distractibility and working memory and inhibitory control at 27 ± 2 mo (Gould et al. 2014), or cognition, language, and fine motor skills in a cohort at 12 y of life (Meldrum et al. 2015).

The DOMInO study presented data from 726 18-month-old infants with no benefits of the supplementation on cognitive, language, and motor development (Makrides et al. 2010)[1]. Researchers presented follow-up data of 646 children at 4 y (Makrides et al. 2014). In 2017, Gould et al. published 7-year follow-up data from 543 children. Findings were largely consistent, providing strong evidence for the lack of a benefit of prenatal long-chain PUFA supplementation on these neurodevelopmental outcomes. These results contribute new insights to the research question as this RCT stands out due to its well-powered study design (Gould et al. 2017).

In summary, more than 30 publications/abstracts investigating the effect of DHA supplementation during pregnancy or pregnancy and lactation (range: 200–3300 mg long-chain PUFA/day) on neurodevelopment outcomes in the offspring are available, with the latest publication coming from our department (Brei et al. 2017). The sample sizes among studies ranged between 27 and 797, with different outcome parameters between studies, making studies difficult to compare. They have mostly included global tests to analyze skills across major neurologic domains (Dunstan et al. 2008; Mulder et al. 2014; Campoy et al. 2011), but also sensitive tests for the detection of specific domains have been applied (Colombo et al. 2016; Gould et al. 2014). Trials have been performed at ages from birth to 12 y, with mixed results. However, most of them report negative findings.

It is worth noting that most studies have been performed in high-income countries. As per capita estimated daily intakes of AA and DHA are positively associated with gross national income (Forsyth et al. 2017), a distinction between results obtained from high- and low-income countries should be made. It cannot be excluded that infants/children from low-income communities might benefit from maternal prenatal/early postnatal supplementation of n-3 long-chain PUFAs (Campoy et al. 2012).

Another determinant is the current FA status when data is collected. Heaton et al. state that an association between DHA status at birth (e.g. cord blood) and child neurocognitive status does not always demonstrate causality due to potential confounding variables (Heaton et al. 2013). Meldrum and coworkers observed that long-chain EPA and DHA concentrations in cord blood were not significantly correlated with concentrations at 12 y postpartum (measured in erythrocytes). Further, they found no evidence of a difference between concentrations of EPA and DHA at 12 y between study groups (duration: 20th wk gestation–delivery; intervention,

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[1] This study is included in the above mentioned meta-analysis

2.2 g DHA and 1.1 g EPH/day; control, olive oil), suggesting that the prenatal effect of a daily high-dose n-3 FA supplementation is diluted by its daily nutritional intake. Further, the analysis showed a positive correlation of n-3 FA status at 12 y and neurodevelopmental performance. This finding points towards an important role of the present n-3 long-chain PUFA status when the neurological tests are performed. Unfortunately, authors did not present results for the association of cord blood FAs and neurodevelopmental performance at 12 y (Meldrum et al. 2015). Our findings on cord blood long-chain PUFAs point to a potential influence on offspring development. In addition to the limitations in Chapter III, it would have been worthwhile to measure FA status in our cohort at the date of testing and assess its association with neurodevelopment outcomes.

Albeit literature points towards no impact of maternal n-3 long-chain PUFA supplementation during pregnancy and lactation on neurodevelopmental outcomes, there is currently no conclusive evidence (Chmielewska et al. 2016). Future studies (with large sample sizes and a high dosage) could be designed to determine which domains of the brain are mostly affected by an n-3 long-chain PUFA supplementation and then subsequently identify appropriate test procedures for possible effects (Demmelmair and Koletzko 2015; Nyaradi et al. 2013). For this, the use of MRI-technology is considered to be helpful (Brenna and Carlson 2014). Further, future directions could consider the interactive effects of nutrients, and, if an overall healthy and balanced diet enhances cognitive development (Nyaradi et al. 2013).

4.4 Related research

In considering outcomes beyond the central research questions, there is consistent evidence that n-3 long-chain PUFA supplementation during pregnancy increases gestational age (Chen et al. 2016), also proven in the INFAT study (Hauner et al. 2012). It is recognized that an extension of pregnancy length is associated with a reduction in early preterm birth[1] (Yelland et al. 2016), but also with obstetric interventions aiming to limit the risk associated with post-term birth (Makrides et al. 2010). Besides, it is worth adding that no potential harms of a high-dose supplementation were observed (Rogers et al. 2013).

As mentioned briefly in the introduction, prenatal n-3 long-chain PUFA supplementation may play a role in the immune response to inflammation and have a prophylactic potential for the prevention of related diseases (Demmelmair and Koletzko 2015). A recently published study investigated the effect of a high-dose n-3 long-chain PUFA supplementation (duration: 24th wk

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[1] Early preterm is defined as born alive before 34 completed wk of gestation (Yelland et al. 2016)

of gestation–1 wk after delivery; intervention, 2.4 g n-3 FAs; control, olive oil) during the third trimester of pregnancy on wheeze and asthma up to 5 y of age. The intervention resulted in a significant relative risk reduction of 30.7 % to develop wheeze and asthma (Bisgaard et al. 2016). However, due to inconsistent results between studies, further work is needed (Best et al. 2016). For this purpose, obtained data from the INFAT study[1] could be analyzed in a future project.

5   Conclusion

In recent years, it has been increasingly recognized that during the early life period (periconceptional, prenatal and early postnatal) maternal and environmental factors can have an impact on long-term consequences on health outcomes later in life (Godfrey et al. 2016).

The randomized controlled INFAT study provides an excellent opportunity to advance the understanding of a possible programming role of n-3 long-chain PUFAs during pregnancy and lactation on offsprings' short- and long-term outcomes. The intervention aimed to reduce the n-6/n-3 long-chain PUFA ratio from the 15th wk of gestation until 4 mo postpartum in the diet of healthy, pregnant women who live in Germany. In conclusion, findings do not show evidence for short- and long-term effects on offsprings' body composition up to 5 y of age, assessed with direct and indirect measuring techniques. In this context, US was verified as a non-invasive and feasible approach for the assessment of abdominal fat distribution in early infancy. Obtained data showed age- and sex-dependent development of subcutaneous and preperitoneal fat compartments from 6 wk–5 y of age. Further, data do not provide convincing evidence regarding an effect of n-3 long-chain PUFA supplementation on offspring's neurodevelopment at 4 and 5 y of age. However, findings on cord blood long-chain PUFAs suggest a need for further elucidation.

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[1] Questionnaire for the assessment of atopic eczema

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Appendix

A-1 3-day dietary records (1 day as example)

Else Kröner-Fresenius-Zentrum für
Ernährungsmedizin
des Klinikums rechts der Isar
der Technischen Universität München
Direktor: Univ.-Prof. Dr. Hans Hauner

+
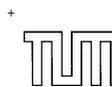
Else Kröner-Fresenius-Zentrum für Ernährungsmedizin
Klinikum rechts der Isar der TU München
Ismaninger Str. 22, 81675 München

Direktor: Univ.-Prof. Dr. Hans Hauner

Ernährungsprotokoll

für 3 Tage

Von:_____

Datum von:_____bis:_____

Besondere Ernährungsform (bitte ankreuzen)

- Vegetarisch ❑
- Vegetarisch mit Fisch ❑
- Laktosefrei ❑
- Glutenfrei ❑
- Sonstiges _____

Ernährungsprotokoll

Name:_____   Datum: _____

Frühstück / Zwischenmahlzeit:

Zeit und Ort	Menge	Lebensmittel/Getränk	Zubereitung

Mittagessen / Zwischenmahlzeit:

Zeit	Menge	Lebensmittel/Getränk	Zubereitung

Abendessen / Spätmahlzeit:

Zeit	Menge	Lebensmittel/Getränk	Zubereitung

A-2 Parents' questionnaire to assess child physical activity

Eltern-Fragebogen zur körperlichen Aktivität

Follow-up

Untersuchungsdatum: |__|__| . |__| |__| |__| . |__||__||__||__|
            Tag   Monat   Jahr

Teilnehmer-ID: |__||__||__||__|

Geschlecht: männlich ❑   weiblich ❑

Geburtsdatum: |__|__| . |__| |__| |__| . |__||__||__||__|
            Tag   Monat   Jahr

Lebensmonat des Kindes: |__||__|

1. Wie häufig spielt Ihr Kind im Freien?

Fast jeden Tag   ❑
3-5 mal pro Woche ❑
1-2 mal pro Woche ❑
seltener   ❑
nie   ❑

2. Wie häufig treibt Ihr Kind Sport in einem Verein?

Fast jeden Tag   ❑
3-5 mal pro Woche ❑
1-2 mal pro Woche ❑
seltener   ❑
nie   ❑

3. Wie häufig treibt Ihr Kind Sport außerhalb eines Vereins?

Fast jeden Tag   ❑
3-5 mal pro Woche ❑
1-2 mal pro Woche ❑
seltener   ❑
nie   ❑

Weitere Fragen zu Fernsehkonsum und Computerspielen

4. Wie lange sieht Ihr Kind **durchschnittlich pro Tag** Fernsehsendungen oder Videofilme?
(Bitte kreuzen Sie an, was am ehesten zutrifft.)

An einem Wochentag
Gar nicht   ❑
Ca. 30 min pro Tag   ❑
Ca. 1-2 Stunden pro Tag ❑
Ca. 3-4 Stunden pro Tag ❑
Mehr als 4 Stunden pro Tag ❑

An einem Samstag/Sonntag
Gar nicht   ❑
Ca. 30 min pro Tag   ❑
Ca. 1-2 Stunden pro Tag ❑
Ca. 3-4 Stunden pro Tag ❑
Mehr als 4 Stunden pro Tag ❑

5. Wie lange spielt Ihr Kind **durchschnittlich pro Tag** an einem Computer?
(Bitte kreuzen Sie an, was am ehesten zutrifft.)

An einem Wochentag
Gar nicht   ❑
Ca. 30 min pro Tag   ❑
Ca. 1-2 Stunden pro Tag ❑
Ca. 3-4 Stunden pro Tag ❑
Mehr als 4 Stunden pro Tag ❑

An einem Samstag/Sonntag
Gar nicht   ❑
Ca. 30 min pro Tag   ❑
Ca. 1-2 Stunden pro Tag ❑
Ca. 3-4 Stunden pro Tag ❑
Mehr als 4 Stunden pro Tag ❑

Appendix

A-3 BMI/BMI percentiles and AT development by age and gender

Age	Parameter	All Mean (SD)	n	Female Mean (SD)	n	Male Mean (SD)	n	Estimated mean difference (95% CI)	P
6 wk		59.2 (28.0)[1]	180	61.6 (28.5)	86	57.0 (27.6)	94	−4.6 (−12.8, 3.6)	0.274
4 mo		57.5 (28.7)	173	59.0 (28.5)	83	56.2 (29.0)	90	−2.9 (−11.5, 5.6)	0.500
1 y		52.4 (30.0)	170	51.6 (30.7)	84	53.1 (29.5)	86	1.2 (−7.8, 10.2)	0.797
2 y	BMI percentile[2]	52.9 (28.2)	170	50.3 (28.4)	83	55.4 (27.9)	87	4.7 (−3.7, 13.1)	0.270
3 y		50.0 (26.2)	162	46.9 (26.3)	79	53.0 (25.9)	83	5.0 (−2.8, 12.8)	0.208
4 y		47.2 (26.7)	159	44.6 (27.0)	77	49.6 (26.4)	82	3.2 (−4.9, 11.3)	0.438
5 y		45.8 (25.8)	152	41.8 (26.0)	74	49.6 (25.3)	78	5.2 (−2.6, 13.0)	0.193
6 wk		15.2 (1.3)	180	15.1 (1.3)	86	15.4 (1.3)	94	0.3 (−0.1, 0.7)	0.140
4 mo		16.3 (1.4)	174	16.1 (1.3)	84	16.6 (1.3)	90	0.5 (0.1, 0.9)	0.015
1 y		16.8 (1.4)	170	16.6 (1.5)	84	17.0 (1.3)	86	0.5 (0.0, 0.9)	0.028
2 y	BMI (kg/m^2)	16.2 (1.3)	170	16.0 (1.3)	83	16.5 (1.3)	87	0.4 (0.0, 0.8)	0.041
3 y		15.7 (1.2)	162	15.5 (1.1)	79	15.9 (1.2)	83	0.3 (−0.0, 0.7)	0.055
4 y		15.4 (1.2)	159	15.2 (1.2)	77	15.5 (1.1)	82	0.3 (−0.0, 0.6)	0.123
5 y		15.3 (1.2)	153	15.1 (1.3)	75	15.5 (1.2)	78	0.3 (−0.0, 0.7)	0.070
6 wk		22.1 (3.8)	180	22.2 (3.6)	86	21.9 (3.9)	94	−0.4 (−1.5, 0.8)	0.532
4 mo		25.2 (4.1)	174	25.8 (4.6)	84	24.7 (3.7)	90	−1.0 (−2.3, 0.2)	0.092
1 y		24.1 (4.2)	165	24.4 (4.4)	80	23.7 (4.0)	85	−0.8 (−2.0, 0.5)	0.238
2 y	Sum 4 SFTs (mm)[3]	23.7 (3.4)	110	24.4 (3.6)	46	23.2 (3.2)	64	−1.4 (−2.6, −0.3)	0.016
3 y		23.3 (3.6)	113	24.7 (3.8)	53	22.2 (3.1)	60	−2.2 (−3.4, −1.0)	<0.001
4 y		23.5 (3.7)	102	24.6 (3.8)	47	22.6 (3.3)	55	−2.3 (−3.6, −1.0)	<0.001
5 y		24.2 (4.8)	112	25.8 (5.5)	51	22.8 (3.8)	61	−3.0 (−4.6, −1.4)	<0.001
6 wk		19.0 (3.0)	180	19.2 (2.8)	86	18.9 (3.2)	94	−0.3 (−1.2, 0.6)	0.468
4 mo		21.1 (2.8)	174	21.4 (3.0)	84	20.8 (2.5)	90	−0.6 (−1.4, 0.2)	0.121
1 y		19.7 (2.9)	165	20.0 (3.0)	80	19.5 (2.8)	85	−0.5 (−1.4, 0.4)	0.246
2 y	Fat mass (%)[4]	19.1 (2.3)	110	19.6 (2.3)	46	18.7 (2.2)	64	−1.0 (−1.8, −0.2)	0.017
3 y		18.4 (2.6)	113	19.6 (2.5)	53	17.3 (2.2)	60	−2.1 (−2.9, −1.3)	<0.001
4 y		18.1 (2.8)	102	19.5 (2.5)	47	16.9 (2.4)	55	−2.8 (−3.7, −1.9)	<0.001
5 y		18.0 (3.5)	112	20.0 (3.4)	51	16.3 (2.6)	61	−3.7 (−4.7, −2.7)	<0.001

[1] Data are presented as mean ± SD (n) along with the nonadjusted mean difference (95% confidence interval) from mixed models containing time, sex, and an interaction between sex and time.
[2] Calculated according to Kromeyer-Hauschild et al. 2001.
[3] Sum 4 SFTs was calculated as biceps + triceps + subscapular + suprailiac SFTs; SFT, skinfold thickness.
[4] Calculated according to Weststrate and Deurenberg 1989.

A-4 Approval letter – Pediatric Research 78(3): 342–50, 2015

Brei, Christina

Von:	Permissions@nature.com
Gesendet:	Freitag, 3. Februar 2017 11:17
An:	Brei, Christina
Betreff:	RE: Permission request, Ped Res

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From: Brei, Christina [mailto:christina.brei@tum.de]
Sent: 02 February 2017 14:48
To: ajpermissions
Subject: Permission request, Ped Res

Dear Sir or Madam,

I am writing to you concerning the manuscript entitled "Sonographic assessment of abdominal fat distribution during the first year of infancy", published in Pediatric Research in 2015 (Ped Res. 2015 Sept; 78(3):342-50).
I am a PhD student at the Technical University of Munich (TUM) and I'm aiming towards a PhD by publication. For this, I would like to include the aforementioned publication in my thesis in its original form.
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Kind regards,
Christina Brei

Christina Brei, M. Sc. Ern. wiss.

Institut für Ernährungsmedizin
Technische Universität München
Uptown München Campus D
Georg-Brauchle-Ring 60/62, 5. Stock
80992 München
Tel.: 089/ 289 249 18
christina.brei@tum.de

2

Appendix

A-5 Approval letter – American Journal of Clinical Nutrition 103(6): 1472–81, 2016

Brei, Christina

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On a personal note, and as a former resident of Germany, I would like to offer my condolences for the loss of life at the Christmas Market in Berlin yesterday.

Best regards,

Sarah L. McCormack
Assistant Director of Editorial and Production Operations
American Society for Nutrition
9211 Corporate Blvd., Suite 300
Rockville, MD 20850
P: 240-428-3616/F: 240-404-6798

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From: AJCNSubmit Account
Sent: Wednesday, December 14, 2016 12:54 PM
To: Sarah McCormack
Subject: FW: Permission request, AJCN

From: Brei, Christina [mailto:christina.brei@tum.de]
Sent: Wednesday, December 14, 2016 12:40 PM
To: AJCNSubmit Account
Subject: Permission request, AJCN

Dear Sir or Madam,

I am writing to you concerning the manuscript entitled "Reduction of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring body composition: follow-up results from a randomized controlled trail up to 5 y of age", published in the American Journal of Clinical Nutrition in 2016 (Am J Clin Nutr. 2016 Jun;103(6):1472-81). I am a PhD student at the Technical University of Munich (TUM) and I'm aiming towards a PhD by publication. For this, I would like to include the aforementioned publication in my thesis in its original form.
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Christina Brei

Christina Brei, M. Sc. Ern. wiss.

Institut für Ernährungsmedizin
Technische Universität München
Uptown München Campus D
Georg-Brauchle-Ring 60/62, 5. Stock
80992 München
Tel.: 089/ 289 249 18
christina.brei@tum.de

Appendix

A-6 Approval letter – European Journal of Clinical Nutrition, 2017 [epub ahead of print]

Brei, Christina

Von:	Journalpermissions <journalpermissions@springernature.com>
Gesendet:	Freitag, 26. Mai 2017 13:27
An:	Brei, Christina
Betreff:	RE: Permission request, EJCN

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From: Brei, Christina [mailto:christina.brei@tum.de]
Sent: 26 May 2017 09:07
To: Permissions@nature.com
Subject: Permission request, EJCN

Dear Sir or Madam,

I am writing to you concerning the manuscript entitled "Impact of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring neurodevelopment: 5-year follow-up of a randomized controlled trial", published in the European Journal of Clinical Nutrition in 2017.
I am a PhD student at the Technical University of Munich (TUM) and I'm aiming towards a PhD by publication. For this, I would like to include the aforementioned publication in my thesis in its original form.

Under the terms of the TUM Regulations for the Award of Doctoral Degrees, the electronic version will be archived and openly available at mediaTUM (https://mediatum.ub.tum.de), the TUM's media server.

I would therefore be very grateful if you could grant permission for the reproduction of the manuscript in my thesis.

Kind regards,
Christina Brei

Christina Brei, M. Sc. Ern. wiss.

Institut für Ernährungsmedizin
Technische Universität München
Uptown München Campus D
Georg-Brauchle-Ring 60/62, 5. Stock
80992 München
Tel.: 089/ 289 249 18
christina.brei@tum.de

A-7 Einverständniserklärung zur publikationsbasierten Promotion

TUM

Technische Universität München

Einverständniserklärung zur publikationsbasierten Promotion[1]

Anlage 6 (für § 6 Abs. 2)

Hiermit erkläre ich mein Einverständnis, dass die Dissertation als publikationsbasierte Dissertation eingereicht wird. Sie erfüllt die nachfolgenden Kriterien:

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12. 5. 2017
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Datum

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1 Zur Vorlage bei der Einreichung der Dissertation.

Acknowledgment

Curriculum vitae

Persönliche Daten

Name	Christina Brei
Geburtsdatum	4. Juni 1987
Nationalität	deutsch

Promotion

seit 04/2013	**Technische Universität München (TUM)**
	Promotion am Else Kröner-Fresenius-Zentrum für Ernährungsmedizin, Lehrstuhl für Klinische Ernährungsmedizin
	Thema der Doktorarbeit: „Effect of changing the n-6/n-3 fatty acid ratio in the maternal diet during pregnancy and lactation on child body composition and neurodevelopment: long-term results from the INFAT study"

Studium

10/2009 – 08/2012	**Justus-Liebig-Universität Gießen**
	Abschluss: Master of Science (M.Sc.) Ernährungswissenschaften
	Thema der Masterarbeit: „Vitamin A-Versorgung von Patienten mit Mukoviszidose"
10/2006 – 10/2009	**Justus-Liebig-Universität Gießen**
	Abschluss: Bachelor of Science (B.Sc.) Ökotrophologie
	Thema der Bachelorarbeit: „Ernährungstherapie bei Morbus Crohn"

Schulausbildung

09/1997 – 06/2006	**Karl-von-Closen-Gymnasium Eggenfelden**
09/1993 – 07/1997	**Grundschule Eggenfelden**

List of publications and congress contributions

Original articles, in English

Brei C, Simon A, Krawinkel MB, Naehrlich L. 2013. Individualized vitamin A supplementation for patients with cystic fibrosis. *Clin Nutr* 32 (5): 805–10 [not associated with the present study].

Brei C[1], Much D[1], Heimberg E, Schulte V, Brunner S, Stecher L, Vollhardt C, Bauer JS, Amann-Gassner U, Hauner H. 2015. Sonographic assessment of abdominal fat distribution during the first year of infancy. *Pediatr Res* 78 (3): 342–50.

Brei C, Stecher L, Much D, Karla MT, Amann-Gassner U, Shen J, Ganter C, Karampinos DC, Brunner S, Hauner H. 2016. Reduction of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring body composition: Follow-up results from a randomized controlled trial up to 5 y of age. *Am J Clin Nutr* 103(6): 1472–81.

Meyer DM, **Brei C**, Stecher L, Much D, Brunner S, Hauner H. 2016. The relationship between breast milk leptin and adiponectin and child body composition from 3 to 5 years: a follow-up study. *Ped Obes* [epub ahead of print].

Meyer DM[1], **Brei C[1]**, Stecher L, Much D, Brunner S, Hauner H. 2017. Cord blood and child plasma adiponectin levels in relation to childhood obesity risk and fat distribution up to 5 y. *Pediatr Res* [epub ahead of print].

Visscher TL, Lakerveld J, Olsen N, Küpers L, Ramalho S, Keaver L, **Brei C**, Bjune JI, Ezquerro S, Yumuk V. 2017. Perceived Health Status: Is Obesity Perceived as a Risk Factor and Disease? *Obes Facts* 10(1): 52–60 [not associated with the present study].

Brei C, Stecher L, Brunner S, Ensenauer R, Heinen F, Wagner PD, Hermsdörfer J, Hauner H. 2017. Impact of the n-6:n-3 long-chain PUFA ratio during pregnancy and lactation on offspring neurodevelopment: 5-year follow-up of a randomized controlled trial. *Eur J Clin Nutr* [epub ahead of print].

---

[1] The first two authors contributed equally to this work

Review articles, in German

Brei C[1], Amann-Gassner U[1], Langhans W, Wolfram G. 2016. Milch und Milchfrischprodukte. Teil 1: Konsum von Milchfrischprodukten und Adipositas. *Ernährungs Umschau* 63(3): M173–M178 [not associated with the present study].

Brei C[1], Amann-Gassner U[1], Erbersdobler HF, Baerlocher K. 2016. Milch und Milchfrischprodukte. Teil 2: Wachstum bei Kindern und Körperzusammensetzung bei Erwachsenen. *Ernährungs Umschau* 63(5): M297–M301 [not associated with the present study].

Brei C[1], Amann-Gassner U[1], de Zwaan M, Hauner H, Schrezenmeir J. 2016. Milch und Milchfrischprodukte. Teil 3: Konsum von Milchfrischprodukten und Diabetes mellitus. *Ernährungs Umschau* 63(9): M536–M541 [not associated with the present study].

Brei C, Amann-Gassner U, Heseker H, Schrezenmeir J. 2017. Milch und Milchfrischprodukte. Teil 4: Konsum von Milchfrischprodukten und Dyslipoproteinämie. *Ernährungs Umschau* 64(1): M42–M44 [not associated with the present study].

Brei C, Heseker H, Erbersdobler HF. 2017. Milch und Milchfrischprodukte. Teil 5: Konsum von Milchfrischprodukten und Hypertonie. *Ernährungs Umschau*, 64(5): M288–M292 [not associated with the present study].

---

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Congress contributions

<u>Talks</u>

29. Jahrestagung DAG, 03–05.10.2013, Hannover. **Brei C**, Brunner S, Pusch K, Much D, Amann-Gassner U, Hauner H. Impact of nutritional fatty acids during pregnancy and lactation on infant body composition up to 3 years of life – Results of the INFAT study.

22nd European Congress on Obesity (ECO) 06–09.05.2015, Prague. **Brei C**, Brunner S, Pusch K, Much D, Stecher L, Amann-Gassner U, Hauner H. Impact of long-chain polyunsaturated fatty acids during pregnancy and lactation on infant body composition up to 5 years of life – Results of the INFAT study

12th Congress of the International society for the study of fatty acids and lipids (ISSFAL), 05–09.09.2016, Stellenbosch. Brei C, Brunner S, Much D, Stecher L, Amann-Gassner U, Hauner H. Effect of long-chain polyunsaturated fatty acids during pregnancy and lactation on children's body composition: Follow-up results of the INFAT study.

<u>Posters</u>

Herbsttagung der DDG und 30. Jahrestagung der DAG, 21./22.11.2014, Leipzig. Brei C, Brunner S, Pusch K, Much D, Cresswell L, Amann-Gassner U, Hauner H. Impact of long-chain polyunsaturated fatty acids during pregnancy and lactation on infant body composition up to 4 years of life – Results of the INFAT study.

The 8th International DIP Symposium Diabetes, Hypertension, Metabolic Syndrome & Pregnancy 15–18.04.2015, Berlin. **Brei C**, Brunner S, Pusch K, Much D, Stecher L, Amann-Gassner U, Hauner H. Impact of long-chain polyunsaturated fatty acids during pregnancy and lactation on infant body composition up to 5 years of life – Results of the INFAT study.

12th European Nutrition Conference FENS, 20–23.10.2015, Berlin. **Brei C**, Brunner S, Pusch K, Much D, Stecher L, Amann-Gassner U, Hauner H. Long-chain polyunsaturated fatty acids during pregnancy/lactation and children's body composition: 5-year follow-up data (INFAT study).

Liesel Beckmann Symposium, 27.11.2015, Munich. **Brei C**, Brunner S, Pusch K, Much D, Stecher L, Amann-Gassner U, Hauner H. Long-chain polyunsaturated fatty acids during pregnancy/lactation and children's body composition: 5-year follow-up data (INFAT study).

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