Original Paper

Annals of Nutrition & Metabolism

Ann Nutr Metab 2009;54:97–103
DOI: 10.1159/000209267

Received: September 22, 2008
Accepted after revision: December 23, 2008
Published online: March 19, 2009

# The Impact of Nutritional Fatty Acids during Pregnancy and Lactation on Early Human Adipose Tissue Development

## Rationale and Design of the INFAT Study

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**Key Words**
Body fat · Childhood obesity · Dietary intervention · n–3 fatty acids · Pregnancy

**Abstract**
Recent observational studies suggest that mean birth weight and body fat growth in the first year of life have increased continuously over the last decades. Both elevated birth weight and early fat mass are potential risk factors for childhood obesity. Experimental and limited clinical data suggest that the dietary ratio of n–6 to n–3 fatty acids (FAs) during pregnancy is critical for early adipose tissue growth. The aim of this randomized controlled study is to examine the effect of the supplementation with n–3 long-chain polyunsaturated FAs and reduction in the n–6/n–3 ratio in the diet of pregnant women/breast-feeding mothers on adipose tissue growth in their newborns using various methods for the assessment of body fat mass. Measurement of skinfold thickness in the newborn is the primary outcome parameter. Two hundred and four pregnant women will be recruited before the 15th week of gestation and randomly assigned to either active intervention or an isocaloric control diet. This upcoming study will explore the potential of this dietary approach to limit early adipose tissue growth and may contribute to the development of a new strategy for the primary prevention of childhood obesity.


### Introduction and Rationale of the Study

Obesity has become a global epidemic and reports from many countries indicate a further increase in the years to come [1]. In a recent population-based survey, 6.3% of the German children and adolescents aged between 3 and 17 years were obese, defined by a body mass index (BMI) ≥97th percentile, and in total 15% were overweight, defined as BMI >90th percentile (results of the German Health Interview and Examination Survey for Children and Adolescents) [2]. As obesity is a powerful risk factor for a variety of diseases, this development represents a tremendous challenge to the healthcare systems worldwide. Such complications are particularly

The study protocol is registered at the ClinicalTrials.gov Protocol Registration System (NCT00362089).

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serious if obesity starts early in life and warrants long-term exposure of organs to excess body fat [3]. For example, a recent study showed that childhood obesity increases the risk of developing coronary heart disease in adult life [4]. This has led to numerous political initiatives to develop strategies to prevent obesity particularly in children and adolescents.

Despite many efforts to prevent childhood obesity using various strategies, the results are disappointing so far. There is little evidence from a number of intervention studies that concepts which combine education, dietary counseling and promotion of physical activity are effective under real-life conditions [5]. In addition, weight reduction programs for obese children and adolescents are only moderately effective in the short term, but fail to induce long-term weight control [3].

Recent data indicate that weight gain of the mother during pregnancy as well as high birth weight are predictors for the development of early childhood obesity [6, 7]. Several pieces of evidence suggest that the fetal environment can have a programing effect and may contribute to the risk of developing obesity not only during childhood but even during the whole lifetime [8, 9]. This hypothesis is best supported by animal experiments and there is still weak evidence that the same holds true for humans. However, there is at least one condition that supports this concept in humans. Babies delivered by mothers with gestational diabetes mellitus not only are at risk of having an elevated birth weight, but also show a much greater weight gain and risk of obesity in the first years of life [10, 11]. These observations suggest that the intrauterine hyperglycemic milieu has long-term effects on the obesity risk. In another study in non-diabetic pregnant women it turned out that offspring with high birth weight as a marker of fetal overnutrition are at increased risk for childhood obesity [6].

Although our current understanding of the multiple factors and mechanisms which contribute to early childhood obesity is rather limited, there is some evidence that the composition of fatty acids (FAs) in the diet during pregnancy may play a role in determining the risk of the offspring of becoming overweight. This observation is mainly based on animal experiments [12]. Wild-type mother mice have been fed before mating and during the gestation/suckling period with either a high-fat diet rich in linoleic acid (LA) or the same isocaloric diet enriched in LA and α-linolenic acid (LNA). The corresponding ratios of n–6 polyunsaturated FAs (PUFAs) versus n–3 PUFAs were 59/1 and 2/1, respectively. Body weight and fat mass of the pups at 8 weeks of age were found to be significantly higher with the LA diet than with the LA/LNA diet. This difference in body weight persisted until the pups reached adulthood [13]. This and other animal data suggest a role of the n–6/n–3 ratio for early adipose tissue growth [12].

Corresponding research looked at the molecular mechanisms controlling adipocyte differentiation. Studies over the last 20 years demonstrated that FAs are acting on adipogenesis via activation of the peroxisome proliferator-activated receptor-γ, which is the master regulator of fat cell formation [14], thereby providing a molecular link between FA uptake and fat cell development [15]. Of particular importance for adipogenesis is arachidonic acid (AA), a naturally abundant FA of the n–6 series. AA is converted into prostacyclin, which finally stimulates adipose differentiation of primary preadipocytes in rodents and humans [16]. In contrast, eicosapentaenoic acid (EPA) and to a lesser extent docosahexaenoic acid (DHA) were found to inhibit the stimulatory effect of AA on cAMP production, which is the central pathway mediating the adipogenic action of AA [13]. This and other findings led to the hypothesis that a high intake of PUFA of the n–6 series is a potent promoter of both adipogenesis in vitro and adipose tissue development in vivo during the gestation/lactation period [17].

The formation of adipocytes from specific precursor cells is a key step in the expansion of adipose tissue and can be found already between the 14th and 16th week of prenatal life in human fetuses. Thereafter, fat lobules develop and are detectable in the main fat depots [18]. After birth, during the first year of life, body fat accumulates substantially due to fat cell hypertrophy [19]. Other studies in newborns revealed elevated proliferation of adipocyte precursor cells in adipose tissue samples in the first year of life [20, 21]. It is plausible from these and other data that early life is a highly sensitive period for adipose tissue growth.

Ailhaud and Guesnet [17] collected other more indirect evidence from epidemiological studies in humans for a role of PUFAs in human adipose tissue development. One interesting observation in this context is that the content of n–6 PUFAs in breast milk of US women increased steadily from 6–7 to 15–16% of total FAs between 1945 and 1995, whereas the content of LNA has remained unchanged at approximately 1%. Thus, the LA/LNA ratio has progressively increased in this time period. Similar changes, although less pronounced, have been observed in Europe, with an increase in the LA content in breast milk [22]. In Germany, the current ratio of n–6 to n–3 PUFAs in the diet is 7–8:1, clearly above recommended

levels due to changing eating patterns towards a diet rich in meat and meat products over the last decades [23]. In young German women, the current AA intake is in the range of 100–150 mg/day, the intake of long-chain (LC) n–3 PUFAs, mainly DHA and EPA, is on average 200–300 mg/day [23].

Despite growing evidence from in vitro and animal data indicating that the ratio of EPA and DHA versus AA during the gestation/suckling period may lead to changes in the pattern of adipose tissue development, data on this topic in humans are scarce although supplementation with LC-PUFAs has become popular due to reports of a beneficial effect of these FAs on neural development [24]. In a study in term infants, feeding an LC-PUFA-supplemented formula had no effect on growth [25], whereas premature infants fed an LC-PUFA-supplemented formula showed a reduction in fat mass and an increase in lean body mass [26]. In a study of children whose mothers received DHA during lactation, the BMI of their children was increased at 30 months [27]. In contrast, in a recent retrospective analysis of a small study, DHA intake by pregnant and lactating mothers was associated with a reduced BMI at 21 months [28].

Thus, at present, it remains to be determined whether supplementation of n–3 LC-PUFAs during pregnancy, i.e. a decrease in the ratio of n–6 versus n–3 FAs, affects early adipose tissue development in humans. Therefore, it appears attractive to examine the hypothesis introduced by Ailhaud and Guesnet [17] that a reduced n–6/n–3 FA ratio may help to limit adipose tissue growth and may thereby represent a novel strategy for the primary prevention of childhood obesity. The following study design will address and test the above-mentioned hypothesis to be applied in forthcoming human intervention studies.

## Study Design

The study is designed as a prospective, randomized, controlled dietary intervention study in pregnant and lactating women and their newborns.

*Hypothesis*

A decrease in the ratio of n–6 versus n–3 FA intake in pregnant and lactating women by supplementing n–3 LC-PUFAs and normalizing the AA intake is associated with less expansive adipose tissue growth in newborns and may be a useful strategy for the primary prevention of childhood obesity.

*Aims of the Study*

The aim of this study is to examine this hypothesis in a prospective human intervention study in pregnant women. The effect of an isocaloric diet supplemented with n–3 LC-PUFAs and a low ratio of n–6/n–3 FAs is compared with the effect of a usual diet, with early adipose tissue growth as primary endpoint. This study also includes secondary objectives to better define possible risk factors for weight gain during early infancy and to obtain new data on the underlying mechanisms.

*Primary Objective.* The primary outcome parameter is adipose tissue mass in the newborn assessed by skinfold thickness. To fully analyze the time course of adipose tissue development, skinfold measurements are planned at the following time points: 3–5 days after birth, and 6 weeks, and 4 and 12 months post partum (PP). In a subgroup of infants, the different fat mass types (subcutaneous vs. visceral) are additionally measured by ultrasonography at the same time points except for 3–5 days after birth and by magnetic resonance imaging (MRI) at 6 weeks and 4 months PP.

*Secondary Objective.* Secondary outcome parameters include: birth weight and height; head and upper arm circumference, and the development of body weight, body length and head and upper arm circumference 3–5 days, 6 weeks, and 4 and 12 months after birth. The following parameters are determined at the time of randomization (15th week of gestation), 32 weeks of gestation, delivery, and 6 weeks and 4 months PP: blood lipid concentrations (triglycerides, total cholesterol, and high- and low-density lipoproteins) in pregnant and lactating women; FA pattern in erythrocytes and plasma in maternal blood as well as in umbilical cord blood samples, and adipokines in the maternal plasma as well as in umbilical cord plasma samples.

*Study Criteria and Follow-Up*

*Inclusion Criteria.* Patients meeting the following inclusion criteria are included in the study: gestational age <15th weeks of gestation; age between 18 and 43 years; BMI at conception between 18 and 30 kg/m$^2$; sufficient German language skills, and written informed consent. Inclusion criteria for follow-up of the newborns are gestational age at birth between 37th and 42nd weeks; appropriate size for gestational age, and an APGAR score >7 at 5 min PP.

*Exclusion Criteria.* Exclusion criteria are a high-risk pregnancy (multiple pregnancy, hepatitis B or C infection, or parity >4); hypertension; chronic diseases such as diabetes or gastrointestinal disorders; psychiatric diseas-

es; supplementation with n–3 FAs before randomization; alcohol abuse; hyperemesis gravidarum, and smoking. Exclusion criteria for follow-up of the newborns are severe malformations or diseases; chromosomal anomaly, and inborn metabolic diseases.

*Patient Recruitment.* Gynecologists in private practices as well as in outpatient clinics in the catchment area of Munich are contacted and invited to refer pregnant women before the 14th week of gestation to the study center (University Hospital Klinikum rechts der Isar). In addition, the study is advertised in local newspapers and on baby-specific internet pages as well as in a German monthly journal (Baby & Familie), which is freely offered in pharmacies to pregnant women and young families to give specific advice on topics related to pregnancy and baby care. The first screening is usually done by telephone or personally using a structured questionnaire.

*Screening.* The screening examination includes a detailed history. Current body height and weight are retrieved from the 'Mutterpass' (mother's passport) together with the blood pressure and the para/gravida status. An initial laboratory investigation, including hemoglobin, hematocrit, thrombocyte and leukocyte counts, concentrations of blood glucose, triglycerides, total cholesterol, and high- and low-density lipoproteins, and coagulation parameters (Quick, international normalized ratio and partial thromboplastin time), is performed. Average daily dietary intake of energy, protein, carbohydrates, lipids and AA is recorded on a 7-day dietary questionnaire after detailed instruction of the women. If a woman fulfils all criteria and provides written informed consent, randomization is performed using a random envelope prepared by the Institute of Medical Statistics and Epidemiology of the Technical University of Munich. Randomization is conducted by varying block lengths to ensure balanced group sample sizes about the whole accrual period using SAS software (version 9.1).

*Study Groups*

*Intervention Group.* The intervention protocol combines two components: (1) to increase n–3 PUFA intake by asking the participants to take 3 capsules of the n–3 PUFA preparation Marinol D-40™ produced by Lipid Nutrition (Loders Croklaan, Wormerveer, The Netherlands), containing 180 mg EPA and 1,020 mg DHA as well as 9 mg vitamin E as antioxidant, and (2) to reduce AA intake to the recommended range of 50–90 mg per day. For this purpose, the women in the intervention group are advised to keep a healthy balanced diet with a reduction in the consumption of AA-rich foods, particularly meat, meat products and eggs. The latter is achieved by advising the women to limit their meat intake to 500 g (2–3 portions) per week. Individual dietary counseling is based on the 7-day dietary record. A previous intervention study in patients with rheumatoid arthritis provides information on the AA content of common foods [29]. To support the intake of fish oil capsules and to maintain the compliance for the low-AA diet, the women are asked to keep capsule intake records and they are additionally called every 4–8 weeks by a member of the study team.

*Control Group.* Participants of the control group receive a brief semistructured counseling on a healthy diet according to the guidelines of the German Nutrition Society for a healthy balanced diet.

Participants of both groups are also offered individual nutrition counseling based on the 7-day dietary record.

*Visits*

Figure 1 shows the procedures during the course of the study including all visits and information on the samples that are taken under standardized conditions from both the mothers and their newborns.

*Visit 1* represents the screening examination.

*Visit 2* (14th–16th week of gestation) includes the baseline collection of blood samples from the pregnant women. A first 7-day dietary questionnaire is filled in. This assessment also includes information on dietary supplements taken by the participants. Treatment is started as described for both treatment arms.

*Visit 3* (week 32 of gestation): blood is collected and the women of the intervention group are advised once again to maintain a healthy, balanced diet and to reduce their AA intake to the recommended range. Furthermore, the women are asked to fill in a second 7-day dietary record.

*At birth*, gestational age, birth weight and height, placental weight, mode of delivery, APGAR score and other data are recorded in a standardized manner. Blood from the mother and the umbilical cord blood, a piece of the umbilical cord and defined placenta samples are collected and immediately frozen at –80°C.

*Visit S1* (3–5 days after delivery): skinfold thickness is measured at four defined sites (triceps, biceps, suprailiac and subscapular) using a Holtain T/W Skinfold Caliper with defined pressure according to Schmelzle and Fusch [30]. Additionally, the upper arm circumference of the infant is measured.

*Visit S2* (6 weeks after birth): blood and breast milk samples are collected from the mothers. A third 7-day dietary record is requested. In the newborn, skinfold

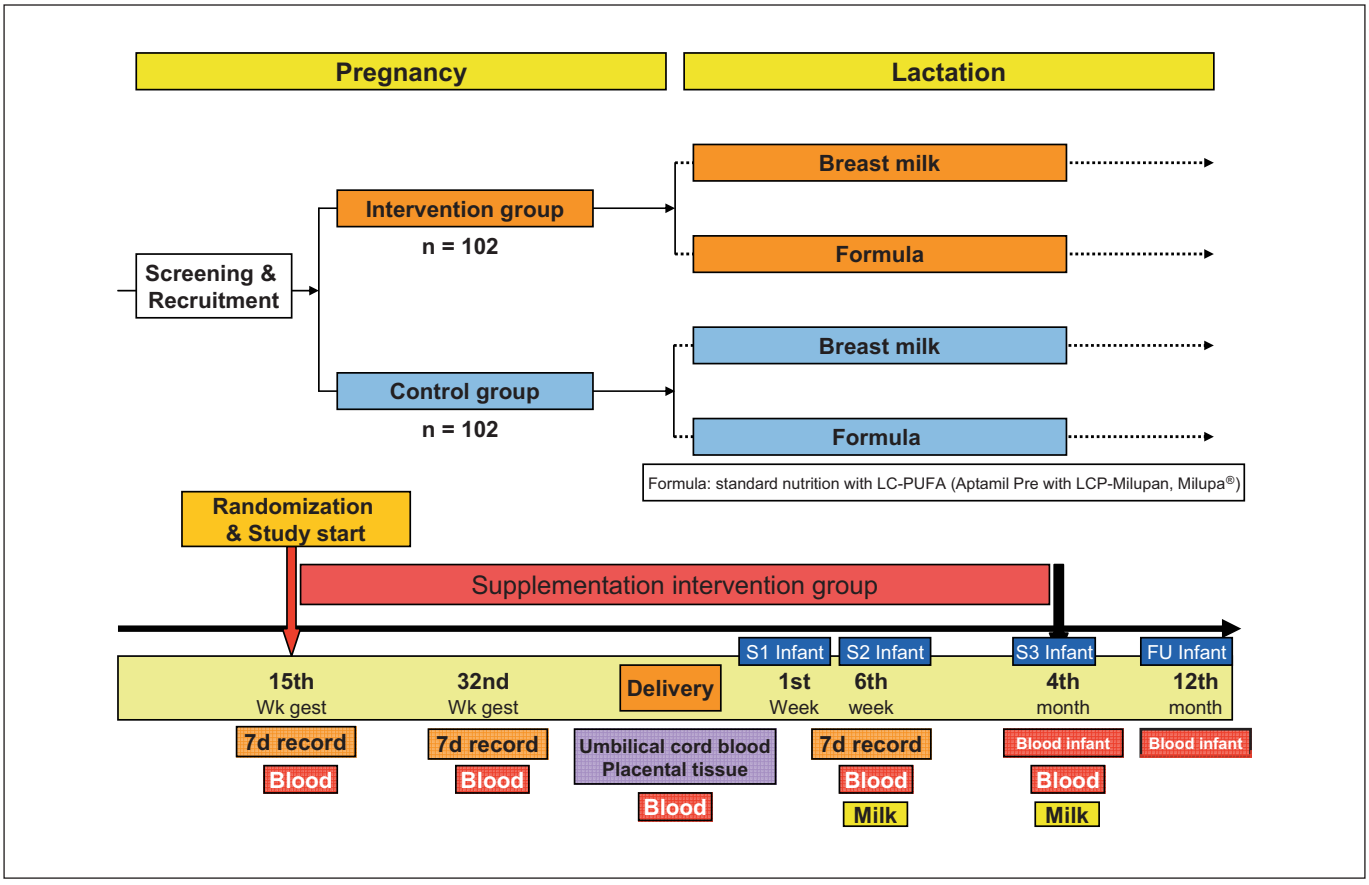


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**Fig. 1.** Study design. 7d = 7-day; FU = follow-up; Wk gest = weeks of gestation.

thickness is measured and body fat is assessed by ultrasound. Furthermore, body height and weight, and head and upper arm circumferences are measured. In a subsample of the newborns and depending on the informed consent given by the mother and/or father, whole body composition is assessed by MRI, giving the unique opportunity to monitor the effect of the intervention on the visceral fat depots.

*Visit S3* (4 months after birth): the same anthropometric measurements are done as described for visit S2. In addition, in a subgroup of infants, 1- to 4-ml blood samples are collected depending on the consent of the mother.

*Visit S4* (12 months after birth): except for MRI, the same measurements described for visit S3 are performed.

Beyond the first year of life, follow-up examinations are planned (18, 24, 36, 48 and 60 months PP) for the assessment of defined anthropometric parameters.

*Tests*

*Assessment of Skinfold Thickness and Subcutaneous and Visceral Fat by Ultrasound.* The biceps, triceps, suprailiac and subscapular skinfold thickness is measured using a Holtain T/W Skinfold Caliper with defined pressure according to a previously described method [30]. Ultrasonography is used to assess the subcutaneous fat mass and visceral adipose tissue separately, as the latter is considered to be an important predictor of metabolic disturbances [31]. Therefore, a 3.5-mHz ultrasound probe is used by a trained investigator. The measurement allows the assessment of subcutaneous and visceral fat according to an established technique [32].

*Analysis of FA Composition by Gas Chromatography.* The FA pattern in the plasma and erythrocytes of the fetus and newborn are closely correlated with the FA composition in the plasma and erythrocytes of their mothers. FAs are transported via the placenta to the fetus, or later via breast milk to the newborn [33]. Thus, the FA compo-

sition of the newborn can be modified by the diet of the mother [34]. Firstly, to monitor the compliance with the intervention program and, secondly, for a precise statistical analysis, repeated FA analysis is performed from the blood samples of both the mother and the newborn. The FA pattern of maternal and fetal plasma phospholipids and erythrocytes is assessed by capillary gas chromatography according to published methods [35, 36].

*Safety Issues*

Supplementation with marine n–3 PUFAs in pregnant women has been done in a number of intervention studies. The daily intake of n–3 PUFAs was between 200 mg and 4 g per day. In all these clinical studies, supplementation with n–3 LC-PUFAs was safe, with no evidence of adverse effects on fetal development. However, a modest prolongation of gestation was observed [28, 37, 38].

Although AA is an important nutrient for the fetus and newborn, studies using formula diets with different n–3/n–6 FA ratios do not indicate that a 50- to 100-mg reduction in the intake is associated with any disturbance in fetal development and later growth characteristics [24]. In addition, an observational study in pregnant women on a vegetarian diet did not indicate that a low intake of AA (40 mg/day) is critical, possibly due to increased endogenous LA synthesis [39].

*Data Management*

*Documentation of Adverse Events.* All adverse events reported by the participating women and regularly assessed during the visits are carefully documented as requested in the CRF.

*Data and Quality Management.* Data management is performed considering current data protection laws and using professional support by the Center for Clinical Studies of the Faculty of Medicine. A data source verification procedure will be established.

*Calculation of Sample Size.* The sample size is calculated based on skinfold measurements. It is assumed that there will be a difference of at least 5 mm in the sum of the four defined skinfolds (subscapular, suprailiac, triceps and biceps) between the intervention and control groups at 4 months PP. According to recent publications, a mean sum of the four skinfolds of 30 $\pm$ 5 mm is expected [30, 40]. To be able to detect a clinically significant difference of 5 mm between the groups at $\alpha = 0.05$ and a power of 80%, and assuming a dropout rate of 30% over the study period, a total of 102 women in each group is required. Thus, a total of 204 women will be recruited for the study. The calculation is based on the nQuery (version 5) software program.

## Conclusion

In conclusion, this study design will form the basis for the first human intervention trial on the effect of a dietary change in the n–6/n–3 FA ratio in pregnant women on adipose tissue growth of their newborns as primary outcome variable. A careful long-term follow-up of the infants is planned to get additional insight into the interaction between potential early programming and environmental influences during early childhood. Thus, the results of this study will provide valuable information on the potential of a nutritional intervention on perinatal programming towards primary prevention of childhood obesity.


## Acknowledgments

This study is funded by grants from the Else Kröner-Fresenius Foundation, Bad Homburg, the International Unilever Foundation, Hamburg, the EU-funded EARNEST consortium (subcontractor of Numico, Frankfurt) and the German Ministry of Education and Research via the Competence Network Adiposity (Kompetenznetz Adipositas).


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