PROGRAMMING OF ADULT METABOLIC HEALTH BY DIETARY LIPIDS IN EARLY LIFE

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Keep your head up
Keep your heart strong
Keep your mind set in your ways

- Ben Howard –

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Chapter 1

General introduction

Obesity, a global threat

Global prevalence of obesity has almost doubled from 1980 to 2008 with rates accelerating in the last decade [1]. The World Health Organization (WHO) estimated in 2008 that 900 million adults were overweight and another 500 million were obese [2]. Although obesity numbers and trends vary among countries, obesity is not limited to high income or to industrialized countries [1]. Predictions indicate that if the current trend persists, 57% of the world's population will be overweight (BMI 25-29.9) or obese (BMI > 30) in 2030 [3]. The obesity epidemic poses a global economic and health burden due to high medical costs, loss of productivity and loss of health-related quality of life [4]. Obesity increases morbidity and mortality from non-communicable diseases (NCD) worldwide, including diabetes, metabolic syndrome, cardiovascular disease and certain cancers [5]. Approximately 2.8 million die due to obesity and its co-morbidities each year, ranking it the number five cause of death worldwide [2]. Annual per capita medical costs are on average 30-40% higher for obese than for normal weight individuals[6]. The high prevalence in the USA resulted in medical costs equivalent to 1.3% of the Gross Domestic Product in 2000 [7] and accounted for 4-7% of the total health care costs, i.e. $75 billion, in 2003 [8]. Prognoses suggest that medical costs will rise with $22-28 billion/y by 2020 and $48-66 billion/y by 2030 [4].

Obesity increasingly emerges at an early age as childhood obesity prevalence is rising fast [9]. In 2010, 43 million children under the age of 5 were overweight or obese and numbers are expected to increase to 60 million by the year 2020 [10], which is particularly alarming because early onset obesity is associated with adult obesity risk [11] and with early onset and severe metabolic disease [12]. Weight management programs with high intensity lifestyle interventions, including education, diet and exercise, show only moderate and short term effectiveness and regular relapses at follow-up [13-16]. Therefore, prevention is of the upmost importance to prevent further increase of obesity and NCD incidence.

Excess intake of energy-dense foods and sedentary lifestyle are considered the two most important contributors to the energy imbalance underlying obesity. Public health strategies target these two factors in order to prevent and treat obesity [17, 18]. However, neither contemporary lifestyle factors nor established genetic factors can fully explain the rapid increase in (childhood) obesity over the past few decades [18, 19]. Other factors have been suggested to contribute

including for instance pharmaceutical agents, endocrine disruptors, reduced sleep duration and changes in early life (nutritional) environment [20]. Although less extensively investigated, these factors could have a considerable impact on obesity incidence. Insights in these alternative mechanisms may help to design more effective public health policies and intervention programs.

Nutritional programming of metabolic health

The Developmental Origins of Adult Health and Disease (DOHaD) concept was originally put forward by David Barker and colleagues as the "fetal origins of adult health" hypothesis. Their notion was based on the observation that low birth weight, suggested as a proxy for impaired fetal growth, was associated with increased mortality due to ischemic heart disease at middle age [21]. From this initial finding, the hypothesis originated that early environmental factors, in particular nutrition, "programs" health and disease risk later in life. Since then, many epidemiological and experimental studies confirmed that nutritional disturbances during critical periods of early life development predispose to obesity and metabolic disease later in life [22-29]. Retrospective birth cohort studies provide evidence for detrimental effects of maternal undernutrition during gestation on health of the adult offspring. Historical epidemiological data from the 1944-45 Dutch Famine birth cohort showed that famine during gestation was associated with impaired glucose tolerance, dyslipidemia and an increased risk for coronary artery disease [30]. In rural China, offspring of women pregnant during the famine between 1959 and 1961 were at higher risk of hyperglycemia in adulthood compared to non-exposed offspring. This effect was aggravated by subsequent exposure to a western dietary pattern [31]. Although gestational undernutrition is not necessarily reflected by birth weight [32], both low and high birth weight have been associated with metabolic disturbances in adult life [26, 28, 29, 33]. Taken together, these observations underline the importance of a sufficient supply of nutrients in early life. The epidemiological findings in humans have been supported by comparable outcomes after environmental manipulations in early life in experimental animal models [27, 34, 35].

These early findings on fetal programming led to the "thrifty phenotype" hypothesis: A poor nutritional intrauterine environment causes the fetus to reduce growth to secure immediate survival at the expense of developing

tissues, organs and neuroendocrine systems less critical for acute survival. According to the hypothesis, this results in adult maladaptation and thus an increased risk for chronic (non-communicable) disease later in life [36]. The fetal environment generated a phenotype adapted to function in a poor nutritional environment. A (relative) nutrition-rich postnatal environment may cause an accelerated ("catch-up") growth, that by itself or in combination with the initially nutritionally poor environment, aggravates long-term negative effects on metabolic health [37].

As research in the field advanced, others considered the "thrifty phenotype" hypothesis too limited to fully cover the DOHaD concept and proposed the "predictive adaptive response" (PAR) hypothesis [38]. Rather than fetal adaptations as a strategy to counter an acute threatening nutritional insult with an adult health trade-off, the PAR hypothesis concerns (normal)adaptations to a range of nutritional signals in anticipation of the future (nutritional) environment. An individual with a certain genotype can generate a variety of different phenotypes depending on environmental cues during critical periods of development, i.e. periods of developmental plasticity. PARs can only be induced during these critical periods of development [38]. In turn, timing of these critical periods depends on the organ systems involved. For example, the capacity of human adipocyte precursors to proliferate and differentiate is highest directly after birth and in pre-adolescence, representing two distinctive sensitive periods for this specific tissue [39]. Critical periods are not accurately defined in most species, but changes in phenotype are considered irreversible when the period of developmental plasticity ends [40]. If the developmental PARs are appropriate, theoretically, the individual has the (metabolic) flexibility to respond to environmental challenges throughout life, resulting in a low disease risk. In contrast, inappropriate PARs result in a high disease risk because the individual is incapable of generating an adequate physiological response required by (elements of) the adult environment [38]. Thus, the match or mismatch between the phenotype after developmental plasticity and the environment determines disease risk (Figure 1). According to the PAR hypothesis, appropriate early (preventive) interventions will be far more effective than therapeutic strategies aimed at the moment of manifestation of the disease. The suggested preventive benefits according to the hypothesis have collected wide spread attention, including that of policymakers. Awareness of a "so-called" window of opportunity is increasing and even the United Nations take action to prevent

non-communicable disease by improving nutrition during the first 1000 days of life, from conception to the age of 2 years [41].

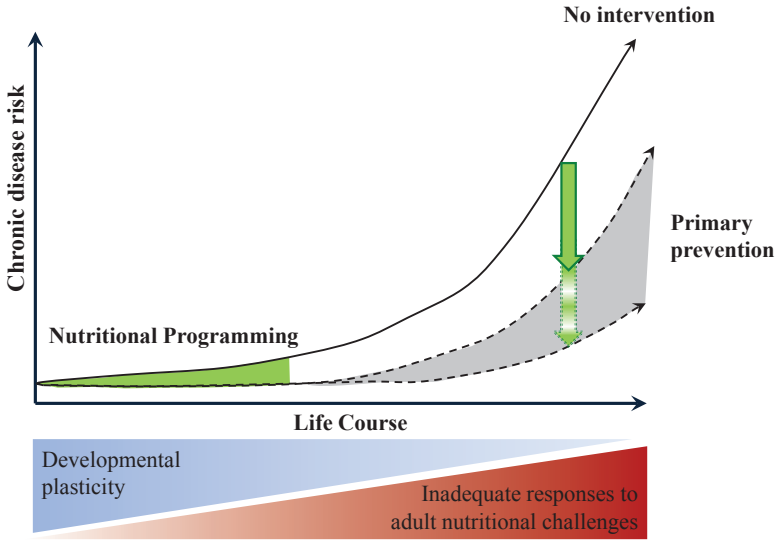

FIGURE 1. Manifestation of non-communicable disease occurs later in life, but originates from early development trajectories. Increased disease risk results from reduced plasticity preventing adjustment to the environment and subsequent accumulating inadequate responses to environmental challenges. Prevention in early life is therefore more likely to be effective than treatment of disease. Adapted from Godfrey *et al* (2010) [42].

The precise mechanism underlying nutritional programming remains unclear, although several mechanisms have been proposed. These mechanisms include irreversible changes in organ and tissue structure, epigenetic regulation of gene expression, altered set-point of homeostatic neuroendocrine systems and changes in mitochondrial function [43]. Adult phenotype and underlying mechanisms can vary depending on the severity of the early life nutritional insult (e.g. mild versus severe energy restriction) and the type of nutrients (e.g. low energy versus low protein). Additionally, duration (e.g. 1st trimester versus entire pregnancy) of the nutritional signal, timing (e.g. pregnancy versus lactation) of the nutritional signal and the organ systems implicated also play a key role (Figure 2). Epidemiological data from the Dutch Famine cohort provide a clear example of the importance of timing on adult phenotype: Maternal undernutrition during early gestation, a period in which vital organs are formed and placentation occurs, did not affect birth weight, but did increase the incidence of obesity and cardio-metabolic disease in the adult offspring. Maternal undernutrition during mid-gestation, a period of organ maturation

and tissue remodeling, was associated with micro-albuminuria, renal and obstructive airway disease. Maternal undernutrition during late gestation, a period of fetal hyperthrophic growth, was associated with low birth weight, crown-to-heel length and head circumference and with an impaired glucose tolerance [30, 32].

Heijmans and colleagues [44] showed that at least part of these detrimental programming effects were induced through epigenetic mechanisms. DNA methylation of 7 loci implicated in growth and metabolic disease were associated with fetal undernutrition during the famine. Differences in methylation between exposed individuals and their non-exposed siblings were gender-specific and depended on the timing of exposure [45]. In addition, the famine may have altered organ structures permanently, such as nephron number and alveolar tissue of individuals exposed to the famine at mid gestation [46,47]. Invasive techniques such as biopsies to obtain indisputable proof of changes in organ structures as underlying mechanism could not be included since individuals from the birth cohort were healthy volunteers. However, other human and animal studies did show sustained effects of prenatal undernutrition on kidney [48] and lung[48] structure and function. Data from animal studies were in accordance with epidemiological findings of nutritional programming from cohort studies, contributed to a better understanding of underlying mechanisms and provided insights in differential effects of different nutritional signals [35, 40, 49, 50]. For instance, a rat study by Dumortier and colleagues demonstrated differential mechanisms of type of nutrient (low energy versus low protein) and duration of the nutritional signal (one week versus three weeks) during gestation on offspring pancreatic β-cell mass. Both diets reduced β-cell mass, but energy restriction increased corticosterone levels which limited differentiation of progenitors to dedicated endocrine β-cells whereas protein restriction reduced β-cell mass through reduced proliferation and islet vascularisation. Interestingly, protein restriction during the last week of gestation was more detrimental than exposure throughout gestation, whereas the opposite was true for energy restriction [49].

Apart from nutrient quantity, e.g. energy restriction or low protein, also nutrient quality can have programming effects on later life health. For instance, iron deficiency during late pregnancy impairs the auditory system after birth and iron deficiency in late infancy impairs cognitive function later in childhood[51]. In addition, high folic acid and low vitamin B12 status in pregnant Indian women was associated with insulin resistance in their children at 6 years of age.

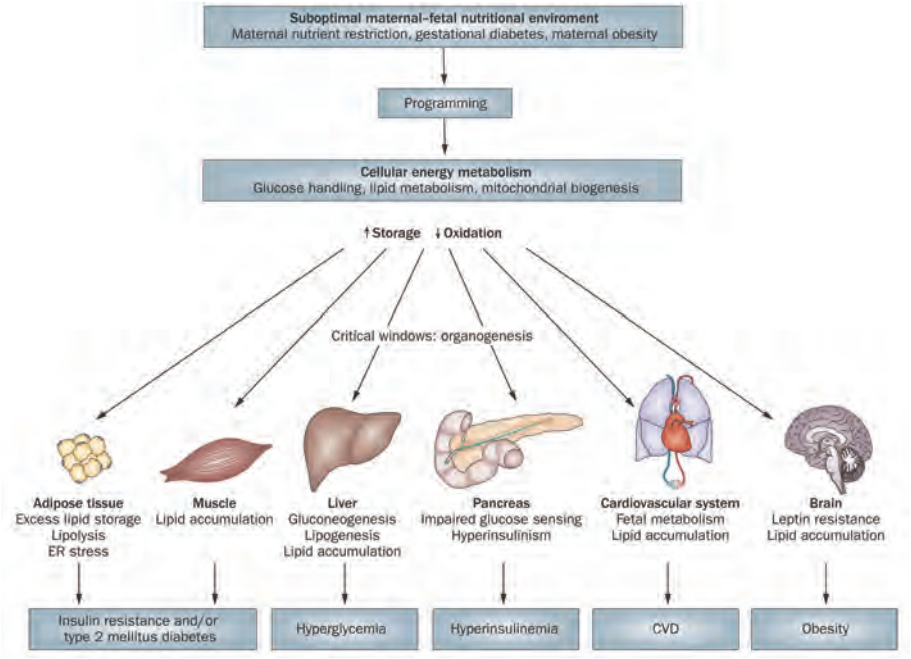

FIGURE 2 Early life nutritional environment contributes to development of cardio-metabolic disease through several organ systems. Reprinted with permission [52].

Early postnatal life: a critical window

Initial DOHaD studies almost exclusively focused on the role of the *fetal* environment on obesity and NCD risk at adult age, but it has become increasingly acknowledged that the window of programming extends into the (early) postnatal period [53-55]. Although the timing of the critical period for individual organs is not accurately established, the development of many (metabolic) organs including gastrointestinal tract [55], brain [56], pancreas [57] and adipose tissue [58] continues for a considerable time after birth. Some interesting findings from birth cohorts and animal studies indicate specific importance of the postnatal period as contributor to disease risk. Women exposed to the Dutch famine between the age of 0 and 9 years showed increased type 2 diabetes and overweight compared to unexposed women [59, 60]. In addition, famine at the age of 10-18 years was associated with a higher risk of coronary heart disease whereas famine at the age of 0 to 9 years and older than 18 years was associated with reduced risk of stroke [48].

Zambrano and colleagues [61] showed that postnatal low protein reduced adult fat mass whereas fetal low protein increased leptin and reduced insulin sensitivity in male rats and increased body fat in female rats compared to adult controls. Moderate energy restriction during lactation protected against enhanced adult fat accumulation in rats, whereas energy restriction during gestation had the opposite effect [62]. These observations illustrate that growth depends on different fuels in fetal and postnatal life, which in turn depends on the timing of development of individual organs (Figure 3) and on their nutritional needs at different stages of development. For example, insulin release from fetal pancreatic β cells is responsive to amino acids, but not to glucose signaling. Shortly after birth these fetal cells become apoptotic and are replaced by postnatal glucose sensitive β cells, preparing for postnatal metabolism [63]. One could hypothesize that fetal protein restriction has a more severe detrimental effect on adult β cells functionality and increases risk on diabetes compared to postnatal protein restriction. As remodeling of the pancreas continues into early childhood [57], it is not unlikely that also factors like carbohydrate intake in this period could affect the postnatal maturation and adult function of the endocrine pancreas.

Observational studies investigating postnatal growth velocity provide evidence that the period after birth can be considered a critical window of plasticity for specific organs independent of the prenatal period. A meta-analysis of individual-level data of more than 47 thousand individuals from 10 cohort studies showed independent positive association of weight gain in the first year of life with childhood obesity [64]. Postnatal growth velocity across all birth weight tertiles predicted abdominal adiposity at 2 years of age in a prospective birth cohort [65]. In accordance, Wells and colleagues [66] showed that enhanced postnatal weight gain rather than birth weight was strongly associated with abdominal fat mass in a pediatric obese population.

White adipose tissue development

Although many organ systems can contribute to the early origins of obesity and NCDs, adipose tissue development might be a key determinant. The excessive expansion of adipose tissue in obesity results in dysregulation of lipid and glucose metabolism and chronic low grade inflammation, all implicated in the

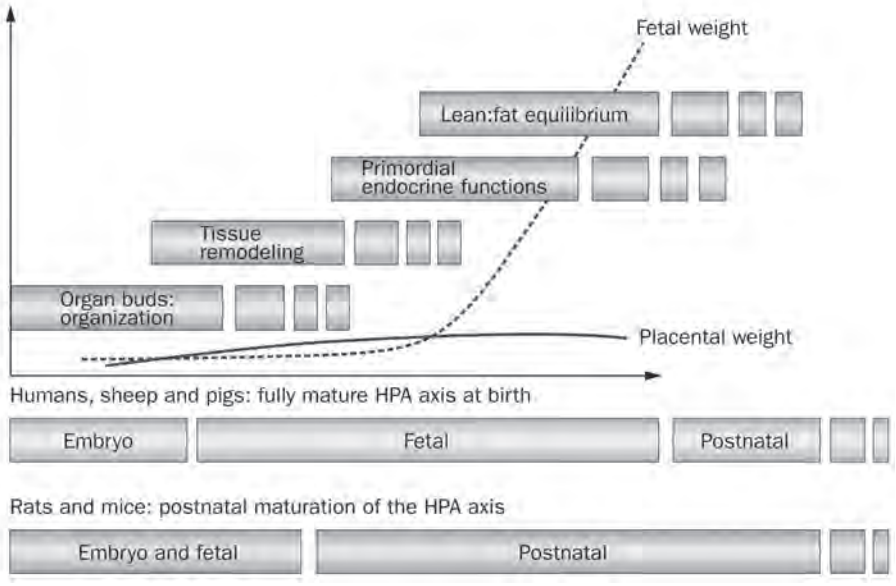

FIGURE 3. Critical windows for fetal and early postnatal organogenesis. HPA axis: Hypothalamus-pituitary-adrenal axis. Reprinted with permission (52).

pathophysiology of NCDs [67]. Early life modulation of adipose tissue development may affect its adult function. Spalding and colleagues [68] showed that adipocyte number is a major determinant of adult fat mass and that obese individuals have a higher number of adipocytes than lean individuals, even after considerable weight loss which reduced adipocyte size but not number. This difference in adipocyte number already emerged early in childhood suggesting that this is a critical period with respect to adipose tissue development with potentially sustained effects on adipose tissue function in adulthood. Indeed, animal studies show that maternal obesity enhances expression of fetal genes regulating lipogenesis, i.e. fatty acid synthesis, and adipogenesis, i.e. differentiation of preadipocytes to mature adipocytes. The net result is enhanced lipid storage in adipocytes (hypertrophy), and increased adipocyte number (hyperplasia), both predisposing to enhanced fat accumulation later in life [69, 70].

In humans, adipose tissue development starts in the third trimester of gestation through proliferation of pre-adipocytes and their subsequent differentiation into mature adipocytes. Mammals have two types of adipose tissue: White adipose tissue (WAT) and brown adipose tissue (BAT). BAT dissipates energy in order to generate heat, i.e. non-shivering thermogenesis, to maintain body

temperature. It is abundant in infants but decreases shortly after birth and was traditionally considered insignificant in adults, both in amount and functionality. However, although human data are scarce, recent findings suggest that brown adipocytes reside in white adipose tissue and skeletal muscle of adults and may play a role in regulation of energy balance throughout life [71-73]. In contrast, WAT stores excess energy in the form of triglycerides and can expand throughout life. White adipocyte number increases throughout childhood and adolescence to plateau and remain relatively constant in adulthood [68, 74]. Fat mass accounts for approximately 15% of the (term) birth weight. Fat deposition accounts for 40-65% of the body weight gain between 0 and 6 months after birth and peak adiposity (up to 26% of body weight) is reached between 6 to 9 months after birth [75].

In contrast to humans, development of WAT in most mammals, including rodents, only starts after birth [76]. Comparable to human ontogeny, WAT development in rodents is initially driven by hyperplastic growth and subsequent hypertrophic growth [77]. Total cell number increases from birth and plateaus between 9-18 weeks of age on *ad libitum* standard rodent diet [78-80], whereas adipocyte size can increase until senescence [81]. Adipocyte size and number are higher in obese compared to lean rats [82] and hyperplastic and hypertrophic growth dynamics differ according to fat depot, gender and diet [83, 84].

Both hypertrophic and hyperplastic adipose tissue growth are regulated by the concerted actions of several transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ), which heterodimerizes upon activation with retinoid X receptor α (RXRα), and the CCAAT/enhancer binding protein (C/EBP) family members [85, 86]. N-6 and n-3 polyunsaturated fatty acids (PUFA) and their eicosanoid metabolites act as endogenous PPAR ligands [87], enabling sensing of nutritional signals and translating these into a metabolic response to maintain homeostasis.

Arachidonic acid (ARA; C20:4 n-6) is a very potent adipogenic factor as precursor of prostacyclin, in contrast to other PUFA. After its release from preadipocytes, prostacyclin binds to its receptor, which activates the protein kinase A pathway through cAMP production, thereby enhancing C/EBPβ and C/EBPδ expression [88,89] (Figure 4). N-3 PUFAs eicosapentaenoic acid (EPA; C20:5 n-3) and to a lesser extent docosahexaenoic acid (DHA; C22:6 n-3) inhibit ARA effects on cAMP production and can thereby counteract pro-adipogenic effects of ARA [88].

Apart from adipogenesis, n-3 and n-6 PUFA have differential effects on

transcription factors involved in WAT lipogenesis with n-3 inhibiting and n-6 PUFA stimulating expression of lipogenic transcription factors [90]. Although PUFA could exert their effects on WAT transcription factors throughout life, early life exposure may enhance their effects due to the high capacity of adipocyte precursors to proliferate and differentiate in this period [39]. Moreover, early PUFA exposure may program lipogenic genes towards enhanced expression, for instance through modulation of DNA-methylation [91].

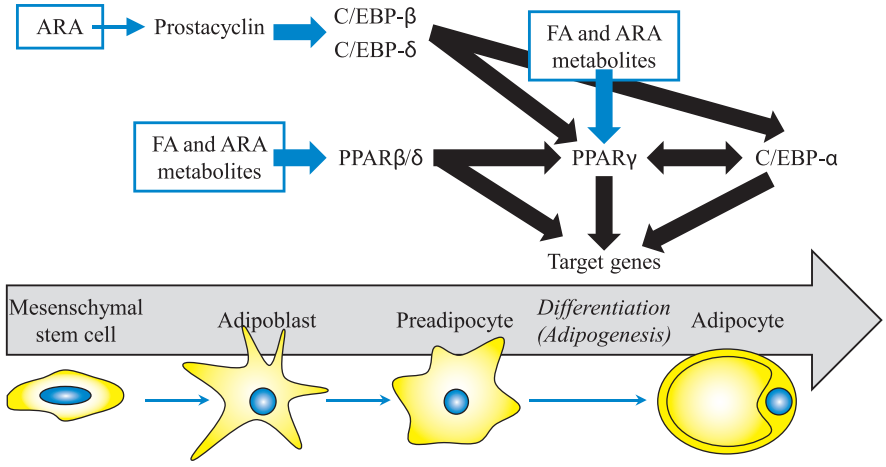

FIGURE 4. Arachidonic is a potent adipogenic factor as it is the precursor for prostacyclin, which upregulates C/EPB-β and δ, which subsequentely upregulate PPARγ to activate genes involved in adipogenesis. Additionally, other PUFAs and other ARA metabolites are ligands of PPARβ/δ and PPARγ and thereby regulators of adipogenesis. Adapted from Massiera *et al* (2006) and Avram (2007) [92, 93].

Protective effects of breastfeeding

Epidemiological studies indicate that breastfeeding is associated with a moderately reduced risk of later life obesity and metabolic disease [94-97]. Specifically longer duration (> 6 months) of breastfeeding is associated with a reduced risk of childhood obesity [98]. It has been hypothesized that the reduced growth velocity of breastfed (BF) infants compared to formula fed (FF) infants might underlie the reduced obesity risk[99]. The PIAMA birth cohort showed that infants who were BF for more than 16 weeks had a lower BMI at 1 year of age, suggesting slower growth, compared to FF infants. BMI at 1 year of age was positively associated with BMI at 7 years of age [100]. A systematic review and meta-analysis by Gale and colleagues showed that differences in weight

gain between BF and FF infants were accompanied by differences in body composition trajectories [99]. BF infants had lower lean body mass throughout infancy compared to FF infants, whereas fat mass was higher before 6 months of age but lower between 6 and 12 months of age [99]. Additionally, breastfeeding for more than 4 months was associated lower visceral adiposity at 2 years of age [101]. Long-term implications of these different growth trajectories remain to be elucidated, but the reduced growth velocity and altered lean and fat mass development are compatible with a better "quality of growth" in BF infants, resulting in less deposition of visceral adipose tissue.

It is presently unknown which specific aspects of infant feeding contribute to BF and FF growth trajectories. Many factors could be implicated, including feeding behaviour (meal frequency and size, on demand or fixed schedule, breast or bottle) as well as nutritional composition (macronutrient composition, energy content, biological active compounds). Human milk (HM) contains several biologically active compounds for optimal growth and development in order to secure health during infancy as well as later in life [102-106] which are not present in infant milk formula (IMF). Dietary lipids, a component differing considerably in HM of and IMF [107-109], but also highly variable in HM due to maternal diet, may be an important determinant for the development of body composition and metabolic homeostasis as illustrated by the effects of n-6 and n-3 PUFAs on adipose tissue.

Dietary lipids during infancy

An important part of the daily energy intake is derived from dietary fat. During the first 4-6 months of life, HM or IMF is the sole source of nutrition for the infant. Both provide 40-50% energy as fat. Dietary lipids provide energy for growth, supply the essential fatty acids (EFA) linoleic acid (LA; C18:2 n-6) and α-linolenic acid (ALA; C18:3 n-3), and ensure adequate absorption of the fat-soluble vitamins required for a healthy growth and development. Between 6 months and 2 years of age, the WHO recommends 30-40% energy from fat, although it is recently suggested that the energy derived from fat should be gradually reduced to maximal 30% to better match energy requirements and reduce weight gain velocity according to the latest reference growth standards [110].

EFA have to be acquired through dietary sources, because mammals lack the

ability to insert double bonds at position 3 and 6 [111]. In order to fulfill their physiological function, the 18-carbon EFA need to be converted to 20 to 22-carbon long chain polyunsaturated fatty acids (LCP) by (delta-5 and delta-6) desaturases and elongases [112] (Figure 5).

LA, 18-carbon precursor of the n-6 series, is metabolized to ARA. ALA, 18-carbon precursor of the n-3 series, is metabolized to EPA and DHA. Because LA and ALA compete for the same set of enzymes, the absolute amount of dietary EFA as well as the n-6/n-3 ratio determines the relative abundance of ARA and EPA derived eicosanoid metabolites [113] and ARA and DHA incorporation in biological membranes [114, 115].

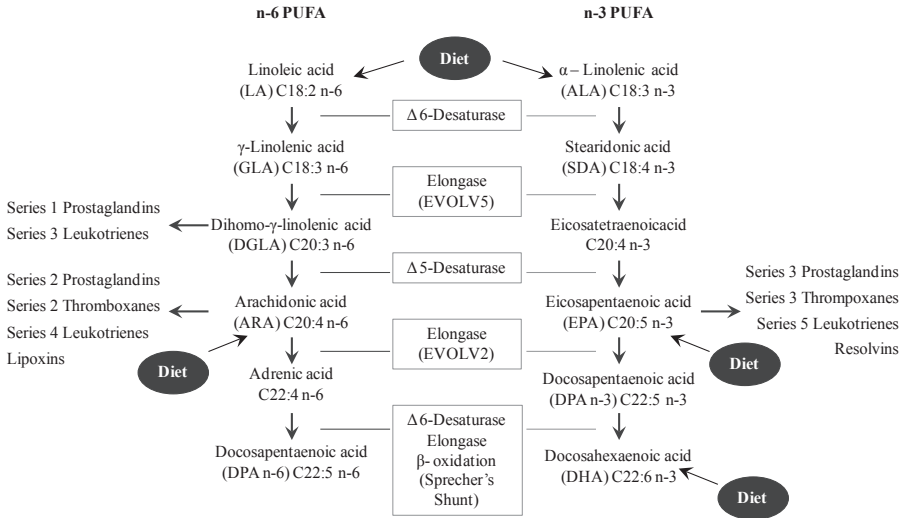

FIGURE 5. Metabolic pathways of n-6 and n-3 series of fatty acids. Adapted from Schmitz and Ecker (2008), Lorente-Cebrian *et al* (2013) and Massiera *et al* (2006) [93, 116, 117]

PUFA, EFA as well as LCP, play an important role in growth and development during the last months of gestation and the first months of postnatal life [118, 119]. LCPs can be considered conditionally essential during this period of rapid growth, because although both term and preterm infants are able to convert EFA in LCP, this synthesis capacity may be too limited to obtain tissue LCP levels as high as found in infants fed preformed DHA and ARA [120-122].

HM lipid content is affected by maternal diet and body composition, stage of lactation (colostrum, transitional or mature milk), interval between feeds during 24h and volume ingested per feed, but even changes during a single

feed (fore- versus hind-milk) [123-125]. The average lipid content of mature milk is 39 g/L, but can vary between 25 and 59 g/L. HM lipid globules are formed in the mammary epithelial cells and secreted through exocytosis in the alveolar lumen resulting in globules with a core consisting of triglycerides and cholesteryl-esters surrounded by a native biological membrane composed mainly of phospholipids, proteins and enzymes, free cholesterol and glycoproteins. The lipid globule size ranges between 1 and 10 µm and increases in the first 4 weeks of lactation with an average mode diameter of 4 µm in mature milk [108, 126]. The amount of cholesterol in HM varies between 0.1 and 0.35 g/L (0.25-0.5 g/100g total lipids) and is correlated to milk lipid content and changing lipid droplet size during different stages of lactation. The same applies for phospholipids, which fluctuate between 0.1 and 0.4 g/L (0.3-1.3 g/100g total lipids) [109, 126-128]. In contrast, IMF lipid globules have a diameter of approximately 0.3-1 µm and consist of a core of triglycerides with milk proteins adhering to the globule surface due to the use of vegetable oils, and homogenization and emulsification during processing [108, 129, 130]. IMF lipid composition is maintained constant and uniform to adhere to (inter)national legislation [131, 132]. Depending on formulation, IMF lipid content ranges between 29-40 g lipid/L. IMF may contain low concentrations lecithin as emulgator, but contains no cholesterol unless dairy lipids are used as ingredient [108, 133].

Smit and colleagues [134] have evaluated recommendations for IMF PUFA of different (inter)national regulatory bodies. IMF LA content should range between 11 and 20 wt% of total fatty acids according to the EU Commission directive, but margins set by others range from 8 and 35 wt%. ALA should range 1 and 4 wt% and the LA/ALA ratio is set between 5 and 15. Addition of LCPs is recommended by most authorities, but limits are set to maximal 1 wt% n-3 LCP and 2 wt% n-6 LCP. Additionally, DHA content may not exceed ARA content. PUFA limits were set based on human milk content, data on infant PUFA status and data on functional outcome such as growth and visual acuity [110]. HM PUFA content vary considerably across geographies and often do not comply with these recommendations [134]. DHA content is most variable and is strongly defined by the maternal diet, ranging between 0.2 and 1 wt% of total fatty acids, whereas ARA ranges between 0.3 and 0.7 wt% and is less responsive to maternal diet [110]. LA, ALA and LA/ALA ratio content of mature milk of women from 5 different countries ranged between 3.5 and 30 wt%, 0.7 and 2.7 wt%, and 3.5 and 101, respectively. Some consider that IMF PUFA

recommendations based on contemporary HM composition originating from (limited) data in Caucasian women on a typical Western diet should be reconsidered [135]. The marked increase in dietary LA intake and decrease in n-3 LCP over the last decades [136, 137] is reflected in HM FA composition and has been hypothesized to induce nutritional programming during lactation resulting in increased obesity risk [138].

Aim and outline of the thesis

It has become increasingly recognized that developmental trajectories established in early life determine an individual's ability to cope with environmental challenges throughout life. Adverse early nutritional programming together with the contemporary obesogenic environment in many countries may contribute to the rapid increase in NCDs over the past few decades. Scientists endorsing the "predictive adaptive response" (PAR) hypothesis suggest that relative modest early life nutritional interventions can have large beneficial effect on disease risk later in life [42]. If indeed early manipulations would have long-term preventive potential, it would evidently offer great perspectives for targeted interventions by changing the early environment (including early nutrition). Until now, however, most studies investigated detrimental effects of more extreme adverse nutritional challenges in early life to demonstrate the contribution of early life programming in the current obesity epidemic. This approach is far from more subtle nutritional interventions that may help to counter the high obesity incidence worldwide. At present, the possible contribution of moderate dietary changes in early life on metabolic outcomes during adulthood has remained largely unexplored.

The overall aim of this thesis is to determine whether modest changes in lipid quality during early postnatal life can influence the risk of obesity in adult life in mice.

To manipulate diet in mice directly after birth, one could use artificial feeding techniques. However, these approaches are not considered physiological and are rather stressful for mouse pups. An alternative approach could be to manipulate the milk composition of lactating dams. In **chapter 2** we determined whether and to what extent manipulation of the dietary FA composition of lactating

dams is translated into the milk FA composition, and thus whether it affects dietary FA intake of the mouse pups.

Changes in food processing, sourcing of dietary lipids and dietary intake patterns have resulted in a contemporary global increase in exposure to dietary LA and a decrease in n-3 LCP over the last decades [136, 137]. The net result is a high dietary n-6/n-3 ratio, which has been suggested to contribute to the pathogenesis of cardiovascular disease, cancer, inflammatory and auto-immune diseases [139-141]. Ailhaud and colleagues hypothesized that the contemporary high dietary LA intake and the concomitant increased n-6/n-3 ratio, is a determinant in the high obesity incidence worldwide. The proposed underlying mechanism includes the stimulatory effect of high ARA, synthesized from dietary LA, on adipose tissue expansion through enhanced adipogenesis and lipogenesis especially during early development [142]. According to this hypothesis, decreasing the dietary n-6/n-3 ratio in early life would protect against obesity later in life. In **chapter 3**, we aimed to establish in mice whether decreasing the n-6/n-3 ratio by increasing postnatal n-3 LCP during lactation and early weaning, could program adult body composition towards reduced body fat accumulation. Additionally, we aimed to determine whether a potential programming effect on body composition was caused by changes in adipose tissue development, i.e. changes in cell size and/or cell number. However, as specifically high dietary LA is thought to be a contributor to the current high obesity incidence, decreasing the n-6/n-3 ratio towards a "healthier" dietary PUFA composition can also be achieved by reducing LA. Therefore, we reduced dietary LA in preweaning mice by 50% and investigated whether this would have a beneficial effect on adult body composition (**chapter 4**). In a rat study with similar design, we aimed to investigate effects of lowering postnatal LA on adult metabolic phenotype, specifically insulin sensitivity, and to identify whether programming effects on body composition were mediated though modulation of adipocyte size and/or number (**chapter 4**).

Breastfeeding (duration) has been associated with a moderately reduced obesity risk [95] compared to formula feeding, which may arise from many different factors implicated in milk composition as well as feeding behavior. One striking difference between IMF and HM is the physical lipid structure. HM contains large lipid globules (average mode diameter of 4 µm) and is surrounded by a biological PL membrane whereas IMF lipid globules have a diameter of approximately 0.3-1 µm and consist of a core of triglycerides with milk proteins

adhering to the globule surface. This difference could cause differential absorption and digestion kinetics of HM and IMF lipids, which in turn may affect growth and body composition development with potential long-term effects on adult obesity risk. In **chapter 5** we determined whether an IMF concept with a lipid structure closer to the HM lipid structure would program adult body composition. Since BF and FF infants have different growth trajectories in the first year of life, with distinct differences in fat mass development [99], effects of infant feeding on adipose tissue development may underlie sustained effects on later life obesity risk. Therefore, we established the effect of dietary lipid structure on adipose tissue development and function in **chapter 6**, which may give insight in potential mechanisms underlying programming of adult body composition by early life lipid structure.

Finally, we discuss the findings of the various experimental chapters including potential health implications and future perspectives (**chapter 7**).

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Chapter 2

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Rapid and selective manipulation of milk fatty acid composition in mice through the maternal diet during lactation

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Abstract

Dietary fatty acid (FA) composition in early postnatal life can modulate growth and development and, ultimately, body composition and metabolic health in adult life. Manipulation of dietary intake in early life can be accomplished by artificial feeding techniques. However, this approach has proven to be stressful and complicated in rodents. It has remained largely unknown to what extent milk fatty acid composition can be directly manipulated by the dietary fat intake of the lactating mother. In the present study we aimed to assess this in mice.

We exposed dams to diets with different FA compositions from postnatal day (PN)2 until PN28. C57/BL6 dams with litters were randomly assigned to a control (CTRL) diet, a diet high in medium chain FA (MCFA), low in linoleic acid (Low LA), high in n-3 long chain polyunsaturated FA (n-3 LCP), or high in both n-3 LCP and MCFA (n-3 LCP/MCFA). All diets contained 10 wt% fat. At three separate days, between PN7 and 15, dams were milked and fatty acid compositions were determined in milk samples and, at day PN28, in the erythrocytes (RBCs) of male pups.

The results showed that the MCFA content in milk was essentially independent from maternal dietary MCFA intake: neither the MCFA nor the n-3 LCP/MCFA diet increased milk MCFA content. In contrast, the Low LA diet reduced milk LA content by ~50% compared to CTRL, already within 1 week of the maternal diet change. Finally, the maternal n-3 LCP intake strongly influenced milk n-3 LCP content: the milk of dams fed the n-3 LCP or n-3 LCP/MCFA diet for 1 week contained ~6-fold more n-3 LCP than the CTRL diet. Besides some gradual shifts in FA classes, no further changes in FA composition were seen over the lactation period. At PN28, the RBC FA composition of the male pups correlated with the milk FA profiles.

We conclude that manipulation of the diet of lactating mice can strongly and rapidly affect breast milk FA composition, in particular of n-6 polyunsaturated fatty acids (PUFA) and n-3 LCP. Targeted manipulation of dietary FA composition of milk enables modulation of nutrition in early postnatal life of mouse pups without artificial feeding techniques. We anticipate that our present findings will strongly facilitate mechanistic studies on the programming of adult metabolic health by dietary FA in early nutrition.

Introduction

Infants derive dietary fatty acids (FA) from either human milk (HM) or infant milk formula (IMF). In this early period of life, dietary lipids are the main source of energy (~50 en%) and the main supplier of fat-soluble vitamins and essential fatty acids linoleic acid (LA; C18:2 n-6) and α-linolenic acid (ALA; C18:3 n-3). The latter FA are precursors for long-chain polyunsaturated fatty acids (LCP) arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosaenoic acid (DHA), constituents of biological membranes and precursors for eicosanoid biosynthesis [1].

The FA composition in infant milk formula is constant and relatively uniform to adhere to (inter)national legislation [2, 3]. In Europe, the LA content of the different infant formulas must range between 11 and 20 wt% of total FA according to the EU Commission directive. ALA should range 1 and 4 wt% and the LA/ALA ratio must be between 5 and 15. The addition of LCPs is recommended by most authorities including the European Union and World Health Organization, but limits are set to maximal 1wt% n-3 LCP and 2 wt% n6 LCP [4]. DHA content may not exceed ARA content [4].

The FA composition of infant formulas have been based on FA composition of human milk and, for PUFA content, on data concerning infant PUFA status and on functional outcome such as growth and visual acuity [5]. Interestingly, however, human milk FA composition and content is not very constant: HM lipid content and FA composition is affected by maternal diet and body composition, stage of lactation (colostrum, transitional or mature milk), interval between feeds during 24h and volume ingested per feed, and finally, it even changes during a single feed (fore- versus hind-milk) [6-8]. The variation of HM lipid composition is related to the origin of milk FA. Milk FA can originate from different sources. Milk FA are either derived from recent dietary FA intake, mobilized from adipose tissue depots or liver, or synthesized endogenously (i.e. *de novo* lipogenesis, from glucose and other dietary precursors) [9]. Medium chain fatty acids (MCFA) up to a chain length of 14 carbon-atoms (C14) are largely synthesized *de novo* in the mammary gland [10, 11]. Approximately 50% of the C16 is synthesized in the mammary gland and 50% is derived from dietary C16, mobilized from adipose tissue or synthesized in other tissues, specifically liver. FA with a chain length of 18 carbon atoms and longer are derived from circulating plasma lipids, mainly chylomicrons and VLDL, either originating from maternal fat stores or recent

dietary intake [9, 12]. Studies with stable isotopes showed that approximately 30% of the milk LA and LCPs was derived from the diet and 60% from maternal stores [12, 13].

The marked increase in dietary LA intake and decrease in n3 LCP over the last decades [14,15] is reflected in HM FA composition [16] and translated to contemporary IMF FA composition[17]. This increase in LA intake has been hypothesized to induce adverse nutritional programming during lactation, thereby contributing to the current high global obesity incidence [16, 18, 19]. In contrast, a high n-3 LCP exposure in early life has been considered beneficial for later life body composition and metabolic phenotype [20-22]. In other words, dietary fatty acid (FA) composition in early postnatal life is considered to modulate growth and development and ultimately to affect later life metabolic health [18]. Yet, proof of causal relations of early FA intake on later metabolic life is still rather scarce.

In order to establish the role of different dietary fatty acids in postnatal life on adult body composition and metabolic phenotype, it would be helpful to modulate dietary FA intake of pups, without inducing stress by artificial feeding. We reasoned that the most natural way to modulate dietary FA intake of pups would be via the milk FA composition. In the present study we aimed to establish whether and to what extent FA composition of the maternal diet during lactation affects FA composition of murine breast milk (BM). In order to prevent the effects of the maternal diets on the pups mediated via the placenta, we exposed dams to diets with different FA compositions *after* delivery, i.e. from postnatal day (PN)2 onwards. Using different diets fed to the dams, we could address the effects and kinetics of manipulation of medium chain FA, of LA and alpha-linolenic acid (ALA), and of n-3 LCP on milk FA composition.

Methods

Animals and procedures. All experimental procedures were approved by the Animal Experimental Committee (DEC consult) and complied with the principles of good laboratory animal care. Mice were conventionally housed in a temperature- and humidity-controlled room (21 ± 2 °C and 50 ± 5%, respectively) on a 12h light/dark cycle with lights on at 6 a.m. Food (AIN93-compliant semi-synthetic chow) and water were available *ad libitum*.

Female multiparous C57/BL6 mice were obtained from the breeding facility of

Harlan laboratories (Horst, NL) and mated with males of the same strain. Males were introduced in the home cage of two females and removed from the cage after three days. After two weeks, females were checked for pregnancy and housed individually. On postnatal day (PN) 2, the dams were assigned to one of the five experimental diets, each containing 10 wt% fat (Table 1; 4-5 litters per diet). Litters were not culled and were left undisturbed to assure sufficient milk yield for subsequent analyses. Milk samples were taken three times during lactation. Dams and pups were anesthetized (isoflurane/N_2O/O_2) and killed with cervical dislocation after blood sampling through heart puncture at PN28. Blood samples were collected in K 3 EDTA-coated 1-mL microtubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Erythrocytes (RBCs) of male pups were was obtained by centrifugation at 1350×g for 12 min at 4°C (Biofuge fresco, Heraeus, Hanau, Germany), the supernatant was removed and RBC samples were stored at -80 °C until FA analysis. Samples were exclusively collected from male pups, because planned studies at our laboratory concerning metabolic programming by postnatal dietary lipids involved only long-term follow up of the male offspring.

Diets. Diets were AIN93-compliant[23] and hence comprised 18 wt% protein, 60 wt% carbohydrates, and 5 wt% cellulose. All diets contained 10 wt% fat. Fatty acid composition of the diets was based on the human diet distribution of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), with 42 wt% SFA, 41 wt% MUFA and 17 wt% PUFA (Table 1). FA composition of the experimental diets varied due to use of different oil blends comprised of vegetable and fish oils. Litters were exposed to either a control diet (CTRL), with a FA composition comparable to that of infant milk formula, a diet high in medium chain FA (MCFA; C8:0-C12:0; 21 wt% of total FA, 55% increase compared to CTRL), a diet with high n-3 long chain polyunsaturated FA (n-3 LCP; 5 wt% DHA of total FA, 1,2 wt% EPA of total FA; this diet also contained a low amount of the n-6 LCP ARA: 0.28 wt%), a diet with 57% reduced linoleic acid (Low LA; 6,4 wt% of total FA) and a diet combining high n-3 LCP and high MCFA (n-3 LCP/MCFA). Table 1 shows the FA composition of the CTRL and experimental diets according to recipe as calculated by the Department of Processing and New Technologies of Nutricia R&D.

TABLE 1 Dietary fatty acid composition of the experimental diets (g/100 g fat)

FA composition	Abbreviation	CTRL	Low LA	MCFA	n-3 LCP	n-3 LCP/MCFA
Medium Chain Fatty Acids (C8:0 - C12:0)	MCFA	13.6	15.7	21.5	8.6	21.5
Docosahexaenoic Acid (C22:6 n-3)	DHA	0.0	0.0	0.0	5.0	5.0
Eicosapentaenoic Acid (C20:5 n-3)	EPA	0.0	0.0	0.0	1.2	1.2
Arachidonic Acid (C20:4 n-6)	ARA	0.0	0.0	0.0	0.3	0.3
Linoleic Acid (C18:2 n-6)	LA	14.8	6.4	14.3	11.9	11.8
a-Linolenic Acid (C18:3 n-3)	ALA	2.6	1.6	2.6	1.1	2.0
Saturated Fatty Acids	SFA	38.8	41.8	38.8	36.3	36.4
Mono Unsaturated Fatty Acids	MUFA	38.6	41.9	39.2	36.7	35.8
Poly Unsaturated Fatty Acids	PUFA	17.4	8.0	16.9	20.0	20.8
Long chain Poly Unsaturated Fatty Acids	LCP	0.0	0.0	0.0	6.5	6.5
	C18 n-6/n-3	5.7	4.1	5.5	11.1	5.9
	LCP n-6/n-3	0.0	0.0	0.0	0.04	0.04
	total n-6	14.8	6.4	14.3	12.2	12.1
	total n-3	2.6	1.6	2.6	7.8	8.7
	total n-6/n-3	5.7	4.1	5.5	1.6	1.4

Milk collection. Milk samples (30-550 µL) were obtained three times during the second week of the lactation period from lactating dams with litters (4-8 pups): on PN day 7-9, 10-12, and 13-15. Dams were separated from their litters for at least three hours; litters were kept warm on a temperature-controlled surface. Ten minutes after an s.c. injection with 0.3 mL oxytocin (1 IU/mL, Eurovet Nederland, Bladel, NL), dams were milked using an adjusted human lactation pump. Milking occurred at a fixed time (between 10 and 12 a.m.) to avoid diurnal rhythm confounding, and took about 10 min, after which dams were returned to their litters. Milk samples were frozen (-80°C) until analysis for FA composition.

FA analysis. Lipid FA composition in milk and erythrocytes was determined after lipid extraction according to Bligh & Dyer [24]: Milk samples (10 µL) or erythrocytes (200 µL) were transferred to glass tubes, 1 mL EDTA (1%) solution, 2.2 mL MeOH and 1 mL dichloromethane were added and vortexed for at least 5 min. Subsequently, 1 mL EDTA solution and 1 mL dichloromethane was added and the tube was vortexed again for 5 min. Tubes were centrifuged at ~2000*g* for 10 min. Subsequently, 400 µL of the bottom (dichloromethane) layer was collected and transferred to another, high quality glass tube and evaporated. Upon addition of 2 mL MeOH and 40 µL concentrated H_2SO_4, tubes were placed in a heating block at 100°C for 1 h. To the cooled tubes 2 mL hexane and subsequently 0.5 mL 2.5 M NaOH were added, whereupon tubes were vortexed for 2 min. The top layer (hexane) was transferred to a new tube and evaporated. Residues were reconstituted in 200 µL iso-octane and FA composition was analyzed on a gas chromatograph (GC) equipped with a flame ionization detector (FID).

Statistical analyses. All data are expressed as means ± SEM. Statistical analyses were performed using SPSS 12.0.1 (SPSS Benelux, Gorinchem, The Netherlands). Repeated measures ANOVA was performed to analyze effects of experimental diets on milk composition with time (PN day 7-9, PN day 10-12, PN day 13-15) as within-subject factor and diet (CTRL, MCFA, n-3 LCP, n-3 LCP/MCFA and low LA) as between-subject factor. Effects of experimental diet on male pup RBC FA was analyzed by means of Univariate ANOVA. Post hoc analyses of significant main Diet effects and Time*Diet interactions were performed using multiple comparisons with Fisher's least significant difference (LSD) correction.

Results

Change in milk fatty acid composition due to maternal diet. Our primary aim was to determine to what extent FA composition of the maternal diet during lactation affects the milk FA composition in mice from PN7-9 onwards. Figure 1 and 2 depict the correlation between dietary FA and milk FA at PN7-9. Increasing the dietary MCFA content by 50% compared to the CTRL diet did not affect the milk MCFA content (Figure 1A). Interestingly, the ~2.5 fold variation in dietary MCFA content for the dams (between ~8 and 22 wt%) was associated with a stable, ~15% milk MCFA content. The results were quite different for LA and ALA. Milk LA closely reflected dietary LA content (Figure 1B). The 57% reduction of dietary LA in the Low LA group compared to the CTRL group (6.4 versus 14.8 wt%, respectively) resulted in a 45% lower milk LA content (3.7 versus 6.8 wt% in Low LA and CTRL group, respectively; $p< 0.001$). Maternal ALA content also strongly influenced that of ALA in milk, in an apparent linear fashion (Figure 1C; $p<0.001$). To address the effect of maternal LCP, we supplemented diets with 5 wt% DHA, 1.2 wt% EPA and 0.28 wt% ARA (the n-3 LCP and n-3 LCP/MCFA diet groups) . The increase in DHA and EPA in the diet of the lactating dams corresponded with significantly higher levels of these FA in the milk. Milk DHA content increased by 35% in dams fed n-3 LCP and n-3 LCP/MCFA, compared with dams fed either the CTRL, MCFA or low LA diet ($p<0.001$; Figure 1D). Milk EPA content even doubled, from 0.16% to 0.33%, in dams fed n-3 LCP and n-3 LCP/MCFA, compared with dams fed either the CTRL, MCFA or low LA diet ($p<0,001$, data not shown). In contrast to the n-3 LCPs DHA and EPA, a higher dietary content of the n-6 LCP ARA decreased milk ARA levels (Figure 1E) from 0.67% in milk of CTRL dams compared to 0.52% and 0.62% in milk of n-3 LCP and n-3 LCP/MCFA dams ($p<0,001$). Milk ARA content was lower in low LA dams (0.54%) than in CTRL dams (0,67%, $p= 0.018$).

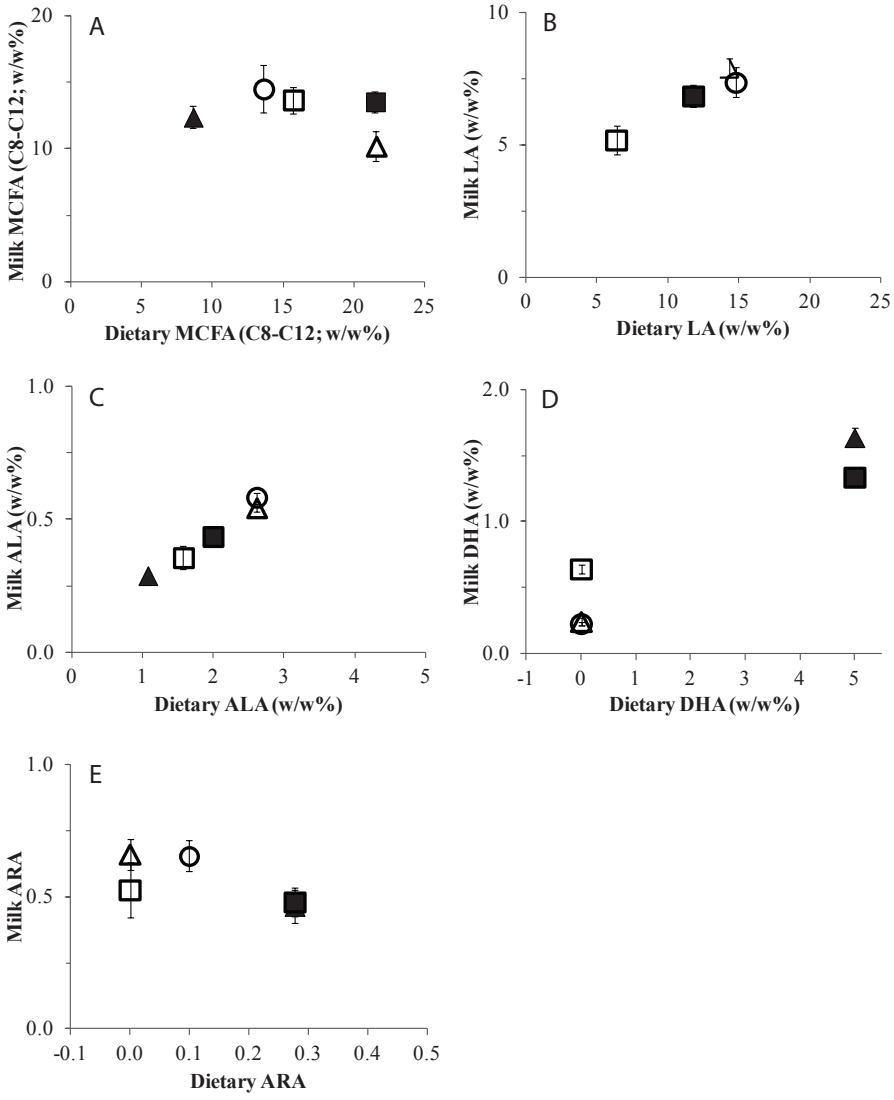

FIGURE 1 The effect of maternal dietary FA intake in lactating mice on milk FA composition; The correlation of mouse milk MCFA (A), LA (B), ALA (C) and DHA (D) at PN 7-9 and dietary MCFA, LA, ALA and DHA of dams fed a CTRL (○), MCFA (△), n-3 LCP (▲), n-3 LCP/MCFA (■) or Low LA (□) diet between PN2 and 28. Concentrations are represented as wt% of total FA. n=5/group

The dietary C18 n-6/n-3 ratio was rather similar between the experimental diets, except for an approximately twofold higher ratio in the n-3 LCP diet (Figure 2A). The latter diet also strongly increased the C18 n-6/n-3 ratio in the milk FA (Figure 2A; $p<0.001$). The LCP n-6/n-3 ratio in milk was higher in the two LCP-

containing diets, comparable to the results of C18 n-6/n-3 ratio (Figure 2B). The total n-6/n-3 ratio in the diets was manipulated more evenly across the experimental groups. Figure 2C shows that the milk total n-6/n-3 ratios strongly (and linearly) correlated with those in the maternal diets.

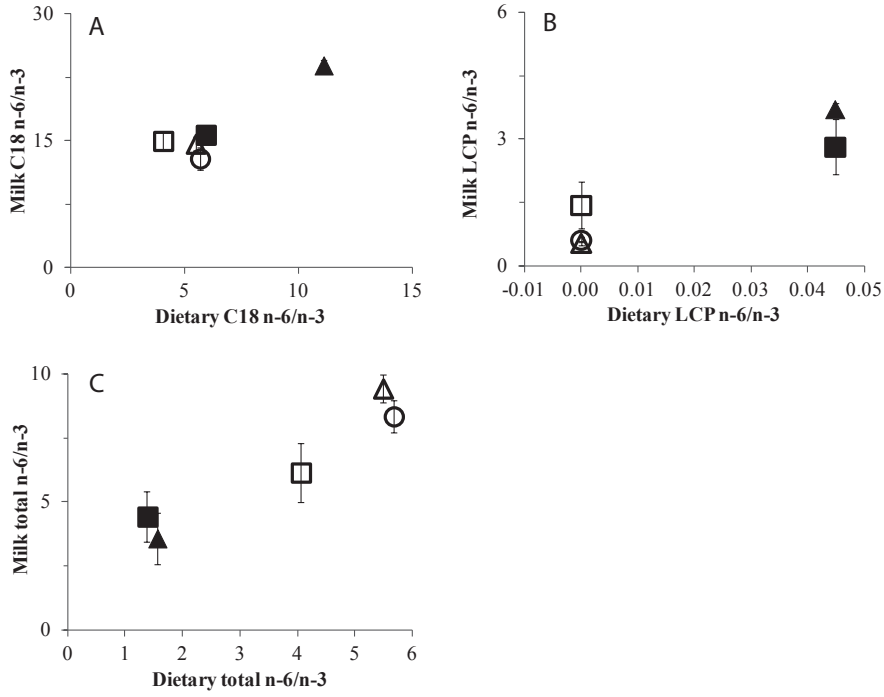

FIGURE 2 The effect of maternal dietary FA intake in lactating mice on milk FA composition; The correlation of LA/ALA (A), LCP n-6/n-3 (B) and total n-6/n-3 ratio (C) in milk at PN7-9 compared to dietary ratios of dams fed CTRL (○), MCFA (△), n-3 LCP (▲),n-3 LCP/MCFA (■) or Low LA (□) diet between PN2 and 28. Concentrations are represented as wt% of total FA. n=5/group

Time dependent changes in milk fatty acid composition. The relative milk SFA content, which accounted for approximately 50% of the total FA in all groups, increased by 5-10% from ~48% to ~55% from PN7 to 15 in all groups ($p<0.001$, data not shown). The relative increase in SFA coincided with a slight, but significant decrease of milk levels of MUFA (approximately 40% of total FA) and PUFA (<10% of total FA; each -2 to -5%, $p<0.001$, data not shown). These effects were observed in all experimental groups, and thus seemed independent of the maternal dietary FA composition.

In all experimental groups, milk MCFA increased significantly by 30% between PN7-9 and PN13-15 ($p<0.001$; Figure 3A). In contrast, milk LA decreased in all groups between PN7 and PN15 (Figure 3B; $p<0.001$). Milk ALA content was rather constant over the time period studied (Figure 3C; $p= 0.333$), whereas the kinetics of DHA were mixed (Figure 5C, $p=0.881$). Finally, milk EPA content remained constant from PN7 to PN15 ($p= 0.633$) and milk ARA decreased by ~28% from PN7 to PN15 ($p< 0.001$, data not shown)

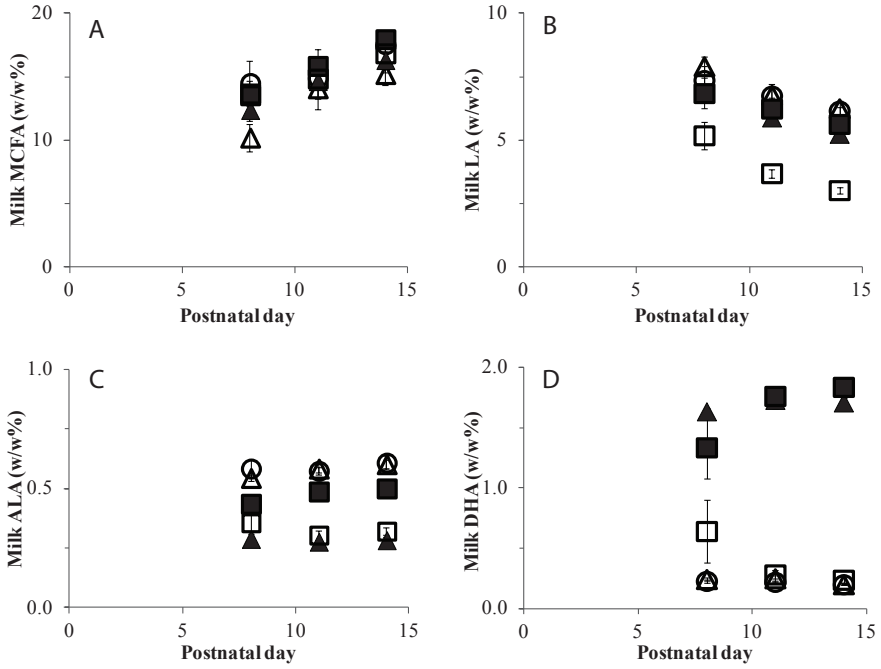

FIGURE 3 Changes in milk FA composition over time in lactating mice fed different dietary FA composition; Milk MCFA (A), LA (B), ALA (C) and DHA (D) concentration during lactation (from PN7-15) of dams fed CTRL (○), MCFA (△), n-3 LCP (▲), n-3 LCP/MCFA (■) or Low LA (□) diet between PN2 and 28. Concentrations are represented as wt% of total FA. n=5/group

Effect of milk and dietary FA composition on pups RBC FA composition. Finally, we determined to what extent the milk composition at PN13-15 and the continued dietary manipulations after lactation from PN16 onwards, influenced RBC FA composition of the male pups at PN28 (Figure 4 and 5). The FA composition of the RBC membrane consisted of ~45% SFA, ~22% MUFA and ~33% PUFA for all experimental groups. Of the dietary FA that differed between the experimental diets, LA, DHA and ARA were the main RBC membrane

constituents with 6-8%, 6-13% and 7-14% of total FA in the experimental groups, respectively. MCFA (<0.1%) and ALA (0.1-0.2%) were barely incorporated in the RBC membrane. Although to a lesser extent, the same applied for EPA: only 0.5-2.7% of the total RBC FA was composed of EPA. Comparable to the results on dietary and milk MCFA composition (Figure 1A), RBC MCFA content did not significantly correlate with milk MCFA (Figure 4A), nor with dietary MCFA (figure 5A). In contrast, the differences in milk LA (Figure 6B) and in dietary LA (Figure 7B) between the experimental groups were reflected in male pup RBC LA content at PN 28. Despite low absolute levels, similar correspondences were observed for ALA (Figure 6C and 7C). RBC DHA content correlated with DHA content of milk and maternal diet (figure 6D and 7D) similarly as RBC EPA content did (data not shown). Milk ARA content was partly reflected in RBC ARA: CTRL and MCFA groups had a higher milk ARA compared to Low LA, which was reflected in RBC ARA (Figure 4E). However, the Low LA group had comparable milk ARA as n-3 LCP and n-3 LCP/MCFA group, whereas RBC ARA was lower in the latter two groups (Figure 4E). As stated above, dietary ARA was inversely correlated with RBC ARA. Correspondingly, RBC ARA was lower in n-3 LCP and n-3 LCP/MCFA pups concurrent with a higher dietary ARA content in these groups (Figure 5E).

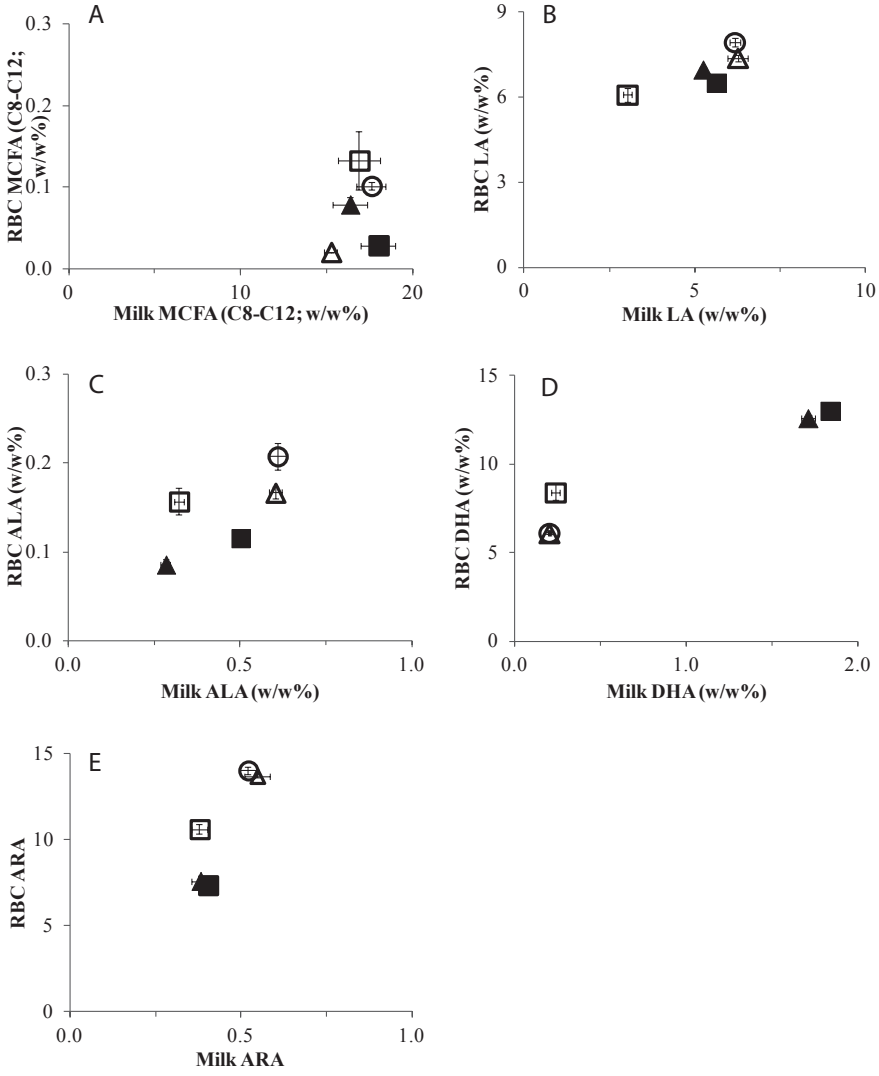

FIGURE 4 The effect of milk FA composition during lactation on FA status of male pups at weaning; MCFA (A), LA (B), ALA (C) and DHA (D) concentration of RBCs of male pups at PN28 (n= 4-9) compared to milk MCFA, LA, ALA, DHA, EPA and ARA at PN13-15 (n=5) of dams fed CTRL (○), MCFA (△), n-3 LCP (▲), n-3 LCP/MCFA (■) or Low LA (□) diet between PN2 and 28. Concentrations are represented as wt% of total FA.

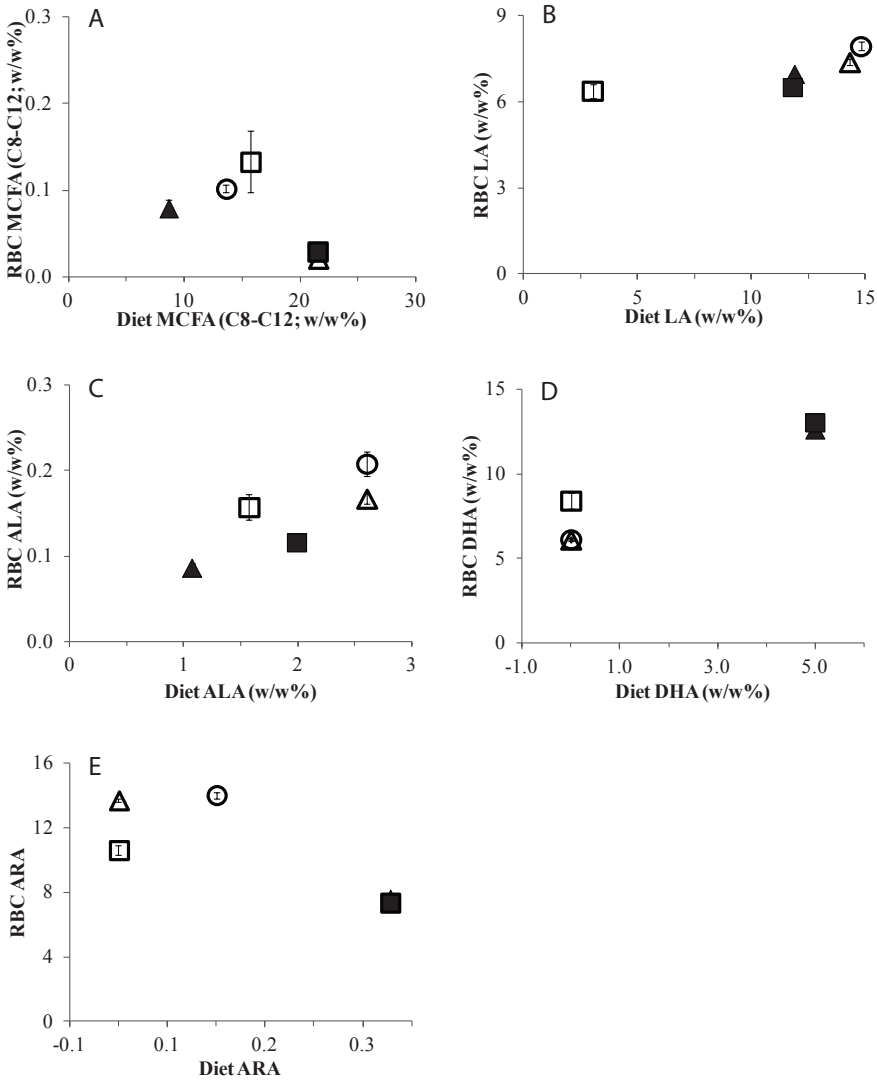

FIGURE 5 The effect of dietary FA composition from PN2 to 28 on FA status of male pups at weaning; MCFA (A), LA (B), ALA (C) and DHA (D) concentration of RBCs of male pups at PN28 (n= 4-9) compared to dietary MCFA, LA, ALA, DHA, EPA and ARA of CTRL (○), MCFA (△), n-3 LCP (▲), n-3 LCP/MCFA (■) or Low LA (□) diet fed to litters between PN2 and 28. Concentrations are represented as wt% of total FA.

Discussion

We aimed to determine whether changing the FA composition of the maternal diet during lactation in mice allows for a rapid manipulation of the milk FA composition and thus of the dietary FA supply to the pups. This approach would selectively change the FA quality in the early diet of mice in a non-stressful manner. Our data clearly show that in particular the LA, ALA and the n-3 LCP content of the milk can be rapidly manipulated by the maternal diet composition (i.e. within 1 week), and that these changes are propagated into the RBC FA composition of the pups after weaning. In contrast, milk MCFA content appears very resistant to manipulation of the dietary FA composition. Our data indicate that modulating dietary PUFA intake by newborn pups is feasible via the maternal diet and strongly support the concept that this approach can be used in mouse models to study nutritional programming.

The resistance of milk MCFA content against the manipulation of the dietary FA composition was evident across a wide range of maternal MCFA contents. The lack of effect of dietary MCFA are likely to be explained by the fact that milk MCFA are mainly synthesized *de novo* from carbohydrate and short chain fatty acid (SCFA) precursors [10]. Indeed, studies in rats [25, 26] and dairy cows [27] have demonstrated that the dietary carbohydrate: lipid ratio determines MCFA content in milk: a higher carbohydrate content increases de novo MCFA synthesis in the mammary gland and reduces uptake of longer chain FA (LCFA; ≥ C18) from plasma, whereas a high fat diet decreases mammary MCFA synthesis in rats and humans [26-29]. Novak and Innis (2011) suggest that the availability of plasma LCFA determines MCFA synthesis, because lowering plasma TGs due to a low fat or high n-3 LCP diet increased milk MCFA content in rats [30]. Additionally, the FA composition of these high fat diets influenced the extent by which the MCFA synthesis was suppressed in rats; PUFA were more effective than MUFA whereas SFA were least effective [31]. In accordance, milk MCFA content in lactating women on a low fat, high carbohydrate diet was significantly higher than milk MCFA content of women on a high fat, low carbohydrate diet [32]. MCFA biosynthesis pathways in the human mammary gland are similar to that of other non-ruminants, including mice and rats [10]. However, human MCFA synthesis is quantitatively low compared to rats, presumably due to a higher lipid contribution in the human diet [10, 29]. Our present observations indicate that investigating the role of early life MCFAs in programming of later life metabolic

health in an animal model would either need artificial feeding of pups during lactation, an increase in dietary carbohydrate: lipid ratio of the maternal diet during lactation, or would implicate a start of the dietary intervention after the lactation period. Modulating milk MCFA by exposure of lactating mice to a high carbohydrate/low fat versus low carbohydrate/high fat diet might be most effective. However, this change in dietary macronutrient composition might affect total lipid content of the milk[33-35] although studies in rats showed that low fat/high carbohydrate increased the percentage of MCFA, but did not affect total FA content of rat milk [30, 36].

In contrast to MCFA, modulation of dietary n-6 and n-3 FA in dams was highly effective in changing milk n-6 and n-3 FA content, including LA, ALA, DHA, EPA and (although negatively) ARA. These findings were in accordance with an observation study in human volunteers, showing that 42% of the variation observed in milk PUFA in the first month of lactation correlated with variations in dietary PUFA intake in this period [37]. In addition, a significant increase in milk ALA, LA, EPA, DHA was found in lactating women within 6 hours after ingestion of a single bolus of various vegetable and fish oils, which correlated with the FA composition of the respective oils [38]. In our present study, lowering dietary LA effectively reduced milk LA. Demmelmair and colleagues demonstrated that 23 to 30% of milk LA was directly derived from dietary LA [13, 39]. A single bolus of canola oil, high in LA, increased milk LA within 6 hours after ingestion and peak values were obtained in 14 hours in healthy lactating women [38]. A rat study with comparable experimental design as our present study showed that dietary LA supplementation of rat dams from PN2 to PN15 increased milk LA at PN15 [40]. Taken together these data suggest that the observation is rather generic and species independent.

The Low LA diet did not only decrease LA content in the milk but also the milk ARA content. This observation suggests that a quantitative part of the (maternal) dietary LA is metabolized before it is transferred as ARA into the milk. Human studies with stable isotopes, however, indicated that the amount of milk ARA derived from synthesis from LA was very limited [6]. Lactating women on a low fat diet had comparable ARA content as women on a adequate fat diet, but using a ^{13}C-labeled LA tracer, it was demonstrated that only 0.01% could be recovered from the milk ARA fraction, indicating that the majority of the milk ARA was obtained from preexisting maternal fat stores [41]. However, rodents have a higher LCP biosynthesis capacity than humans [31, 42-44], which may explain the significant effect of low LA on milk ARA content in our study.

Our data showed that supplementation of DHA and EPA resulted in a 6-fold increase in milk DHA and EPA compared to the CTRL diet. Milk DHA and EPA levels correspond with approximately 30% and 28% of dietary DHA and EPA levels, respectively. These percentages are in accordance with human intervention studies showing that 30% of milk LA and LCFAs could be derived from dietary sources, whereas 60% was derived from maternal lipid stores [12, 13]. Six weeks of dietary supplementation of ALA and n-3 LCP during lactation, with similar LA and ARA content of the control and supplemented diet, increased milk ALA and DHA and did not affect milk LA and ARA content [45]. Supplementation of 200 mg DHA to lactating women for two weeks doubled milk DHA content, without any effect on milk ARA. The use of a stable isotope tracer showed that approximately 20% of dietary DHA was secreted into the milk [46].

In contrast to the C18 and the n-3 LCP FA, results were different for ARA. Feeding dams the n-3 LCP or the n-3 LCP/MCFA diet reduced milk ARA, compared to maternal CTRL diet. This observation was counterintuitive, since the two experimental diets were supplemented with 0.28% ARA, whereas the other diets did not contain any ARA. We speculate that this may originate from the dietary DHA and EPA that were co-supplemented. Dietary DHA and EPA are known to decrease plasma and tissue ARA[47], presumably because incorporation of n-6 and n-3 LCPs in phospholipids depends on the dietary intake [48]. Also, dietary DHA and EPA inhibit Δ5 and Δ6 desaturase which inhibits the ARA synthesis from LA [49]. In order to determine whether the reduced ARA is indeed caused by the concurrent high dietary n-3 LCP, we would have to supplement ARA in an isolated fashion, which we did not do in the present study. Alternatively, the negative relationship between maternal diet ARA and milk ARA may also be a species-specific effect. Increasing dietary ARA, despite high dietary DHA and EPA, increased in milk ARA in lactating women [50]. These changes in diet and milk LCP content were also strongly correlated with maternal RBC LCP content [50].

The net balance of n-6 and n-3 PUFA in tissues of either dams and their offspring is determined by dietary intake of LA and ALA as well as of intake of their respective LCPs, because LA and ALA depend on the same set of elongases and desaturases for conversion to their respective LCPs, and because dietary LCPs inhibit endogenous LCP synthesis. For instance, supplementation of ALA to a high LA diet may have very limited effects on n-3 LCP status and metabolic health, because LA inhibits both n-3 LCP synthesis from ALA and incorporation in biological membranes [16].

To use the concept of maternal diet manipulation in mouse models for nutritional programming, it needs to be demonstrated that the dietary manipulation is propagated into the tissues of the growing pups. Indeed, dietary and milk FA compositions changed the RBC FA composition of the male pups at PN28. Most evident effects of experimental maternal diet were found in DHA and ARA, and to a lesser extent in LA. We suggest that the explanation for this specificity relates to the fact that these PUFAs are preferentially incorporated in membrane PL and are thus relatively abundant in biological membranes. N-3 EFA ALA is neither incorporated in membranes to a large extent nor stored in adipose tissue depots. Rather, a considerable amount is oxidized to generate energy rather than being substrate for DHA and EPA conversion [9].

In conclusion, our data show that changes in n-6 and n-3 EFA and LCP are rapidly translated in maternal milk, indicating that modulation of PUFA supply to the pups during lactation by changing maternal dietary PUFA content is effective and can be used in nutritional programming mouse studies. If the intention would be to investigate metabolic programming effects of MCFA, alternative dietary or artificial feeding methodologies seem warranted.

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Conflicts of interest. A.O., D.K., B.J.M.H., and E.M.B. are employed by Nutricia Research. H.J.V. is a consultant for Nutricia Research outside the submitted work for which his institution (Department of Pediatric Gastroenterology and Hepatology, University Medical Center Groningen, The Netherlands) is compensated financially.

Authorship. A.O., D.K., B.J.M.H. and E.M.B. designed the research; A.O. and D.K. conducted the research; A.O., B.J.M.H. and D.K. analyzed data, A.O. wrote the paper, H.J.V. reviewed the manuscript, E.M.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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Chapter 3

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N-3 long-chain polyunsaturated fatty acids prevent excessive fat deposition in adulthood in a mouse model of postnatal nutritional programming

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Abstract

The present study investigates whether improved quality of nutrients during early postnatal life has effects on adult metabolic profile and body composition in a murine model of nutritional programming. Male offspring of C57Bl/6j dams received a diet containing 21 En% fat of either 100% vegetable oils (control, CTRL) or 80% vegetable oils/20% tuna fish oil (rich in n-3 Long-chain polyunsaturated fatty acids, n-3 LCP) from postnatal day (PN) 2 to 42. Subsequently, mice of both experimental groups were switched to a western style diet (WSD; 21 En% fat, high saturated fatty acid content and cholesterol) until dissection at PN98. Body composition was analyzed by dual x-ray absorptiometry during the WSD challenge. Results showed that a n-3 LCP-rich diet during postnatal life not only reduced fat accumulation by approximately 30% during the WSD challenge from PN42 to 98 ($p<0.001$), but also led to a healthier plasma lipid profile, healthier plasma glucose homeostasis and less hypertrophic adipocytes compared to CTRL. This study shows that postnatal nutrition has programming effects on adult body composition and metabolic homeostasis. Additionally, it emphasizes that moderate alterations in fat quality during early postnatal life considerably affect adult metabolic health.

Introduction

Overweight has become a major medical and public health problem over the past decades. The prevalence of (abdominal) obesity and impaired glucose tolerance with concomitant morbidity and mortality has risen to epidemic proportions [1, 2] and increasingly starts to emerge at an early age [3, 4]. This exponential increase cannot be explained solely by an increase in fat and energy intake [5]. It is thought to be associated with a shift towards increased dietary n-6 and decreased n-3 fatty acid (FA) intake. Linoleic acid (LA; C18:2n-6) and α-linolenic acid (ALA; C18:3n-3) are essential fatty acids (EFA) from the n-6 and n-3 FA series, respectively, which cannot be synthesized *de novo* by animals and have to be obtained from dietary sources. ALA is substrate for biosynthesis of n-3 long-chain polyunsaturated fatty acids (n-3 LCP) such as docosahexaenoic acid (DHA; C22:6n-3) and eicosapentaenoic acid (EPA; C20:5n-3), which have a biological role as membrane constituent and lipid mediator. Conversely, LA can be converted to arachidonic acid (ARA; C20:4n-6) [6]. N-6 LCP ARA and its eicosanoid metabolites have the capacity to directly stimulate adipogenesis through activation of peroxisome proliferator-activated receptors delta and gamma. As a consequence, a shift towards increased dietary n-6 FA intake, especially in periods of growth and development, would lead to increased fat mass gain [7, 8].

Epidemiological and experimental evidence indicates that cardio-metabolic risk factors not only originate from established genetic and contemporary lifestyle factors but also from nutritional and environmental disturbances during prenatal and early postnatal life [9, 10]. An increased risk of cardiovascular and metabolic disorders based on such disturbances can be either aggravated or ameliorated by early postnatal environment [11, 12]. Indeed, accelerated postnatal growth in preterm infants is associated with increased adiposity at term [13] and higher body mass index (BMI) and fat mass (FM) in young adults [14]. The same was shown for small for gestational age infants in whom postnatal catch-up growth was correlated with higher total FM and more abdominal body fat at age 4 years compared to appropriate for gestational age infants [15].

Most established experimental models concerning dietary programming have a strong focus on fetal development and malnutrition by means of maternal energy or protein restriction or high fat feeding [16-18]. The role of macronutrient quality, as opposed to quantity, is still largely unknown and merits investigation.

Also, the extent to which early nutrition during infancy and childhood can affect adult metabolic health during exposure to a moderate obesogenic environment remains elusive.

In adult human and animal subjects dietary n-3 LCP improve insulin sensitivity and counteract dyslipidemia through the reduction of ectopic triglyceride (TG) accumulation in liver and muscle [19], increasing skeletal muscle glucose utilization [20], increasing mitochondrial and peroxisomal β-oxidation [21] and reducing body weight gain and adiposity [22]. Thus, direct effects of n-3 LCP in adulthood on glucose homeostasis, lipid metabolism and adiposity have been well established. Data on nutritional programming by postnatal n-3 LCP however are restricted to beneficial effects on brain development [23] and allergy prevention [24], whereas data on body weight or composition are scarce. A limited number of experimental programming studies have investigated sustained metabolic effects of EFA during gestation and lactation [25, 26]. The capacity to convert ALA in DHA is rather limited in humans compared to rodents, suggesting that dietary ALA during periods of rapid growth in perinatal life may not suffice and dietary n-3 LCP are required to secure healthy development. This may explain the increased DHA synthesis capacity in women and dams during gestation and lactation [6].

The present study investigated whether a moderate change in dietary fat quality during infancy and childhood affects metabolic health and body composition when subjected to an obesogenic adult environment. With a murine model we examined the programming effect of postnatal n-3 LCP on metabolic response to a western style diet (WSD) in adulthood.

Methods

Animals and Study Design. All experimental procedures were approved by the Animal Experimental Committee DEC-Consult, Bilthoven, the Netherlands and complied with the principles of laboratory animal care. Mice were housed in Macrolon type 2 cages and kept on a 12/12h light/dark cycle (light on 06:00h=Zeitgeber time (ZT) 0h) in a temperature- and humidity-controlled room (21±2°C and 50±5%, respectively). Food and water were available *ad libitum* during the entire experimental protocol. Male and female C57Bl/6j mice were derived from Wageningen University and Research Centre (CKP, WUR, Wageningen, The Netherlands) and time-mated. After birth, litters were culled to four male and two female pups per dam on PN2 and assigned to either an early CTRL or n-3 LCP diet (Research Diet Services, Wijk bij Duurstede, The Netherlands). After weaning, male pups were housed in pairs and continued their respective diets until PN42, a period corresponding with infancy and childhood in humans. All male pups changed to the WSD during adolescence and adulthood from PN42 until dissection on PN98.

A separate experiment comparing body composition development of male C57Bl/6j mice fed WSD or a standard rodent chow (AIN93) from PN42 to 98 showed that especially FM increased due to the WSD. FM increased by 67% whereas lean body mass (LBM) decreased by 8% in WSD fed mice compared to the AIN93 fed mice (unpublished data). These data confirm that the relatively mild WSD indeed affects body composition.

Experimental diets. All diets (Table 1) were semi-synthetic, consisted of AIN93-G ingredients (Research Diet Services) and contained 21 En% fat, 17 En% protein and 62 En% carbohydrates. The composition of mineral and vitamin mix is according to American Institute of Nutrition formulation of AIN93G purified diets for laboratory rodents [27].

The early CTRL diet contained a mixture of vegetable oils with a FA composition similar to human infant milk formula with a total n-6/n-3 ratio of 5.67. The early n-3 LCP diet contained the same vegetable oil blend, but 20% was substituted with tuna fish oil resulting in a 3.7-fold lower total n-6/n-3 ratio compared to the CTRL diet. The WSD contained 21 En% fat, consisting of 11 En% lard, 10 En% vegetable oils and 0.1 En% cholesterol resulting in a high saturated FA content and a relative high n-6/n-3 ratio compared to both early diets.

TABLE 1 Composition of the experimental diets

		Early postnatal diet		WSD
		CTRL	n-3 LCP	
Sodium caseinate	(g/kg)	200	200	200
Cornstarch	(g/kg)	450	450	450
Maltodextrin (DE19)	(g/kg)	150	150	150
Vegetable oilblend*	(g/kg)	100	80	50
Tuna fish oil	(g/kg)	-	20	-
Lard	(g/kg)	0	0	50
Saturated Fatty Acids	SFA (En%)	8.88	7.74	8.94
Mono Unsaturated Fatty Acids	MUFA (En%)	8.26	7.82	9.02
Poly Unsaturated Fatty Acids	PUFA (En%)	3.89	4.28	2.82
Linoleic Acid	C18:2 n-6 (En%)	3.16	2.54	2.54
α-Linolenic Acid	C18:3 n-3 (En%)	0.56	0.27	0.28
Arachidonic Acid	C20:4 n-6 (En%)	-	0.06	-
Eicosapentaenoic Acid	C20:5 n-3 (En%)	-	0.26	-
Docosahexaenoic Acid	C22:6 n-3 (En%)	-	1.07	-
	C18 n-6/n-3	5.67	9.44	9.15
	Total n-6 (En%)	3.16	2.60	2.54
	Total n-3 (En%)	0.56	1.67	0.28
	Total n-6/n-3	5.67	1.55	9.15
Cellulose (Vitacel L 600-20)	(g/kg)	50	50	50
mineral mix	(g/kg)	35	35	35
vitamin mix	(g/kg)	10	10	10
choline bitartrate	(g/kg)	2.5	2.5	2.5
L-cystein	(g/kg)	3	3	3
tert-butylhydroquinone	(g/kg)	0.014	0.014	0.014

*Mixture of oils, among others canola oil, sunflower oil, coconut oil and palm oil.

In a separate study, milk samples were withdrawn from dams on PN7 (n=5/group) using an adjusted human lactation pump 10 minutes after oxytocin injection (0.3 mL s.c., 1 IU/mL, Eurovet) injection, to confirm increased n-3 LCP in milk due to early diet. Additionally, FA composition of erythrocytes of the male pups was analyzed at weaning as index for the effect of early diet on tissue of the offspring [28, 29].

Dual Energy X-ray Absorptiometry (DEXA). On PN42, 70 and 98 FM and LBM were measured by DEXA scan under general anaesthesia (isoflurane/N_2O/O_2)

using a PIXImus imager (GE Lunar, Madison, WI, USA). In addition, crown-rump length (CRL) and body weight (BW) were measured.

Blood Sampling and Dissection. On PN98, mice were put under terminal anesthesia (isoflurane/N_2O/O_2) for final DEXA scan and dissection after four hours fasting during the light phase (from 7:30 AM; ZT=1.5h onwards). Blood samples were taken via cardiac puncture and collected in K_3EDTA-coated 1 mL microtubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Plasma was obtained by centrifugation at 1350*g* for 12 min at 4°C (Biofuge fresco, Heraeus, Hanau, Germany) and stored at -80°C. Liver, pancreas, epididymal, peri-renal and inguinal white adipose tissue (WAT) depots were collected and weighed. Epididymal WAT depots were split and either fixed overnight and embedded in paraffin or snap frozen and stored at -80˚C until further analysis.

Histological and Biochemical Analyses of Adipose Tissue. Embedded epididymal WAT was sectioned (7 µm) and stained with hematoxylin and eosin. Images were captured using an Axioplan 2 Zeiss microscope (Carl Zeiss, Weesp, The Netherlands) and a Sony DXC-950P videocamera (Sony, Badhoevedorp, The Netherlands). Six representative sections and four images per section were used to assess average adipocyte surface area and size distribution per animal with analySIS software (Soft imaging system, Münster, Germany). For DNA analysis, frozen epidydimal WAT samples were ground in liquid N_2, incubated in AL buffer (Qiagen Benelux, Venlo, The Netherlands) and proteinase K (Qiagen Benelux B.V.) for 2 h at 56˚C, subsequently vortexed and lysed in nuclisens lysis buffer (BioMerieux, Boxtel, The Netherlands) for 10 min at 37˚C. DNA was extracted using a NucliSens™ easyMAG automated nucleic acid extraction system (BioMerieux) and incubated with a fluorescent nucleic acid stain for double-stranded DNA (Quant-iT dsDNA HS reagent, Invitrogen, Merelbeke, Belgium). Fluorescence was quantified at 502/523 nm using a Qubit™ fluorometer (Invitrogen). Additionally, WAT homogenate was freeze-dried for colorimetrical analysis of triglyceride content (TG; GPO grinder kit, Sigma Aldrich, Zwijndrecht, The Netherlands). TG/DNA ratio and total DNA per WAT depot (DNA/mg tissue * total depot weight) was calculated from these analyses.

Plasma Analyses. Fasting plasma total cholesterol (TC; cholesterol liquicolor CHOD-PAP , Instruchemie, Delfzijl, The Netherlands), TG (GPO trinder method, Sigma Aldrich), free fatty acids (FFA) (NEFA-C method, Wako Chemicals, Neuss, Germany) and glucose (GOD-PAP method, Roche diagnostics, Almere, The Netherlands) were measured colorimetrically and analysed with a microplate

imaging system (Bio-Rad Laboratories Inc.©, Hercules, CA, USA). Total adiponectin was determined using a mouse adiponectin ELISA kit (Linco Research, Billerica, USA). Plasma insulin, leptin, monocyte chemoattractant protein 1 (MCP-1), total plasminogen activator inhibitor-1 (tPAI-1), interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and resistin were measured simultaneously using a mouse serum adipokine lincoplex kit (Linco Research). Samples, standards and quality control were prepared according to manufacturers' protocol and fluorescence was measured using a Bio-Plex™ 200 Luminex instrument (Bio-Rad Laboratories). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin (glu(mmol/L) * ins(pmol/L)/22.5) as an indirect measure of insulin sensitivity.

Statistical Analysis. Statistical analyses were performed using SPSS 15.0.1 (SPSS Benelux, Gorinchem, The Netherlands). Variables were checked for Gaussian distribution with the Shapiro-Wilkes test. Levene's test for equality of variance was used to estimate the probability that treatment groups had different variances. Direct and programming effects of experimental diet (CTRL, n-3 LCP) on BW, food intake and development of body composition were analyzed using a Repeated-Measures analysis of variance (ANOVA). *Post hoc* analyses were performed on significant Diet*Time interactions by Univariate ANOVA of PN42, 70 and 98, separately. Student's t-test was used to analyze the effect of experimental diets on body composition at PN42 and to analyze effects on WAT weight, organ weight, erythrocyte and milk FA composition, frequency distribution of adipocyte cell surface area, average adipocyte cell surface area, WAT TG, WAT DNA, TG/DNA ratio and plasma parameters at PN98. Data are presented as mean ± SEM unless otherwise indicated. Differences were considered significant at $p<0.05$.

Results

Analysis of milk FA composition at PN7 confirmed a change in FA supply to the pups during lactation. A marked increase in n-3 LCP (t_8=-24.935, $p<0.001$) and decrease of LCP n-6/n-3 ratio (t_8=9.037, $p<0.001$) was observed in milk of n-3 LCP dams. FA composition of erythrocytes in male pups at weaning was changed accordingly (Table 2).

TABLE 2. Milk FA composition (n=5/group) at PN7 and erythrocyte FA composition of male pups (CTRL: n=9; n-3 LCP: n=6) at weaning (% of total FA). Data are presented mean ± SEM.

	Milk		Erythrocytes	
	CTRL	n-3 LCP	CTRL	n-3 LCP
C18:2 n-6	7.37 ± 0.56	6.89 ± 0.29	7.94 ± 0.15	6.97 ± 0.10**
C18:3 n-3	0.58 ± 0.04	0.29 ± 0.01**	0.21 ± 0.01	0.09 ± 0.01**
C20:4 n-6	0.67 ± 0.08	0.52 ± 0.03	14.02 ± 0.19	7.61 ± 0.13**
C20:5 n-3	0.17 ± 0.01	0.27 ± 0.02*	0.47 ± 0.01	2.43 ± 0.04**
C22:6 n-3	0.23 ± 0.01	1.63 ± 0.03**	6.13 ± 0.06	12.61 ± 0.14**
SFA	51.20 ± 3.53	48.57 ± 0.56	42.29 ± 0.10	44.71 ± 0.21**
MUFA	37.44 ± 2.75	39.54 ± 0.56	21.25 ± 0.09	19.60 ± 0.13**
PUFA	11.36 ± 0.85	11.89 ± 0.43	34.50 ± 0.10	33.63 ± 0.23*
n-6 LCP	2.50 ± 0.29	2.25 ± 0.09	18.47 ± 0.16	10.44 ± 0.16**
n-3 LCP	0.59 ± 0.03	2.27 ± 0.06**	7.62 ± 0.06	16.05 ± 0.20**
LCP n-6 / n-3	4.17 ± 0.35	0.99 ± 0.03**	2.42 ± 0.03	0.65 ± 0.02**
Total n-6	10.01 ± 0.83	9.20 ± 0.39	26.59 ± 0.11	17.47 ± 0.24**
Total n-3	1.20 ± 0.04	2.57 ± 0.06**	7.92 ± 0.06	16.17 ± 0.20**
Total n-6 / n-3	8.34 ± 0.64	3.58 ± 0.12**	3.36 ± 0.03	1.08 ± 0.02**

* p<0.01, ** p<0.01 compared to CTRL.

During early dietary intervention from PN2 to 42, a similar increase ($F_{(8, 32)}$=839.063, p<0.001) in BW was observed in both experimental groups ($F_{(8, 32)}$=0.419, p=0.901; data not shown). Food intake did not differ between experimental groups during early diet intervention ($F_{(1, 11)}$=0.018, p=0.895; data not shown).

Body composition however did differ at PN42 due to the dietary intervention during the preceding 40 days (Table 3) with a lower absolute FM ($F_{(1, 22)}$=12.746, p=0.002) and lower relative FM (%FM; $F_{(1, 22)}$=10.011, p=0.004) of n-3 LCP mice compared to CTRL mice. Other body composition parameters at that time, such as CRL ($F_{(1, 22)}$=0.287, p=0.598), BW ($F_{(1, 22)}$=1.798, p=0.194) and LBM ($F_{(1, 22)}$=0.046, p=0.831) were unaffected by early dietary intervention.

TABLE 3 Development of body composition during WSD challenge (PN42–98) of mice fed early CTRL diet or n-3 LCP diet (n=12/group).

	CTRL			n-3 LCP		
	PN42	PN70	PN98	PN42	PN70	PN98
Crown-rump length (mm)	73.0 ± 0.8	75.9 ± 0.6	78.5 ± 0.8	73.5 ± 0.5	76.3 ± 0.8	79.5 ± 0.7
Body weight (g)	22.4 ± 0.3	27.5 ± 0.4	31.0 ± 0.7	21.8 ± 0.4	27.3 ± 0.4	29.2 ± 0.6
Lean body mass (g)	18.1 ± 0.3	21.2 ± 0.4	22.6 ± 0.5	18.2 ± 0.3	21.8 ± 0.4	23.2 ± 0.4
Fat mass (g)	4.3 ± 0.2	6.3 ± 0.3	8.43 ± 0.3	3.5 ± 0.1*	5.5 ± 0.3	5.98 ± 0.4**
%Fat mass	19.2 ± 0.9	22.8 ± 0.9	27.1 ± 0.7	15.8 ± 0.5*	20.2 ± 1.1	20.4 ± 1.2**

* p<0.01, ** p<0.001 compared to CTRL

Between PN42 and 98, during which all mice were switched from their respective early diets to the WSD, no differences in BW gain were observed between CTRL and n-3 LCP mice ($F_{(1, 22)}$=0.265, p=0.612; data not shown). Food intake was also similar between experimental groups during WSD challenge ($F_{(1, 11)}$=0.299, p=0.182). Additionally, food intake within each experimental group did not change with transition from early diet to WSD ($F_{(1, 23)}$=0.623, p=0.439).

During WSD challenge, CRL, BW, LBM, FM and %FM increased significantly over time (Table 3). Development of CRL, BW and LBM was similar between experimental groups. However, both absolute and relative FM accumulations were reduced in n-3 LCP mice compared to CTRL mice. This was confirmed by a significant Time*Diet interaction for FM and %FM ($F_{(2, 44)}$= 6.756, p< 0.01 and $F_{(2, 44)}$= 3.586, p< 0.05, respectively). Subsequent *post hoc* analysis showed that FM and %FM were significantly lower in n-3 LCP mice on PN42 (FM: p < 0.01; %FM: p < 0.01) and PN98 (FM: p < 0.001; %FM: p < 0.001).

In line with the observed lower total body FM at PN98 in n-3 LCP mice, epididymal ($t_{(22)}$=2.585, p=0.017) and inguinal ($t_{(6)}$=2.775, p=0.032) fat depot weights were lower in n-3 LCP mice compared to CTRL mice. Weight of organs involved in metabolism, such as liver ($t_{(22)}$=1.365, p=0.186) and pancreas ($t_{(14)}$=-0.662, p=0.519) were similar between the two experimental groups (Table 4).

TABLE 4 Average weight of WAT depots and organs at PN98 of mice fed CTRL or n-3 LCP diet from PN2-42[§]

		CTRL	n-3 LCP
WAT	Epididymal (mg)	968.2 ± 93.1	632.5 ± 90.5*
	Peri-renal (mg)	110.8 ± 11.9	80.9 ± 12.5
	Inguinal (mg)	628.3 ± 72.9	357.3 ± 65.0*
Liver (g)		1.32 ± 0.1	1.20 ± 0.1
Pancreas (mg)		92.4 ± 17.9	115.5 ± 30.0

[§]n=12/group and n=4/group for inguinal WAT. * p<0.05 compared to CTRL.

At PN98, CTRL mice had a greater number of large epididymal adipocytes compared to n-3 LCP mice (Figure 1). This was confirmed by a higher amount of DNA per mg WAT ($t_{(22)}$=2.846, p=0.012) and lower TG/DNA ratio ($t_{(22)}$=-2.949, p=0.007) in epididymal WAT of n-3 LCP mice compared CTRL. TG content per mg WAT and total DNA per WAT depot were comparable between both groups (Table 5).

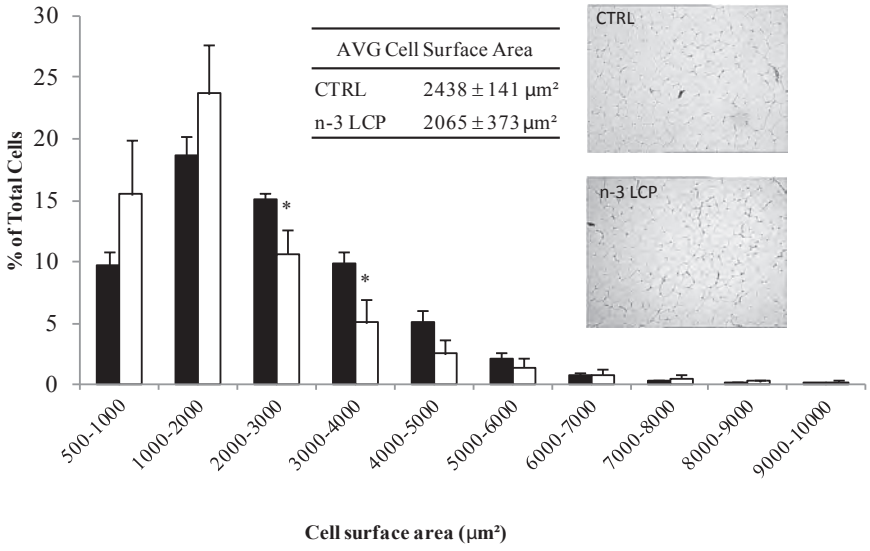

FIGURE 1 Frequency distribution of adipocyte cell surface area from the epididymal fat depot at PN98; CTRL (black bar; n=8) versus n-3 LCP (white bar; n=5) during early neonatal life; * p<0.05 compared to CTRL.

TABLE 5 Average fasting plasma parameters and DNA and TG content of epididymal WAT depot at PN98 of mice fed CTRL or n-3 LCP diet from PN2 to 42[§]

			CTRL	n-3 LCP
Plasma	Adipokines	Adiponectin (mg/L)	8.381 ± 0.114	8.157 ± 0.390
		Leptin (µg/L)	4.971 ± 0.797	2.600 ± 0.638*
		Resistin (µg/L)	4.059 ± 0.251	4.028 ± 0.409
		MCP-1 (ng/L)	9.555 ± 1.342	11.806 ± 0.769
		tPAI-1 (µg/L)	1.837 ± 0.169	2.350 ± 0.412
		TNFα (ng/L)	4.265 ± 0.154	4.289 ± 0.296
		IL-6 (ng/L)	6.357 ± 0.826	16.455 ± 3.123*
	Insulin	Glucose (mmol/L)	16.509 ± 0.710	13.609 ± 1.008*
		Insulin (pmol/L)	114.623 ± 26.314	56.920 ± 9.525
		HOMA-IR ((mmol/L * pmol/L)/22.5)	89.108 ± 22.851	35.437 ± 7.656*
	Lipids	TG (mmol/L)	0.324 ± 0.027	0.282 ± 0.017
		TC (mmol/L)	3.264 ± 0.143	2.719 ± 0.223
		FFA (mmol/L)	0.497 ± 0.056	0.496 ± 0.024
WAT depot		DNA (ng/mg WAT)	32.3 ± 2.6	49.6 ± 5.2*
		TG (ng/mg WAT)	847.7 ± 26.6	876.7 ± 8.2
		TG/DNA	29.3 ± 3.2	19.3 ± 1.4*
		Total DNA (ng/WAT depot)	28.9 ± 1.2	29.3 ± 3.5

MCP-1: Monocyte chemoattractant protein 1; tPAI-1: Total Plasminogen activator inhibitor-1; TNFα: Tumor necrosis factor α; IL-6: Interleukin-6; TC: Total Cholesterol; FFA: Free Fatty Acids. [§] n=12/group. * $p<0.05$ compared to CTRL.

Plasma fasting TC and TG appeared to be lower in n-3 LCP mice compared to CTRL mice, but these differences did not reach significance (TC: $t_{(21)}=2.061$, $p=0.054$; TG: $t_{(21)}=1.355$, $p=0.190$). In addition, no difference was observed in fasting plasma FFA in n-3 LCP and CTRL mice. Most plasma adipokines including MCP-1, adiponectin, TNFα, tPAI-1 and resistin were similar between both treatment groups (Table 5). However, in accordance with the observed reduction in FM, plasma leptin was significantly reduced ($t_{(20)}=2.351$, $p=0.029$) in n-3 LCP mice compared to CTRL mice. Additionally, plasma IL-6 was significantly higher in n-3 LCP mice ($t_{(21)}=-3.126$, $p=0.008$). With regard to glucose homeostasis, n-3 LCP

mice had lower fasting glucose levels ($t_{(21)}$=2.313, p=0.031) and a tendency to have lower fasting insulin ($t_{(21)}$=2.062, p=0.052), ultimately resulting in a lower HOMA-IR index ($t_{(20)}$=2.227, p=0.045) compared to their CTRL fed counterparts.

Discussion

The murine model used in the current study provides an investigative tool for programming effects of nutrition during infancy and childhood on development of body composition and metabolism in a moderate obesogenic environment as present in many western and developing countries. Data on milk and erythrocyte FA profile confirmed that dietary FA supply to the pups changed according to maternal diet during lactation and early diet intervention had a significant impact on tissue FA composition at weaning. Our study is the first to show lasting effects of fat quality of postnatal nutrition on adult body composition and metabolic homeostasis. Indeed, feeding of n-3 LCP for 40 days during postnatal development reduced body FM significantly. When subsequently challenged with a WSD in adolescence and adulthood, fat accumulation was reduced even more in mice previously fed n-3 LCP compared to mice fed CTRL diet. Therefore, n-3 LCP during postnatal development adjust metabolic responses to a WSD later in life and thus limit excessive body fat deposition during adulthood.

Dietary DHA and ARA differentially affect early growth. In human preterm infants dietary DHA reduced growth [30, 31] whereas addition of both DHA and ARA enhanced growth [31]. Rat offspring fed fish oil mainly consisting of n-3 LCP during both pregnancy and lactation had a reduced birth weight and decreased BW gain during lactation [32]. In our mouse model, postnatal n-3 LCP reduced body fat before WSD challenge without significantly affecting LBM or BW gain. Reduced energy intake did not account for this reduction since energy density of the early diets was similar as was food intake in n-3 LCP and CTRL mice. We hypothesize that dietary n-3 LCP have altered adipocyte proliferation and differentiation in the postnatal period. In accordance with this hypothesis, n-3 LCP inhibit adipocyte differentiation *in vitro* and induce early apoptosis in obese rats [33, 34]. Depot-specific effects of n-3 LCP have been demonstrated on expression of genes encoding for lipolytic and lipogenic proteins in rat WAT which may specifically limit adipocyte hypertrophy of abdominal fat depots [35].

Alternately, ARA and its metabolic products stimulate preadipocyte proliferation and adipogenesis [7].

Surprisingly, the effect of postnatal n-3 LCP on body composition did not subside after switching to a moderate obesogenic diet during adulthood. Postnatal n-3 LCP feeding actually decreased WSD induced body fat accumulation by 29% in adulthood compared to postnatal CTRL feeding.

The histological and biochemical analyses of WAT do not allow discerning between direct effects of the postnatal diet on PN42 and subsequent effect of the WSD. However, cell size distribution did show a shift towards smaller adipocytes due to early dietary n-3 LCP. This is in line with a decreased TG/DNA ratio suggesting less hypertrophic cells. Both groups had equal total DNA amounts per epididymal WAT depot. Therefore, programming effects of n-3 LCP on adipocyte number cannot be ruled out, because DNA content is determined by both adipocytes and cells of the stromal-vascular fraction. In conclusion, results of the current study suggest that postnatal n-3 LCP affect development of WAT and give rise to sustained effects on adipocyte functionality during adulthood.

Postnatal n-3 LCP improve metabolic state and consequently may reduce susceptibility to metabolic diseases. Indeed, in addition to lowered body FM, insulin sensitivity was preserved and plasma lipid profile improved in adulthood. Similar direct and programming effects have been shown when n-3 LCP were provided during both pregnancy and lactation in rats [32]. Moreover, postnatal dietary n-3 LCP also prevented hyperleptinemia and hypertension due to prenatal dexamethasone exposure [12]. However, in this study dietary n-3 LCP were provided from birth until the end of the study 6 months later, which makes it difficult to discern between acute and programming effects of dietary n-3 LCP. The described protective effect may therefore not have persisted after diet discontinuation. In contrast, we show persisting effects of postnatal n-3 LCP even after eight weeks of WSD, indicating that the period from birth up to childhood represents a critical timeframe during which nutrition programs adult metabolism and body composition.

Consistent with reduced epididymal and inguinal fat depot weight and body FM, plasma leptin levels were significantly reduced by the n-3 LCP diet. No sustained effects on plasma levels of the other adipokines were identified, except for IL-6. Either these levels were not affected by postnatal n-3 LCP or effects were abolished during WSD exposure. However, the lack of a dietary

effect on adipokine levels measured systemically cannot exclude possible paracrine effects at tissue level [36].

Dietary n-3 LCP have anti-inflammatory properties and reduce pro-inflammatory interleukins such as IL-6 in obese rats [37] and human subjects [38]. High IL-6 levels have been correlated with high BMI, adiposity [39] and increased adipocyte size [40]. Thus, higher plasma IL-6 at PN98 in n-3 LCP mice compared to CTRL is in clear contradiction with the reduced adiposity, smaller adipocytes and the overall healthier metabolic profile with improved insulin sensitivity and plasma lipids. To our knowledge, there are no programming studies available at present which could provide better understanding of the cause and consequence of the currently found increased adult IL-6 levels after postnatal n-3 LCP exposure. None of the other measured inflammatory adipokines were affected suggesting that n-3 LCP feeding during infancy and childhood did not result in increased oxidative stress or inflammation in adulthood. Including analyses of IL-6 and other pro-inflammatory markers on tissue level in future studies may elucidate the origin and physiological significance of the higher systemically IL-6 levels found here.

Overall, we show prevention of hypertrophic growth of epididymal adipose tissue by postnatal dietary n-3 LCP. The critical time frame of diet administration is emphasized by the persistence of effects despite an eight week WSD exposure To our knowledge we are the first to show lasting protective effects of modest FA composition changes in postnatal diet on excessive fat accumulation later in life. It is anticipated that even small alterations in postnatal nutrition offered during a critical time-frame may be relevant for improving adult metabolic health. Additionally, this experimental mouse model provides a useful tool to explore potential programming effects of postnatal dietary interventions and may help identify the critical periods in postnatal metabolic development.

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Chapter 4

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Dietary n-6 polyunsaturated fatty acids content in early life programs adult body composition and metabolic response to a western diet challenge in rodents

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Abstract

The global increase in dietary n-6 polyunsaturated fatty acids (PUFA) intake is suggested to contribute to the rise in obesity incidence. In rats and mice, we tested the hypothesis that n-6 PUFA intake during early postnatal life programs adult body composition and metabolic phenotype. Male offspring of C57Bl/6j mice and of Wistar Unilever rats were subjected to a control diet (CTRL, 3.16 En% LA) or a low n-6 PUFA diet (Low LA, 1.36 En% LA) from postnatal day (PN)2 to 42. Subsequently, all animals were switched to a Western style diet until PN98. We monitored body composition by dual x-ray absorptiometry and glucose homeostasis by an intravenous glucose and insulin tolerance test in rats and by the homeostasis model assessment of insulin resistance (HOMA-IR) in mice. After dissection, plasma lipid profile, glucose, insulin and adipokines were measured and adipocyte number and size were analyzed. In mice, the postnatal low LA diet decreased fat accumulation during the adult WSD challenge (- 27% compared to CTRL, $p < 0.001$). Simultaneously, it reduced fasting triglyceride levels, improved insulin sensitivity, and lowered fasting resistin and leptin levels. In rats, the low LA diet did not affect adult fat mass or body composition, but reduced the number of retroperitoneal adipocytes, increased the number of large adipocytes and improved glucose tolerance in adulthood. In conclusion, dietary n-6 PUFA content in early life programs the metabolic response to an obesogenic diet challenge in adult life. Our data underline the importance of early life dietary lipid composition in modulating obesity risk later in life.

Introduction

Incidence of obesity and its co-morbidities has increased rapidly during the last decades [1, 2]. This profound increase cannot solely be explained contemporary lifestyle factors such as an unbalanced diet or reduced physical activity [3]. Accumulating evidence shows that dietary factors in critical developmental periods, including fetal life, infancy and early childhood, are associated with obesity risk later in life [4-8]. Both over- and undernutrition during pregnancy and infancy resulted in higher susceptibility to obesity and in poor metabolic health in adult life [9-12]. Apart from nutrient quantity also nutrient quality, specifically lipid quality, proved to be a key factor in nutritional programming of metabolic phenotype in adult life. Exposure to dietary n-3 polyunsaturated fatty acids (n-3 PUFA) during pregnancy and/or lactation programmed towards reduced fat accumulation and improved metabolic homeostasis in animal [13, 14] and human subjects [15-17], suggesting a role for dietary fatty acid composition.

In contrast to n-3 PUFA, n-6 PUFAs, both arachidonic acid (ARA) and its precursor linolenic acid (LA), promote adipogenesis, lipogenesis and inflammation [18-20]. Exposure of adult rodents to high n-6 PUFAs thereby contributes to enhanced body fat accumulation by both increased adipocyte size and increased adipocyte number, e.g. hypertrophic and hyperplasic expansion of white adipose tissue (WAT) [21, 22]. These findings support the hypothesis that the global shift towards excess dietary n-6 intake and insufficient n-3 intake in the past decades [23, 24] plays a role in the worldwide increased incidence of obesity and its co-morbidities [25, 26]. Especially exposure to a high n-6 PUFA diet in early postnatal development could amplify detrimental effects on metabolic health [27], since this is considered a critical period for WAT development [28, 29]. A recent cohort study by Moon *et al* [17] demonstrated that maternal plasma n-6 PUFA content was correlated to fat mass of the offspring at 4 and 6 years of age. Only very few experimental studies investigated sustained effects of n-6 PUFA exposure on metabolic health. These studies investigated either high n-6 PUFA combined with a high fat diet throughout life (starting during pregnancy) or over multiple generations [30, 31] or tested the effect of the n-6/n-3 ratio, rather than absolute n-6 PUFA content, during the pregnancy and lactation period [32]. As a result, data concerning the role of n-6 PUFA in a diet with normal lipid content, specifically during early postnatal life, are lacking.

White adipose tissue (WAT) development is driven by both proliferation and

differentiation of preadipocytes to mature adipocytes, e.g. adipogenesis, as well as the lipid storage capacity of these mature adipocytes [33, 34]. In humans, adipocyte number is established during childhood and adolescence, whereas adipocyte size can increase throughout life. Both increase in cell number and size are positively correlated with fat mass [29]. In rodents, total adipocyte number is set between 9-18 weeks of age and remains constant on *ad libitum* standard rodent diet [33, 35, 36]. In contrast, adipocyte size can increase until senescence in rodents [37]. Experimental data from *in vitro* and *in vivo* studies suggest that n-3 and n-6 PUFA differentially modulate proliferation and differentiation of preadipocytes via several mechanisms including gene transcription, mRNA processing and posttranscriptional processes [22, 25]. LA can be converted to arachidonic acid (C20:4 n-6). Arachidonic acid and its eicosanoid metabolites have the capacity to stimulate adipogenesis through direct activation of peroxisome proliferator-activated receptors delta and gamma [25]. Additionally, n-3 and n-6 PUFA are known to have differential effects on transcription factors involved in WAT lipogenesis with n-3 inhibiting and n-6 PUFA stimulating expression of lipogenic transcription factors [22]. These findings led us to speculate that exposure to high dietary n-6 PUFA during gestation and lactation without differences in total amount of dietary lipids would lead to increased number of adipocytes in early life, thereby increasing lipid storage capacity. Combined with subsequent exposure to a high fat, high n-6, western diet throughout life, this would result in excessive WAT expansion. Lowering of dietary n-6 PUFA especially during the critical period in early life, may modulate adipose tissue development thereby reducing susceptibility to obesity later in life.

The objective of the present study was to investigate the effect of lowering linoleic acid (LA) content in the diet selectively during the postnatal period on white adipose tissue development, adult body composition and metabolic profile in mice and rats. The mouse model was used to gain insight on programming effects on body composition over time whereas the rat model was used to investigate effects on adult metabolic phenotype, specifically WAT morphology and insulin sensitivity. If dietary LA content in early postnatal life would be able to influence early life growth of WAT, this could result in ways to beneficially modify fat accumulation and metabolic state in a mild obesogenic adult environment.

Methods

Animals. All experimental procedures were approved by the Animal Experimental Committee DEC-Consult, Bilthoven, the Netherlands and complied with the principles of laboratory animal care. Mice and rats were housed at Wageningen University and Research Centre (CKP, WUR, Wageningen, The Netherlands) on a 12/12h light/dark cycle (light on 06:00h=Zeitgeber time (ZT) 0h) in a temperature- and humidity-controlled room (21±2°C and 50±5%, respectively). Food and water were available *ad libitum* during the entire experimental protocol, except before blood sampling when animals were fasted for at least four hours during the light phase (from 7:30 AM; ZT=1.5h onwards). Food intake was measured per cage twice a week. Body weight was measured per litter before weaning and individually after weaning twice a week.

Experimental diets. All diets (Table 1) were semi-synthetic, consisted of AIN93-G ingredients (Research Diet Services) and contained 21 En% fat, 17 En% protein and 62 En% carbohydrates. The composition of mineral and vitamin mix was according to American Institute of Nutrition formulation of AIN93G purified diets for laboratory rodents [38].

Mice and rats were assigned to either a postnatal control (CTRL) diet which contained a mixture of vegetable oils with a FA composition similar to infant milk formula or a diet which contained an oil blend with ~50% less LA (Table 1). Diets were thus different in FA composition but had similar total fatty acid content. Subsequently, all animals were switched to a western style diet (WSD) which contained 21 En% fat, consisting of 11 En% lard, 10 En% vegetable oils and 1 g/kg cholesterol.

TABLE 1 Composition of the experimental diets

		Early diet		WSD
		CTRL	Low LA	
Sodium caseinate	(g/kg)	200	200	200
Cornstarch	(g/kg)	450	450	450
Maltodextrin (DE19)	(g/kg)	150	150	150
Oilblend[†]	(g/kg)	100	100	50
Lard	(g/kg)	0	0	50
Cholesterol	(g/kg)	0	0	1
Saturated Fatty Acids	SFA (En%)	8.88	9.14	8.94
Mono Unsaturated Fatty Acids	MUFA (En%)	8.26	8.93	9.02
Poly Unsaturated Fatty Acids	PUFA (En%)	3.89	1.70	2.82
Linoleic Acid	C18:2 n-6 (En%)	3.16	1.36	2.54
α-Linolenic Acid	C18:3 n-3 (En%)	0.56	0.33	0.28
	C18 n-6/n-3	5.67	4.06	9.15
Cellulose (Vitacel L 600-20)	(g/kg)	50	50	50
mineral mix	(g/kg)	35	35	35
vitamin mix	(g/kg)	10	10	10
choline bitartrate	(g/kg)	2.5	2.5	2.5
L-cystein	(g/kg)	3	3	3
tert-butylhydroquinone	(g/kg)	0.014	0.014	0.014

[†]Mixture of rapeseed, sunflower, butter, coconut, and palm oil.

Mouse study. Male and female C57Bl/6j mice were derived from Wageningen University and Research Centre (CKP, WUR, Wageningen, The Netherlands) and time-mated. After birth, litters were culled to four male and two female pups per dam on postnatal day (PN) 2 and randomly assigned to one of two experimental diets. After weaning, male pups were housed in pairs and continued their respective diets until PN42, a period corresponding with infancy and childhood in humans. All male pups were changed to the WSD from PN42 until dissection on PN98, thus during adolescence and adulthood. Body composition was measured on PN42, 70 and 98 by Dual Energy X-ray Absorptiometry (DEXA; PIXImus, GE Lunar, Madison, WI, USA) under general anaesthesia (isoflurane/N_2O/

O_2; Figure 1). Body composition of a non-challenged chow fed group (Chow) at PN98 were assessed to determine possible effects of the WSD challenge (Supplementary Table 1).

In a separate study, milk samples were taken from dams on PN7 (n=5/group) to assess to what extent the diet that had been administered to the dams had affected the n-6 PUFA content in the milk. Milk was withdrawn using an adjusted human lactation pump 10 minutes after oxytocin injection (0.3 mL s.c., 1 IU/mL, Eurovet). FA composition of erythrocytes of the male pups was analyzed at weaning as index for the effect of early diet on tissue of the offspring [39, 40].

Rat study. Female Wistar Unilever (WU) rats (Harlan laboratories, Horst, The Netherlands) were time-mated. Litters were culled to four male pups and four female pups per dam on PN2 and assigned to CTRL or Low LA diet. Dams and female pups were sacrificed at weaning. Male pups were housed in pairs and continued on their respective diets until PN day 42. All male pups subsequently changed to a WSD until dissection on PN98 (Figure 1).

A blood sample was taken on PN42 after 4 hours of fasting by means of orbital puncture under general anaesthesia (isoflurane/N_2O/O_2). Body composition was measured on PN42, 63 and 98 by DEXA (Discovery A, Hologic Inc, Bedford, MA, USA) under general anaesthesia. Like the mouse study, body composition measurements of a non-challenged chow fed group (Chow) was included at PN 98 to determine effects of the WSD challenge in adolescence and adulthood (Supplementary Table 1).

On PN63, the rats were anesthetized and the right jugular vein was cannulated with a silicone catheter [41] to allow repeated stress free blood sampling in conscious animals. After surgery the rats were housed individually. After full recovery, an intravenous glucose tolerance test (ivGTT) was performed on PN84. Briefly, blood samples were taken at -10, 2, 4, 6, 8, 10, 15, 20 and 30 minutes after i.v. administration of 500 mg/kg glucose (500 mg/ml saline). At PN90, an insulin tolerance test (ivITT) was performed during which 0.5 U/kg insulin (I-5500, Sigma Aldrich Chemie, Zwijndrecht, The Netherlands; 29 U/mg, 27,59 µg/ml saline) was administered and blood samples were taken at -10, 2, 4, 6, 8, 10, 15, 20, 30 and 60 minutes. Samples of both ivGTT and ivITT were collected in K_3EDTA-coated 1 ml microtubes (Greiner Bio-one, Germany), centrifuged during 15 minutes at 4 °C (3500 rpm, Biofuge fresco, Heraeus). Plasma was isolated and stored at -80 °C prior to analysis.

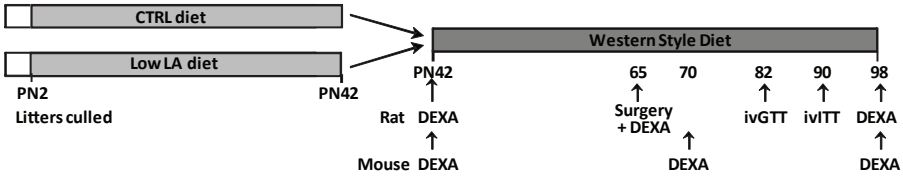


FIGURE 1 Experimental design of mouse and rat study.

Termination and dissection. On PN98, mice and rats were put under terminal anaesthesia (isoflurane/N_2O/O_2) for dissection. Blood samples were taken via cardiac puncture and collected in K_3EDTA-coated 1 mL microtubes. Plasma was obtained by centrifugation at 1350*g* for 12 min at 4°C and stored at -80°C. Liver, pancreas, epididymal, retroperitoneal, peri-renal and inguinal white adipose tissue (WAT) depots were collected and weighed.

Cytological and biochemical analyses of mouse and rat WAT. Adipocyte size distribution, DNA and TG content in mouse epididymal WAT were determined as previously described [13]. Rat adipocyte size distribution was determined in fresh inguinal and retroperitoneal WAT depots according to the optical method of Di Girolamo *et al* [42]. Depots were cut into 1 mm pieces using a McIlwain tissue chopper (Mickle laboratory engineering Co. ltd., Gomshall, UK) and incubated at 37°C for 60 min in a shaking water bath (60-80 strokes/min) in gassed (95% O_2 / 5% CO_2) Krebs Ringer bicarbonate buffer (KRB) at pH 7.4 containing 2 mg/ml collagenase type II (Gibco for Invitrogen, California, USA). The digested tissue was filtered through a cell strainer with 250 µm nylon mesh. The freed adipocytes in the filtrate were allowed to float and washed three times with KRB buffer. The infranatant was filtered through a cell strainer with 40 µm nylon mesh and the remaining cells were added to the collected adipocytes.

70 µL aliquots of the adipocyte suspension were placed on a slide and covered with a cover slip. Images were captured using an Axioplan 2 Zeiss microscope (Carl Zeiss, Weesp, The Netherlands) and a Sony DXC-950P videocamera (Sony, Badhoevedorp, The Netherlands) at 100X magnification. Six representative sections per slide and six slides per depot per rat were used to obtain at least 400 cells and assess mean adipocyte volume and size distribution per animal with analySIS software (Soft imaging system, Münster, Germany).

Adipocyte tissue cellularity was calculated by dividing the lipid content of the fat depot by the mean adipocyte weight. [42]. WAT lipid content was determined according to the method of Folch *et al* [43] using dichloromethane: methanol (2:1, v/v). Mean adipocyte weight was calculated by multiplying mean adipocyte

volume by the lipid density, which was derived from triolein density (ρ= 0,915 g/ml).

Plasma Analyses. Fasting plasma total cholesterol (TC; cholesterol liquicolor CHOD-PAP, Instruchemie, Delfzijl, The Netherlands), TG (GPO trinder method, Sigma Aldrich, Zwijndrecht, The Netherlands), free fatty acids (FFA) (NEFA-C method, Wako Chemicals, Neuss, Germany) and glucose (GOD-PAP method, Roche diagnostics, Almere, The Netherlands) were measured colorimetrically and analysed with a microplate imaging system (Bio-Rad Laboratories Inc.©, Hercules, CA, USA). Total adiponectin in mice was determined using a mouse adiponectin ELISA kit (Linco Research, Billerica, USA). Plasma insulin, leptin, monocyte chemoattractant protein 1 (MCP-1), total plasminogen activator inhibitor-1 (tPAI-1), interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and resistin in mouse plasma were measured simultaneously using a mouse serum adipokine lincoplex kit (Linco Research). Samples, standards and quality control were prepared according to manufacturers' protocol and fluorescence was measured using a Bio-Plex™ 200 Luminex instrument (Bio-Rad Laboratories). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin (glu(mmol/L) * ins(pmol/L)/22.5) as an indirect measure of insulin sensitivity. Rat insulin was analyzed using an Enzyme Linked Immuno Sorbent Assay (Rat Insulin ELISA, DRG Diagnostics International inc., New Jersey, USA), according to manufacturers' protocol.

Statistical Analysis. Statistical analyses were performed using SPSS 15.0.1 (SPSS Benelux, Gorinchem, The Netherlands). Variables were checked for Gaussian distribution with the Shapiro-Wilkes test. Levene's test for equality of variance was used to estimate the probability that treatment groups had different variances. Direct and programming effects of experimental diet (CTRL, Low LA) on BW, food intake, body composition development, glucose tolerance and insulin sensitivity were analyzed using a Repeated-Measures analysis of variance (ANOVA). *Post hoc* analyses were performed on significant Diet*Time interactions by Univariate ANOVA of PN42, 70 and 98, separately. Student's t-test was used to analyze the effect of experimental diets on body composition at PN42 and to analyze effects on WAT weight, organ weight, erythrocyte and milk FA composition, cell size distribution, mean adipocyte volume, cell number and plasma parameters. Data are presented as mean ± SEM unless otherwise indicated. Differences were considered significant at $p<0.05$.

Results

Effects of early diet on milk and pup erythrocyte FA composition. Analysis of milk at PN7 showed that the Low LA diet decreased LA (t_8= 2.804, p=0.023), ALA (t_8= 3.756, p=0.006) and total n-6 PUFA (t_8=2.682, p=0.028) in the milk. The Low LA diet non-significantly increased milk DHA and decreased milk ARA, total n-6/n-3 and LCP n-6/n-3 ratio. The changes in erythrocyte membranes of male pups at weaning corresponded with changes in the milk composition. All erythrocyte n-6 PUFAs were decreased and n-3 PUFAs were increased, except for ALA (Table 2).

TABLE 2. Dam milk FA composition at PN7 and male pup erythrocyte FA composition at weaning (% of total FA) of CTRL and Low LA group†

	Milk				Erythrocytes			
	CTRL		Low LA		CTRL		Low LA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C18:2 n-6	7.37	0.56	5.19*	0.54	7.94	0.15	6.08**	0.24
C18:3 n-3	0.58	0.04	0.36*	0.04	0.21	0.01	0.16	0.01
C20:4 n-6	0.67	0.08	0.54	0.04	14.02	0.19	10.61**	0.27
C20:5 n-3	0.17	0.01	0.16	0.05	0.47	0.01	1.15**	0.11
C22:6 n-3	0.23	0.01	0.64	0.26	6.13	0.06	8.41**	0.46
SFA	51.20	3.53	49.32	2.06	42.29	0.10	42.82*	0.22
MUFA	37.44	2.75	41.95	2.74	21.25	0.09	23.83**	0.47
PUFA	11.36	0.85	8.73	2.14	34.50	0.10	31.44**	0.38
LCP n-6	2.50	0.29	1.95	0.09	18.47	0.16	14.37**	0.30
LCP n-3	0.59	0.03	1.03	0.36	7.62	0.06	10.51**	0.56
LCP n-6 / n-3	4.17	0.35	2.75	0.70	2.42	0.03	1.38**	0.09
Total n-6	10.01	0.83	7.24*	0.62	26.59	0.11	20.63**	0.22
Total n-3	1.20	0.04	1.41	0.38	7.92	0.06	10.81**	0.55
Total n-6 / n-3	8.34	0.64	6.15	1.17	3.36	0.03	1.93**	0.12

†n=4-9/group, * p<0.05, ** p<0.01 compared to CTRL.

Direct effect of early diet on growth, body composition and food intake. Body weight did not differ (t_{22}= -1.448, p=0.162) between mice fed CTRL or Low LA at PN42. There was no difference in FM (t_{22}= -1.448, p=0.162) or LBM (t_{22}= -1.448, p=0.162) between both groups directly after diet intervention at PN42 (Figure 2), However, rats fed Low LA had a lower BW (-16%, t_{22}= 3.533, p=0.002) and LBM (-14%, t_{22}= 4.033, p=0.001) compared to CTRL rats (Figure 2). Food intake (Table 3) was comparable between CTRL and Low LA group in mice (t_{10}= -2.052, p=0.067) and rats (t_4= 1.518, p=0.204).

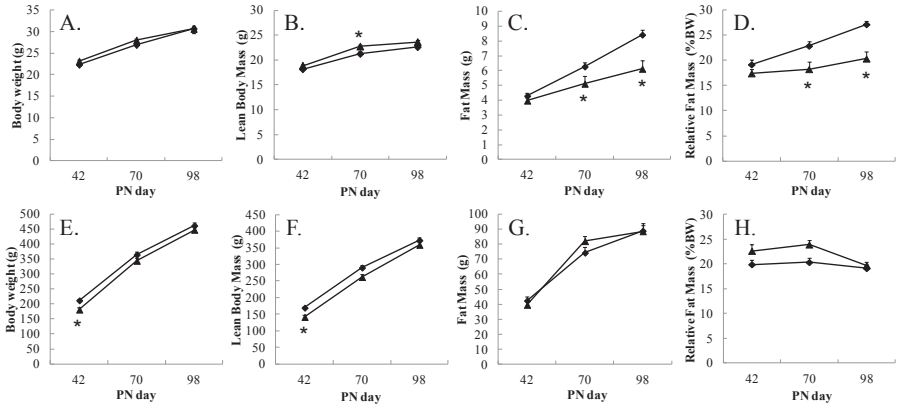


FIGURE 2 Development of Body Weight (BW), Lean Body Mass (LBM), Fat Mass (FM) and Relative Fat Mass (%FM) during WSD challenge (PN 42 – 98) of male mice (A-D) and rats (E-H) fed CTRL (◆) or Low LA diet (▲) in early life. n = 12/group. * $p < 0.05$ compared to CTRL group.

TABLE 3 Mean daily food intake (g) during early CTRL or Low LA diet and WSD intervention.[†]

	Mice				Rats			
	CTRL		Low LA		CTRL		Low LA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Early diet	3.18	0.13	3.48	0.13	21.6	2.6	17.3	1.1
WSD	3.17	0.08	3.53*	0.09	19.6	1.5	20.9	0.6

[†]n=6/group, *$p < 0.05$ compared to CTRL group

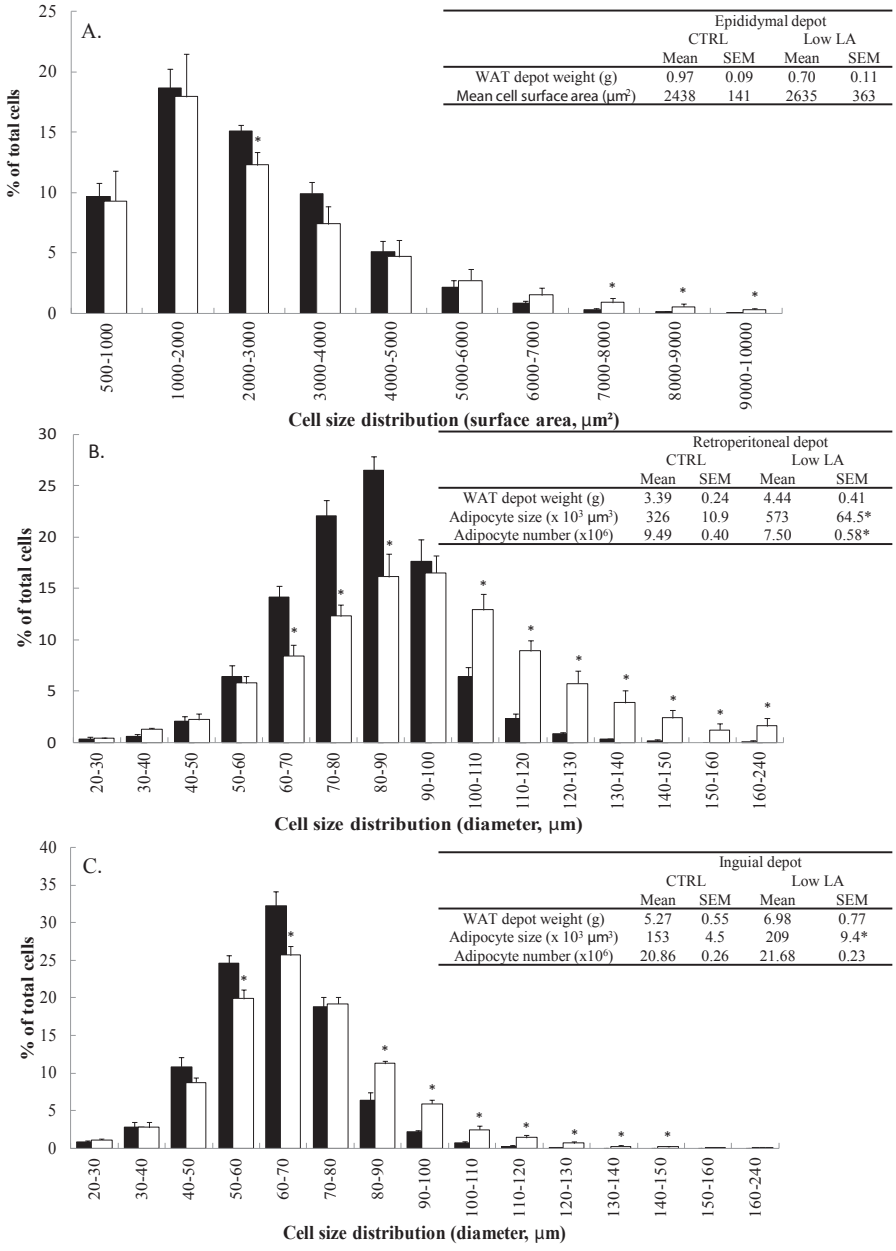
Effect of early diet on food intake and body composition development during WSD challenge. Average daily WSD intake (Table 3) was significantly higher in mice fed Low LA postnatally compared to CTRL fed mice (t_{10}= -2.995, p=0.013). WSD intake did not differ between CTRL and Low LA rats (t_4= -0.114, p=0.915). The WSD challenge induced a similar BW gain in CTRL and Low LA fed mice ($F_{(2,44)}$= 2.015, p=0.145; Figure 2A). The development of the body composition, however, differed between the two groups. Most pronounced effects of postnatal Low LA where found on FM development; Low LA early diet decreased the absolute FM gain by 27% ($F_{(2, 44)}$= 5.396, p= 0.008; Figure 2C), and the relative %FM gain by 25% ($F_{(2, 44)}$= 3.674, p= 0.033; Figure 2D) upon the WSD challenge. LBM gain from PN42 to 98 was not significantly different between groups (Diet*Time: $F_{(2, 44)}$= 1.041, p= 0.362; Figure 2B). However, statistical analysis confirmed a main diet effect ($F_{(2, 48)}$= 6.357, p= 0.019) indicating that Low LA mice overall had a higher LBM, which was significant at PN70 (+7%, $p < 0.05$).

The difference in total FM was in accordance with a reduced inguinal (t_6= 2.898, p=0.027) and perirenal (t_{22}= 2.762, p=0.011) WAT depot weight in Low LA mice compared to CTRL mice (Table 4). Epididymal depot weight appeared to be lower in Low LA mice, but this difference did not reach significance (t_{22}= 1.894, p=0.078). Liver and pancreas weights were comparable between both groups. In rats (Figure 2E-H), body weight gain during WSD challenge was comparable between the CTRL and Low LA group ($F_{(2, 48)}$= 0.975, p=0.385). BW ($F_{(2, 48)}$= 0.975, p=0.385), LBM ($F_{(2, 48)}$= 1.382, p=0.261), FM ($F_{(2, 48)}$= 2.547, p=0.089) gain and %FM ($F_{(2, 48)}$= 2.484, p=0.094) gain during WSD challenge was similar in both groups, despite of differences in body composition at PN42, i.e. directly after the early diet. In contrast to mice, FM gain in rats decelerated over time and LBM increased which resulted in stabilization of %FM at PN98, similar in both groups. The absence of body composition differences corresponded with similar WAT depot weights and liver and pancreas weights at PN98 in CTRL and Low LA rats (Table 4).

TABLE 4 Average weight of WAT depots and organs at PN98 of animals fed CTRL or Low LA diet from PN2-42[§]

		Mouse				Rat			
		CTRL		Low LA		CTRL		Low LA	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
WAT	Epididymal (g)	0.97	0.09	0.70	0.11	6.47	0.49	6.69	0.43
	Peri-renal (g)	0.11	0.12	0.07*	0.01	1.00	0.08	1.20	0.15
	Retroperitoneal (g)	-	-	-	-	3.39	0.24	4.44	0.41
	Inguinal (g)	0.63	0.07	0.35*	0.06	5.27	0.55	6.98	0.77
Liver (g)		1.32	0.06	1.19	0.04	15.72	0.80	16.35	0.84
Pancreas (g)		0.09	0.02	0.10	0.02	1.18	0.06	1.27	0.05

[§]n=12/group, n=4/group for inguinal WAT in mice. Retroperitoneal depot weight was not measured in mice. * p<0.05 compared to CTRL.



	Epididymal depot			
	CTRL		Low LA	
	Mean	SEM	Mean	SEM
WAT depot weight (g)	0.97	0.09	0.70	0.11
Mean cell surface area (µm²)	2438	141	2635	363

	Retroperitoneal depot			
	CTRL		Low LA	
	Mean	SEM	Mean	SEM
WAT depot weight (g)	3.39	0.24	4.44	0.41
Adipocyte size (x 10^3 µm³)	326	10.9	573	64.5*
Adipocyte number (x10^6)	9.49	0.40	7.50	0.58*

	Inguial depot			
	CTRL		Low LA	
	Mean	SEM	Mean	SEM
WAT depot weight (g)	5.27	0.55	6.98	0.77
Adipocyte size (x 10^3 µm³)	153	4.5	209	9.4*
Adipocyte number (x10^6)	20.86	0.26	21.68	0.23

FIGURE 3 Frequency distribution and average epididymal adipocyte surface area in CTRL (n= 8; white bars) and Low LA (n= 5; black bars) mice at PN98 (A). Frequency distribution of adipocyte cell size, average cell size and number of retroperitoneal (B) and inguinal (C) WAT depot at PN98 in CTRL (n= 4; white bars) and Low LA (n= 4; black bars) rats at PN98. *p < 0.05 compared to CTRL.

Effect of early diet on adipocyte size and number in WAT after WSD challenge at PN 98. At PN98, epididymal adipocyte size distribution (Figure 3A) showed that Low LA mice had more cells with a cell surface area higher than 7000 μm^2 compared to CTRL mice. This difference in adipocyte size was very moderate and not significantly reflected in the average adipocyte surface (Figure 3A; t(11)=-0.592, p=0.566), DNA per mg WAT (t(19)=-1.119, p=0.277), TG per mg WAT (t(19)=-0.609, p=0.550) or TG/DNA ratio (t(22)=0.862, p=0.399; Table 5). Only total DNA per epididymal depot (t(19)=1.909, p=0.043) was significantly lower in Low LA mice compared to CTRL mice at PN98.
Low LA rats had a higher average retroperitoneal adipocyte size compared to CTRL rats ($t_{(6)}$=-3.776, p=0.009). This was confirmed by a marked shift in cell size distribution towards a higher amount of large adipocytes in Low LA rats (Figure 3B). Retroperitoneal adipocyte number was lower in Low LA compared to CTRL rats ($t_{(6)}$= 2.829, p=0.03). Although less pronounced, average inguinal adipocyte size was also higher in Low LA rats than CTRL rats. Inguinal adipocyte number was similar in both groups (Figure 3C).

Effect of early diet on metabolic plasma parameters at PN98 after WSD challenge. Fasting plasma TG was 27% lower in Low LA mice compared to CTRL mice ($t_{(21)}$= 3.024, p=0.0023). In contrast, FFA and TC were similar in both groups as were most plasma adipokines (Table 5), except plasma resistin ($t_{(20)}$=2.046, p=0.027) and leptin ($t_{(19)}$= 3.473, p=0.002) which were significantly lower in Low LA mice compared to CTRL mice. Concerning glucose homeostasis, Low LA mice tended to have lower fasting plasma insulin ($t_{(19)}$=1.700, p=0.053) and glucose ($t_{(21)}$=1.638, p=0.056) levels, which resulted in a significantly reduced HOMA-IR ($t_{(19)}$=1.767, p=0.047;-61%) in Low LA compared to CTRL mice.

TABLE 5 Average fasting plasma parameters and DNA and TG content of epididymal WAT depot at PN98 of mice fed CTRL or Low LA diet from PN2 to 42†

			CTRL		Low LA	
			Mean	SEM	Mean	SEM
Plasma	Adipokines	Adiponectin (mg/L)	8.38	0.11	8.43	0.35
		Leptin (µg/L)	4.97	0.80	1.85*	0.42
		Resistin (µg/L)	4.06	0.25	3.24*	0.31
		MCP-1 (ng/L)	9.56	1.34	11.99	1.40
		tPAI-1 (µg/L)	1.84	0.17	1.71	0.22
		TNFα (ng/L)	4.27	0.15	4.44	0.25
		IL-6 (ng/L)	6.36	0.83	5.85	0.78
	Insulin sensitivity	Glucose (mmol/L)	16.51	0.71	14.21	1.17
		Insulin (pmol/L)	114.62	26.31	64.32	11.06
		HOMA-IR ((mmol/L*pmol/L)/22.5)	89.11	22.85	44.13*	8.84
	Lipids	TG (mmol/L)	0.32	0.03	0.24*	0.01
		TC (mmol/L)	3.26	0.14	2.86	0.24
		FFA (mmol/L)	0.50	0.06	0.43	0.03
WAT depot		DNA (ng/mg WAT)	32.3	2.6	37.02	3.4
		TG (ng/mg WAT)	847.7	26.6	868.89	18.6
		TG/DNA	29.3	3.2	25.2	3.0
		Total DNA (ng/WAT depot)	28.9	1.2	21.9*	3.4

MCP-1: Monocyte chemoattractant protein 1; tPAI-1: Total Plasminogen activator inhibitor-1; TNFα: Tumor necrosis factor α; IL-6: Interleukin-6; TC: Total Cholesterol; FFA: Free Fatty Acids. † n=9-12/group. * p<0.05 compared to CTRL.

In rats, fasting plasma glucose, insulin and HOMA-IR were similar in both groups directly after the early dietary intervention at PN42 and during the WSD challenge (Table 6). During ivGTT, Low LA rats had lower absolute peak glucose ($t_{(19)}$=2.549, p=0.020), glucose increase (peak glucose – fasting glucose; $t_{(20)}$=2.691, p=0.014) and area under the glucose curve (AUC30; $t_{(20)}$=2.937, p=0.008) compared to CTRL mice. In contrast, insulin response during ivGTT was similar in both groups. Glucose response to insulin injection during the ivITT was comparable in Low LA and CTRL rats.

TABLE 6 Glucose and insulin dynamics in fasted state and during intravenous glucose and insulin tolerance test in rats fed CTRL or Low LA diet prior to WSD†

		CTRL		Low LA	
		Mean	SEM	Mean	SEM
PN42 Fasted	Fasting glucose (mM)	9.8	0.4	9.2	0.3
	Fasting insulin (pM)	101.4	15.2	141.7	40.0
	HOMA-IR (a.u.)	45.8	7.7	60.7	17.2
PN84 ivGTT	Fasting glucose (mM)	6.9	0.3	6.1	0.3
	Fasting insulin (pM)	269.8	31.3	327.2	42.4
	HOMA-IR (a.u.)	85.4	9.5	88.9	10.8
	AUC30glucose (mM*30min)	206.4	16.7	142.4*	17.0
	peak glucose value (mM)	84.4	7.8	59.86*	5.9
	glucose increase (peak glucose-fasting glucose; mM)	77.3	7.7	54.8*	5.8
	AUC30insulin (pM*30 min)	11.1	1.0	17.2	7.4
	peak insulin value (pM)	1.9	0.1	2.0	0.2
	insulin increase (peak insulin-fasting insulin; pM)	1.6	0.1	1.7	0.2
PN90 ivITT	Fasting glucose (mM)	5.6	0.3	6.1	0.3
	Fasting insulin (pM)	402.4	36.6	348.0	39.0
	HOMA-IR (a.u.)	101.8	13.5	95.1	12.4
	glucose decrease (fasting glucose-lowest glucose value; mM)	-2.4	0.3	-2.5	0.4
	AUC60glucose (mM*60 min)	262.5	12.5	281.8	11.1
PN98 Fasted	Fasting glucose (mM)	12.7	0.7	11.4	0.8
	Fasting insulin (pM)	279.8	61.8	337.1	113.5
	HOMA-IR (a.u.)	169.5	40.9	172.6	57.8

AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance
†n=12/group. * $p<0.05$ compared to CTRL

Effect of WSD challenge during adolescence and adulthood. A non-challenged chow fed group (Chow) was included in the study to assess the effects of the WSD challenge on metabolic phenotype of mice and rats. In contrast to mice which had a 19% higher BW and 2-fold higher FM, the WSD challenge did not affect BW gain and body composition in rats (Supplementary Table 1).

SUPPLEMENTARY TABLE 1 Average body weight and body composition at PN98 of the CTRL and Low LA group challenged with WSD and a non-challenged Chow group†

	Body weight (g)	Lean body mass (g)	Fat mass (g)	%Fat mass
Mice				
CTRL	30.7 ± 0.7*	22.6 ± 0.5	8.4 ± 0.3*	27.1 ± 0.7*
Low LA	30.7 ± 0.4*	23.6 ± 0.4*	6.1 ± 0.6*,‡	20.3 ± 1.4*,‡
Chow	25.7 ± 0.6	21.7 ± 0.5	4.0 ± 0.2	15.5 ± 0.6
Rat				
CTRL	461.6 ± 11.1	373.0 ± 8.3	88.8 ± 4.8	19.1 ± 0.8
Low LA	447.7 ± 8.9	359.2 ± 7.2	88.5 ± 3.9	19.7 ± 0.7
Chow	429.3 ± 20.4	353.1 ± 16.2	76.2 ± 6.2	17.6 ± 0.9

†n=9-12/group, *$p < 0.05$ compared to Chow group, ‡$p < 0.05$ compared to CTRL group

Discussion

In rodents, we tested the hypothesis that LA intake during early postnatal life programs the metabolic response to a dietary fat challenge in adult life. The results show that reduction of postnatal n-6 PUFA intake protects against excessive fat accumulation and induces a beneficial metabolic phenotype in adulthood. The data are in accordance with the hypothesis that an early low LA diet programs the adult body composition and metabolic response via modulated adipose tissue development and function.

It is well established that malnutrition, either over- or under nutrition, in early life can exert a detrimental metabolic programming effects [44, 45]. However, studies concerning disease prevention through moderate manipulation of early life nutrition have been relatively scarce. A limited number of experimental and clinical studies have investigated effects of early pre- and postnatal n-3 LCPs on later life body composition and data have been inconclusive [13, 14, 46-49]. Studies concerning nutritional programming by n-6 PUFA are even more limited and have mainly focussed on negative effects of high n-6 PUFA exposure on adult metabolic phenotype [17, 31]. Our study showed that lowering LA content in early postnatal life can protect against excessive fat accumulation in mice in adulthood. The limited programming effect on body composition in rats could be explained by the fact that the adult WSD challenge did not

elicit increased fat mass in rats compared to standard chow which leaves little window of opportunity for improvement. In comparison, in mice the WSD challenge increased fat accumulation 2-fold compared to chow. Indeed, the WSD challenge can be considered mild, particularly for rats, compared to other experimental obesogenic diets containing 45-60 En% fat [50, 51] and even relatively moderate compared to a diet resembling human western dietary fat intake [52]. To be able to conclude that reduced postnatal LA exposure prevents excessive fat accumulation in rats, future studies should include a more severe obesogenic diet and/or a longer exposure to this diet.

The reduced adult fat mass in mice was accompanied by improved metabolic phenotype since we observed improved HOMA-IR and reduced plasma TG, leptin and resistin levels. Also, despite the absence of an evident programming effect on body composition in rats, insulin sensitivity was improved in Low LA compared to CTRL rats as illustrated by reduced ivGTT glucose levels.

In contrast to mice, reducing postnatal LA did alter body composition development in rats initially which disappeared over the WSD period. At day 42, a low LA diet resulted in reduced rat LBM and BW. DHA and ARA have differential effects on somatic growth in early life. In preterm infants, supplementation of DHA and ARA enhanced growth [53] and specifically increased lean body mass [54], whereas DHA was found to reduce growth [53, 55]. In accordance, exposure of mice to a n-3 PUFA diet compared to a n-6/n-3 and n-6 PUFA diet during gestation and lactation reduced length and BW in the pre-weaning offspring [56]. As dietary LA and ALA compete for the same enzymes for conversion to their respective LCPs, lowering LA and thereby reducing the n-6/n-3 ratio may have reduced ARA biosynthesis [57], reducing growth velocity in our rat study. Our results however clearly indicate that these initial differences in growth and body composition development do not significantly contribute to adult body composition. Nevertheless they may underlie the programming effect on metabolic profile as found in these rats independent of adult fat mass.

Our present study indicates that low LA diet in early life programs the adult body composition and metabolic response to a WSD challenge. The detailed mechanism(s) by which the early life nutrition exerts this effect is still unclear. A recent study by Paschos *et al* [58] suggests that LCPs may impact growth and body composition by changing hypothalamic regulation of satiety and energy expenditure. *Arntl* -knockout mice, carrying an adipocyte specific disruption of a circadian clock component, increased food intake during the light period

and reduced 24h energy expenditure compared to wild type controls. This was mediated through reduced circulating n-3 and n-6 LCP released from WAT, resulting in reduced expression of anorexigenic POMC and CART and increased expression of orexigenic AgRP and NPY. A high n-3 LCP diet restored hypothalamic LCP concentrations, food intake, energy expenditure and fat mass to levels of the wild type control mice on the same diet. In our study, total 24h food intake in mice and rats was similar in Low LA and CTRL groups at PN42. Since the intake pattern over 24h was not monitored, however, potential changes in circadian rhythm cannot be ruled out. At adult age, WSD intake was similar in Low LA versus CTRL rats, and even slightly higher in Low LA mice compared to CTRL mice. A fat balance study in mice showed that isoenergetic diets with different C18 fatty acids elicited differences in partitioning of energy towards storage or expenditure and energy eliminated through excreta [21]. LA tended towards enhanced energy storage in WAT and lower energy expenditure compared to saturated fatty acids, n-3 PUFA and conjugated LA [21]. One might speculate that reducing postnatal LA in our mouse study had sustained effects on adult energy partitioning and feed efficiency resulting in reduced fat mass accumulation despite enhanced food intake. Additionally, Low LA programming of the hypothalamic regulation of energy homeostasis could also play a role, considering the lipid sensing ability of the hypothalamus [59, 60].

In vitro and *in vivo* studies have previously shown that n-6 PUFAs and eicosanoids originating from ARA metabolism, stimulate preadipocyte proliferation and adipogenesis through activation of transcription factors peroxisome proliferator activated receptors (PPAR) and CCAATT/enhancer binding protein-α (C/EBP-α) [25, 31, 61]. Indeed, the n-6 PUFA status in rats has been associated with WAT activation of sterol retinoid binding protein -1c (SREBP1c) activating lipogenic enzymes including fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (G3PDH). In contrast, n-3 PUFAs are negatively associated with SREBP1c, FAS and G3PDH expression [22]. These data indicate that reducing n-6 PUFA would limit both hyperplastic and hypertrophic WAT growth. In our study, there was a marked increase in adipocyte size in Low LA programmed rats despite absence of a programming effect on fat mass. This pattern was more pronounced in the visceral than in the subcutaneous depot. The results showed a decreased retroperitoneal adipocyte number in Low LA rats. Based on known effects of ARA on adipogenesis, we hypothesize that lowering its precursor LA would result in reduced adipogenesis and thus lower adipocyte number. In accordance with

the results on rat adipocyte size, mouse epididymal adipocytes were larger in low LA programmed mice. The lower fat mass combined with larger adipocytes indirectly suggests a reduced adipocyte number, but the methodology does not allow a more reliable estimation of cell number. Nevertheless, these observations support the notion that limitation of cell number by reduced LA in early life could have limited lipid storage capacity of the WAT, and increased lipid storage per adipocyte, thereby increasing average adipocyte size.

The suggested detrimental role of excess n-6 PUFA intake in relation to obesity risk is mostly based on experimental studies by Massiera and colleagues [30, 31]. Mice exposed to a high fat, high LA diet from gestation to 8 weeks of age had increased fat mass, increased adipocyte size but also a decreased adipocyte number in adulthood compared to mice exposed to an isocaloric high fat, LA/ALA diet [31]. Exposure of multiple generations to the same diet resulted in a gradual increase in fat mass with each generation. This was accompanied by increased amount of small adipocytes in the 1st and 4th generation and increased amount of large adipocytes in the 3rd and 4th generation. The authors suggested that this reflects both recruitment of new adipocytes from the pool of precursors in the stromal-vascular fraction and hypertrophic growth by enhanced lipogenesis [30]. The reduced adipocyte number in the former study is unexpected considering promotion of adipogenesis by n-6 ARA *in vitro* described in the same paper. It is also in contrast to our findings of reduced adult adipocyte number and enhanced adipocyte size due to postnatal LA reduction. However, in vitro studies with 3T3 pre-adipocytes have shown that both n-3 and n-6 PUFAs have the capacity to stimulate PPARγ expression and thus adipocyte differentiation [62]. The pro- or anti-adipogenic effects of n-6 ARA appear to depend on intracellular cAMP levels and cyclooxygenase expression which in turn are determined by protein and carbohydrate content of the diet [61]. These data indicate that the overall dietary macronutrient composition may influence the net effect of n-6 and n-3 PUFAs on adult adiposity and metabolic phenotype. Our data suggest that reduction of postnatal LA affect development of white adipose tissue and give rise to sustained effects on adipocyte functionality during adulthood.

In conclusion, reduction of n-6 PUFA intake in early life of rats and mice programs towards a relative metabolic resistance to an obesogenic environment during adolescence and adulthood. The present results underline the importance of early life dietary lipid composition, specifically LA content, for maintaining metabolic health later in life.

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Conflicts of interest. A.O., D.K., B.J.M.H., and E.M.B. are employed by Nutricia Research. H.J.V. is a consultant for Nutricia research outside the submitted work for which his institution (Department of Pediatric Gastroenterology and Hepatology, University Medical Center Groningen, The Netherlands) is compensated financially.

Authorship. A.O., D.K., B.J.M.H. and E.M.B. designed the research; A.O. and D.K. conducted the research; A.O., B.J.M.H. and D.K. analyzed data, A.O. wrote the paper, H.J.V. reviewed the manuscript, E.M.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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Chapter 5

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Size and phospholipid coating of lipid droplets in the diet of young mice modify body fat accumulation in adulthood

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Abstract

Besides contemporary lifestyle factors which contribute to the increased obesity prevalence worldwide, early nutrition is also associated with sustained effects on later life obesity. We hypothesized that physical properties of dietary lipids contributes to this nutritional programming. We developed a concept infant formula (IMF) with large, phospholipid coated lipid droplets (Nuturis®) and investigated its programming effect on metabolic phenotype later in life.
Male C57Bl/6j mice were fed a control formula (Control IMF) or Nuturis® (Concept IMF) diet between postnatal day (PN)16 and 42. All mice were subsequently fed a Western style diet (WSD) until PN126. Body composition was monitored repeatedly by Dual Energy X-ray Absorptiometry between PN42 and 126.
The results showed that the Concept IMF slightly increased lean body mass compared to Control IMF at PN42 but did not affect fat mass. Upon 84 days of WSD feeding, the Concept IMF group showed reduced fat accumulation compared to Control IMF. Additionally, fasting plasma leptin, resistin, glucose and lipids were significantly lower in the Concept IMF group.
In conclusion, large phospholipid coated lipid droplets in young mice reduced fat accumulation and improved metabolic profile in adulthood. These data emphasize that physical properties of early dietary lipids contribute to metabolic programming.

Introduction

Childhood obesity incidence is rising worldwide [1, 2]. Since available treatments have only limited success and specifically early onset obesity increases the risk of early onset of metabolic disease [3], prevention is of the utmost importance. Although evidence is not entirely conclusive, meta-analyses of observational studies have indicated that breast-feeding is associated with a moderately reduced risk of childhood obesity and metabolic disease (reviewed in [4, 5]). The association between infant feeding and long term metabolic health could be related to early life growth trajectory: rapid weight gain during infancy has been associated with increased visceral adipose tissue in young children [6] as well as adults [7]. Since rapid weight gain during early postnatal life in infants is associated with specifically fat mass (FM) gain [8] and a higher risk on obesity [7], breast-feeding may protect by moderating early growth. Indeed, breast-feeding has been shown to result in lower weight-for-length and FM from 7 months onwards compared to formula-feeding [9]. Taken together these data suggest that infant nutrition affects the early development of adipose tissue and has a sustained impact on body composition later in life.

The sustained protective effects of breast-feeding on obesity and metabolic health later in life is probably multi-factorial and could include feeding regimen, maternal behaviour, mode of food delivery and nutritional composition [10]. Human milk (HM) contains both nutrients and bioactive compounds, including endocrine and immune factors, securing healthy growth and development during infancy [11-13]. Dietary lipids are a key source of energy and essential nutrients during infancy. A comparison between lipids in mature HM and those in current infant milk formulas (IMF) shows differences in lipid classes [14], fatty acid (FA) composition [15] and physical structure [16]. HM lipid globules are secreted from the mammary epithelial cells through exocytosis into the alveolar lumen. This process produces droplets with a core consisting of triglycerides and cholesteryl-esters surrounded by a native milk fat globule membrane composed mainly of phospholipids (PL), proteins and enzymes, free cholesterol and glycoproteins. The lipid droplet size ranges between 1 and 10 µm with an average mode diameter of 4 µm in mature milk [17]. In contrast, lipid droplets produced during processing of IMF have a diameter of approximately 0.3-1.0 µm and consist of a triglyceride core with milk proteins adhering to the globule surface [16].

We hypothesized that the specific surface composition and size of the lipid droplets in HM contribute to the long-term benefits of breastfeeding on metabolic health later in life. To test this hypothesis, we developed a concept IMF with lipid droplets with physical properties similar to those in HM, i.e. larger in size and coated with PL (Nuturis®). We compared this concept IMF with a control IMF in a mouse model of nutritional programming [18]. A diet containing either control or concept IMF were provided for 26 days in early life, after which all mice were fed a "moderate" western style diet (WSD; 10 w/w% fat and 0.1 w/w% cholesterol) for 84 days during adolescence and adulthood. We subsequently determined the effects of the control and concept IMF in early life on adult body composition and metabolic health.

Methods

Animals and study design. All experimental procedures were approved by the Animal Experimental Committee DEC-Consult, Bilthoven, The Netherlands and complied with the principles of good laboratory animal care. Mice were housed at facilities of the Wageningen University and Research Centre (Wageningen, The Netherlands) on a 12/12h light/dark cycle (light on 06:00h=Zeitgeber time (ZT) 0h) in a temperature- and humidity-controlled room (21 ± 2°C and 50 ± 5%, respectively). Food and water were available *ad libitum* during the entire experimental protocol, except before blood sampling when animals were fasted for at least 4h during the light phase (from 7:30 AM onwards). Food intake was measured per cage bi-weekly from postnatal day (PN) 42 onwards. Individual food intake could not be measured before PN 42, because the diets were presented in the form of dough in the cage and were provided to the dam and litters from PN 16 onwards. Body weight (BW) was measured per litter before weaning at PN2, 7, 14 and 21 and individually after weaning on a weekly basis. Male and female C57Bl/6j mice were fed standard semi-synthetic rodent chow upon arrival and time-mated. After birth, litters were culled to four male and two female pups per dam on postnatal day (PN)2 and randomly assigned to diet containing a control IMF (Control IMF) or to the Nuturis® concept IMF (Concept IMF) from PN16 onwards. Pups had access to their respective diets but were also allowed to drink milk until weaning at PN21 when male pups were housed in pairs and continued their respective diets until PN42. Subsequently,

male pups of the Control IMF and the Concept IMF group changed to a moderate WSD (10% w/w fat) during their adolescence and adulthood until dissection on PN126 (Figure 1). To confirm effectiveness of the WSD challenge in adolescence and adulthood, we included a separate non-challenged Reference group, which was maintained on a standard semi-synthetic rodent chow (AIN93) during the entire experimental protocol.

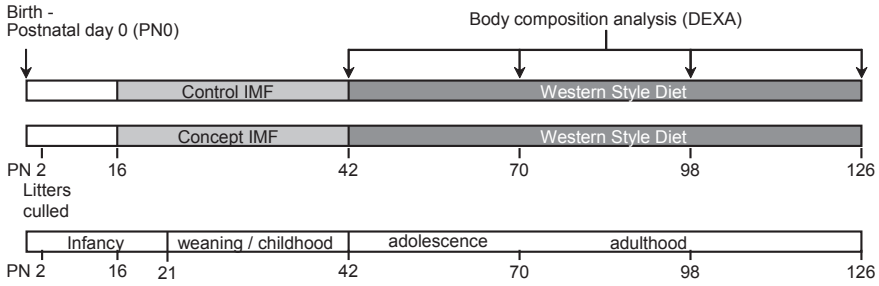

FIGURE 1 Study design from postnatal day (PN) 0 to 126.

IMF production and analyses. The Control IMF was produced according to standard stage I IMF recipe and processing procedure (Danone Research, Wageningen, The Netherlands). The Concept IMF was generated by adding 2 g/l PL of buttermilk serum origin (BAEF powder, Corman Food industry, Goé, Belgium) and adjusted processing to obtain PL coated lipid droplets larger than those in standard IMF. Briefly, the polar PL were added to the aqueous phase and blended with the lipid phase containing the neutral vegetable lipids to generate a PL coating around the lipid droplets. Homogenization pressure during processing was reduced to secure a homogenous mixture of ingredients but also to retain a large lipid droplet size [19].

Lipid droplet size distribution was measured using a Mastersizer laser light-scattering analyser with a refractive index ratio of 1.46 (Mastersizer 2000, Hydro 2000G, Malvern Instruments Limited, Worcestershire, UK). Additionally, lipid droplets were visualized by laser scanning microscopy (Zeiss Laser Scanning Microscope 510, Axiovert 100M, Carl Zeiss Benelux, Sliedrecht, the Netherlands) to obtain high resolution images and dual staining of the fat core and PL coating of the lipid droplets (analyses performed at the Centre for Advanced Microscopy of the Swammerdam Institute for Life Sciences at the University of Amsterdam, courtesy of Professor Dr Gadella). The IMFs were reconstituted in a binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4).

Subsequently fluorescent Annexin V Alexa Fluor®488 (Invitrogen molecular probes, Bleiswijk, The Netherlands) and Nile Red (Sigma-Aldrich, Zwijndrecht, The Netherlands) probes were added and incubated for at least 5 min in the dark at room temperature. Twenty five µl of each sample was spotted into an 8-well chamber slide. Images of representative areas of each sample were taken using a Zeiss Plan-Neofluar 100 x magnification objective with a numerical aperture of 1.3. The stability of the concept IMF lipid structure was analyzed repeatedly over time and was considered stable for at least 6 months.

Experimental diets. All experimental semi-synthetic diets (Research Diet Services, Wijk bij Duurstede, The Netherlands; Table 1) had a macro- and micronutrient composition according to American Institute of Nutrition formulation of AIN93G purified diets for laboratory rodents [20]. The postnatal IMF diets contained 28.3 w/w% Control or Concept IMF and were complemented with protein and carbohydrates. The moderate WSD consisted of 10 w/w% fat (5 w/w% lard and 0.1 w/w% cholesterol).

Body composition measurements. On PN42, 70, 98 and 126 fat mass (FM) and lean body mass (LBM) were measured by Dual Energy X-ray Absorptiometry (DEXA) scan under general anaesthesia (isoflurane/N_2O/O_2) using a PIXImus imager (GE Lunar, Madison, WI). FM was also calculated as percentage of BW (%FM). BW, LBM, FM and %FM gain (dBW, dLBM, dFM and d%FM respectively) between PN42 and 126 was calculated by subtracting PN42 from PN70, 98 and 126 values.

Blood Sampling and Dissection. On PN126, mice were anaesthetized (isoflurane/N_2O/O_2) after 4h fasting (from 7:30 AM) during the light phase for final DEXA scan and killed with cervical dislocation after blood sampling through heart puncture.

Blood samples were collected in K_3EDTA-coated 1 ml microtubes. Plasma was obtained after centrifugation at 1350*g* for 12 min at 4°C and stored at -80°C. Liver, pancreas, *m.tibialis*, brain, epididymal (EPI), retroperitoneal (RP) and inguinal (ING) white adipose tissue (WAT) depots were dissected and weighed.

Plasma Analyses. Total adiponectin was determined using a mouse adiponectin ELISA kit (Linco Research, Millipore Bioscience, Billerica, MA). Plasma insulin, leptin, monocyte chemoattractant protein 1 (MCP-1), total plasminogen activator inhibitor-1 (tPAI-1), interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and resistin were measured using a mouse serum adipokine lincoplex kit (Linco

TABLE 1 Composition of the experimental diets

		Postnatal IMF diet		WSD
		Control	Concept	
Carbohydrates	(g/kg)	645	645	600
Sugars	(g/kg)	235	235	150
Dextrose	(g/kg)	5	5	150
Lactose	(g/kg)	145	145	0
Sucrose	(g/kg)	85	85	0
Polysaccharides	(g/kg)	410	410	450
Maltodextrine	(g/kg)	50	50	250
Starch	(g/kg)	360	360	200
Protein	(g/kg)	179	179	179
Soy	(g/kg)	150	150	179
Whey	(g/kg)	17	17	0
Casein	(g/kg)	12	12	0
Fat	(g/kg)	70	70	100
Oil blend*	(g/kg)	69.1	53.0	50
Milk fat	(g/kg)	0.9	17.0	0.0
Lard	(g/kg)	0	0	50
Saturated Fatty Acids	SFA (g/kg)	28.5	31.4	41.9
Mono Unsaturated Fatty Acids	MUFA (g/kg)	30.5	28.8	42.3
Poly Unsaturated Fatty Acids	PUFA (g/kg)	11.0	9.8	13.2
Linoleic acid (LA)	(g/kg)	9.3	8.4	11.9
α-linolenic acid (ALA)	(g/kg)	1.7	1.5	1.3
LA/ALA		5.5	5.7	9.5
Phospholipids**	(g/kg)	0.1	3.6	0.0
Cholesterol	(mg/kg)	4.7	65	1000
Cellulose (Vitacel L 600-20)	(g/kg)	50	50	50
mineral mix	(g/kg)	35	35	35
vitamin mix	(g/kg)	10	10	10
choline bitartrate	(g/kg)	2.5	2.5	2.5
L-cystein	(g/kg)	3	3	3
tert-butylhydroquinone	(g/kg)	0.014	0.014	0.014

[a] Mixture of oils, among others canola, sunflower, fish, coconut and palm oil; [b] Phospolipids (phosphatidyl choline, 31.1%; phosphatidyl ethanolamine, 24.6%; sphingomyelin, 19.7%; phosphatidyl serine, 11.5%; phosphatidyl inositol, 6.6%) were derived from butter milk

Research). Samples, standards and quality control were prepared according to manufacturers' protocol and fluorescence was measured using a Bio-Plex™ 200 Luminex instrument (Bio-Rad Laboratories Inc.®, Hercules, CA). Fasting plasma total cholesterol (TC; liquicolor cholesterol oxidase-phenol aminophenazone (CHOD-PAP) method, Instruchemie, Delfzijl, The Netherlands), triglycerides (TG) (glycerol phosphate oxidase (GPO) trinder method, Sigma Aldrich) and glucose (glucose oxidase-phenol aminophenazone (GOD-PAP) method, Roche diagnostics, Almere, The Netherlands) were measured colorimetrically and analysed with a microplate imaging system (Bio-Rad Laboratories, Veenendaal, The Netherlands). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin (glu(mmol/l) * ins (pmol/l)/22.5) as an indirect measure of insulin sensitivity.

Statistical Analysis. Statistical analyses were performed using SPSS 15.0.1 software (SPSS Benelux, Gorinchem, The Netherlands). Variables were checked for Gaussian distribution with the Shapiro-Wilkes test. Levene's test for equality of variance was used to assess the probability of different variances among treatment groups. Direct effects of experimental diet (Control IMF, Concept IMF) were analyzed by means of Student's t-test. Programming effects of experimental diet on development of body composition during WSD were analyzed using a Repeated-Measures analysis of variance (ANOVA). *Post hoc* analyses were performed on confirmed Diet*Time interactions by Univariate ANOVA of PN42, 70, 98 and 126, separately. Effects of experimental diets on WAT weight, organ weight, food intake and plasma parameters were analysed by means of Student's t-test. Data are presented as mean ± SEM unless otherwise indicated. Differences were considered significant at $p < 0.05$.

Results

IMF analyses. IMF processing and addition of 2 g/l PL from buttermilk serum powder resulted in the Concept IMF (Nuturis®) with PL-coated lipid droplets (Figure 2). A clear shift was observed towards larger lipid droplets in the Concept IMF compared to Control IMF (Figure 3). In the Concept IMF, more than 50% of the total volume of lipid particles was present in droplets with a diameter ranging between 2 and 12 µm. Less than 35% of the volume was present in particles with a diameter of 2 µm or smaller. In contrast, less than 15% of the particle volume was represented in droplets with a diameter between 2 and 12 µm in the Control IMF and 85% of the total volume was present in the droplets of less than 2 µm. Peak diameter of the particle size distribution was 0.40 and 6.25 µm in the Control and Concept IMF, respectively (Figure 3). FA composition was comparable in Concept and Control IMF. PL content of Concept IMF was 3.6 g/kg compared to 0.1 g/kg in the Control IMF containing diets (Table 1).

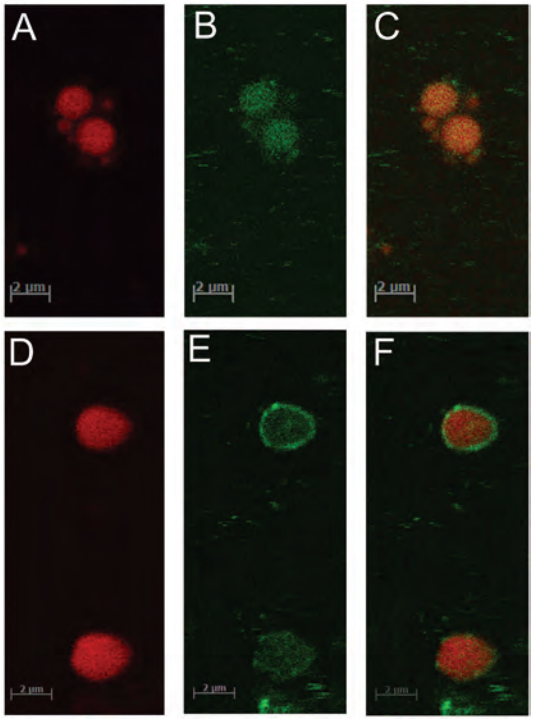

FIGURE 2 Images of confocal laser scanning microscopy of Nile Red stained fat core (A and D), Annexin-V Alexa Fluor® 488 stained PL coating (B and E), and dual staining (C and F) of Control IMF and Concept IMF, respectively. Magnification x4250, scale bar 2 µm.

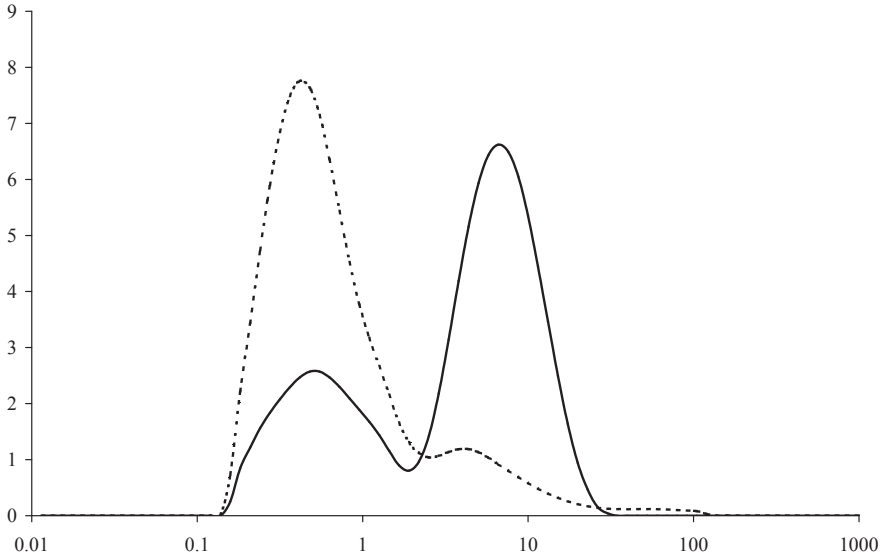

FIGURE 3 Particle size distribution of Control (dashed line) and Concept IMF (solid line).

Direct effects of IMF-based diets on growth and body composition. An early intervention with Concept IMF resulted in a 8% higher LBM at PN 42 ($t_{(20)}$= 5.417, p= 0.031) compared to Control IMF directly after intervention (Figure 4A). In contrast, FM ($t_{(20)}$= 0.007, p= 0.936) and BW ($t_{(20)}$= 1.429, p= 0.246) were similar in both groups.

Programming effects of IMF-based diets on food intake, BW gain and body composition development. Average food intake from PN42 to 126 was comparable between groups (12.7 ± 0.3 and 13.1 ± 0.4 kcal/day/animal for Control and Concept IMF group, respectively). To distinguish between direct and programming effects of early diet on development later in life, we calculated absolute values of BW, LBM, FM and %FM as well as relative changes (e.g. dBW, dLBM, dFM and d%FM) during WSD challenge (Figure 4B). During WSD challenge from PN42 to 126, BW gain was 17% lower in Concept IMF compared to Control IMF group ($F_{(2, 40)}$=4.630, p=0.016). However, differences were moderate since *post hoc* analyses could not confirm significant differences in dBW at individual timepoints.

Relative LBM gain during WSD was similar in Control and Concept IMF group (dLBM: $F_{(1, 20)}$=0.079, p= 0.978). In contrast, fat mass development in adolescence and adulthood was affected by early postnatal diet. Both FM ($F_{(3, 60)}$= 2.974, p< 0.039) and %FM ($F_{(3, 60)}$= 3.914, p= 0.013) gain after WSD challenge was lower

in animals previously fed Concept IMF compared to animals fed Control IMF ($F_{(3, 60)} = 2.974$, $p = 0.039$). *Post hoc* analyses of individual time points confirmed a 24% and 28% lower dFM in Concept IMF compared to Control IMF group at PN98 and 126, respectively. Additionally, d%FM was 31, 30 and 28% lower in Concept IMF compared to Control IMF group at PN70, 98 and 126, respectively.

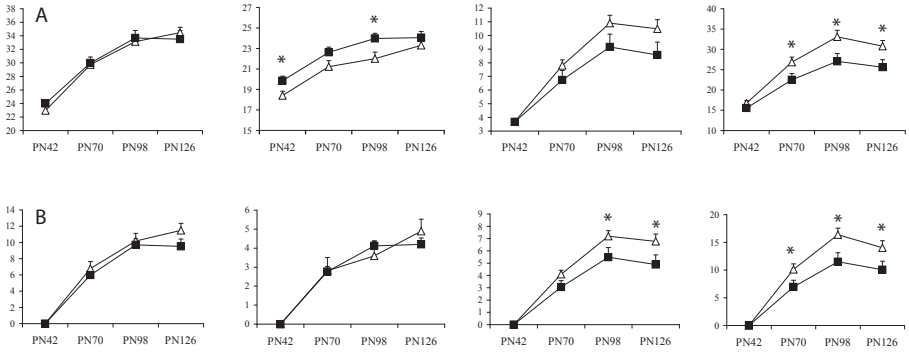

FIGURE 4 Body composition development during WSD challenge (PN42-126) of mice fed Control IMF (△) or Concept IMF (■) until PN42. A) Body Weight (BW), Lean Body Mass (LBM), Fat Mass (FM) and Relative Fat Mass (%FM); B) Relative BW, LBM, FM and %FM gain (dBW, dLBM, dFM and d%FM respectively); n=10-12/group; * $p<0.05$

Organ weight. Weights of WAT depots at PN126 were only moderately affected by postnatal diets (Table 2) and subsequent WSD (data not shown). Comparison of Control and Concept IMF group did not show significant differences in WAT depot or organ weight.

TABLE 2 Average weight of WAT depots and organs at PN 126 of mice early Control (n=10) or Concept IMF (n=14) prior to WSD.

		Control IMF	Concept IMF
WAT	Epididymal (g)	1.4 ± 0.1	1.3 ± 0.1
	Retroperitoneal (mg)	379 ± 32	349 ± 36
	Inguinal (mg)	772 ± 44	666 ± 51
Liver (g)		1.57 ± 0.1	1.6 ± 0.1
Pancreas (mg)		174.2 ± 12.8	174 ± 13
M. tibialis (mg)		94.3 ± 2.1	94 ± 2
Brain (mg)		416.5 ± 7.6	417 ± 8

Plasma parameters. Plasma adiponectin, MCP-1, tPAI-1, IL-6 and TNFα did not differ significantly between both groups at PN126 (Table 3). However, plasma leptin ($t_{(24)}$= 2.324, p= 0.029) and resistin ($t_{(23)}$= 1.913, p= 0.037) were significantly lower in Concept IMF compared to Control IMF group. At PN126, fasting TG ($t_{(25)}$= 3.438, p= 0.002) and TC ($t_{(29)}$= 2.627, p= 0.014) levels were lower in Concept IMF compared to Control IMF group. Moreover, glucose homeostasis during fasting different due to early postnatal diet with a significant lower plasma glucose ($t_{(24)}$=2.531, p=0.018) and HOMA-IR ($t_{(25)}$= 1.741, p=0.047) in Concept IMF compared to Control IMF fed animals. Fasting insulin was similar in both groups.

TABLE 3 Average fasting plasma parameters at PN 126 of mice fed Control (n=10) or Concept IMF (n=14) prior to WSD.

		Control IMF	Concept IMF
Adipokines	Adiponectin (mg/L)	8.95 ± 0.55	7.75 ± 0.50
	Leptin (µg/L)	1.48 ± 0.16	1.26 ± 0.17*
	Resistin (µg/L)	1.55 ± 0.09	1.34 ± 0.05*
	MCP-1 (ng/L)	22.1 ± 5.0	19.1 ± 3.4
	tPAI-1 (µg/L)	2.04 ± 0.19	2.29 ± 0.48
	TNFα (ng/L)	10.3 ± 6.8	5.05 ± 1.0
	IL-6 (ng/L)	8.57 ± 2.3	22.04 ± 10.1
Glucose homeostasis	Glucose (mmol/L)	13.0 ± 0.8	10.4 ± 0.6*
	Insulin (pmol/L)	257.3 ± 28.0	219.2 ± 45.8
	HOMA-IR ((mmol/L * pmol/L)/22.5)	148.8 ± 20.0	102.5 ± 17.7*
Lipids	Triglycerides (mmol/L)	0.57 ± 0.02	0.42 ± 0.04*
	Total Cholesterol (mmol/L)	3.48 ± 0.20	2.68 ± 0.23*

*p < 0.05 compared to Control IMF

Effect of WSD challenge during adolescence and adulthood. Average food intake from PN42 to 126 was comparable between the non-challenged Reference (12.6±0.4 kcal/day/animal) and WSD challenged groups.

Both absolute BW and LBM gain from PN42 to 126 was comparable between the non-challenged Reference group and both WSD challenged (Control and Concept IMF) groups. FM and %FM gain was respectively 51% and 73% higher

in the Control IMF group compared to non-challenged Reference group. In contrast, FM did not differ significantly between Concept IMF group fed WSD from PN42 to 126 and non-challenged Reference group (data not shown). Between PN98 and 126, FM gain in both WSD challenged groups decreased, resulting in %FM values closer to the values of non-challenged Reference mice (data not shown). Additionally, fasting plasma TG and TC were increased due to WSD in adolescence and adulthood (TG: 0.25 ± 0.02 versus 0.57 ± 0.02, TC: 2.44 ± 0.24 versus 3.48 ± 0.20 in non-challenged Reference and Control IMF, respectively). In contrast, plasma adipokines and glucose homeostasis were unaffected by adult WSD.

Discussion

The present study strongly suggests that physical properties of dietary lipids contribute to body composition and metabolic health later in life. Besides distinct differences in FA composition and lipid classes, HM and IMF show a clear difference in the physical structure of lipids: Raw milk lipid droplets are larger and coated with a native milk fat globule membrane compared to lipid droplets in infant formula which are predominantly covered with milk proteins [16].

The most striking and sustained effect of the altered postnatal diet with a lipid structure closer to that of human milk was the difference in fat mass gain and improved metabolic profile once switched to the WSD diet. Absolute and relative FM were similar in all groups directly following the diet intervention at PN42, but postnatal Nuturis® reduced the fat percentage increase due to the WSD challenge by ~30%.

The amount of body fat is strongly related to the prevalence of cardio metabolic risk factors both in children [21] and adults [22], indicating that reducing FM reduces disease risk. Evidence from clinical studies showed that 5% reduction in body fat percentage improved cardio metabolic risk profile in obese children [23] and adults [24]. Very few experimental studies have focussed on prevention of obesity and metabolic disease by nutrients in early life. Most studies on nutritional programming have investigated factors in the fetal and/or postnatal environment that predispose to disease (reviewed in [25]) rather than to *prevention* of disease. Additionally, subsequent postnatal exercise [26] and pharmacological interventions [27] ameliorated adverse metabolic phenotype

caused by intrauterine growth retardation, indicating that early postnatal life is a critical period in development. Few studies have shown that exposure to n3 long-chain polyunsaturated fatty acids (LCP) throughout life protects against the detrimental effects of an adverse fetal environment on blood pressure, inflammation, leptin sensitivity and glucose homeostasis in adult rats [28, 29]. Additionally, n3 LCP supplementation during pregnancy and lactation reduced BW of adult rat offspring on a standard chow by 5% [30]. In accordance, we have shown previously that 5% n3 LCP in postnatal diet of mice lowers adult FM by 28% upon 56 days of WSD feeding [18]. The magnitude of this nutritional programming effect on adult FM closely resembles that of the present study which investigated a postnatal diet with distinct physical and compositional lipid properties without any significant change in FA composition.

Early postnatal life is considered a critical period in development of the metabolic system and programs its functionality and metabolic homeostasis later in life [31-33]. For instance, development of the endocrine pancreas continues until four years after birth in humans and its structure and functionality is affected by nutrition during both fetal and postnatal life [34, 35]. Adult adipocyte number in white adipose tissue, another key endocrine tissue in metabolism, is also set during early life [36] and is affected for instance by dietary FA [37]. Other lipid components may also play a role. For example, dietary cholesterol in infancy affects adult cholesterol metabolism [38, 39]. Moreover, postnatal supplementation of a milk fat globule membrane fraction containing bioactive lipids such as phospholipids, gangliosides and other polar lipids enhanced BW gain and improved brain function in young rats [40]. Thus, dietary lipids during this sensitive period can influence the development of organ structures and physiological systems and thus their functionality.

The mechanism underlying the nutritional programming effects of Nuturis® found in the present study remains elusive. Metabolic development may have been modulated by differences in absorption and digestion kinetics. In adults, small lipid droplets from a fat emulsion were hydrolyzed faster than larger lipid droplets [41], whereas small IMF lipid droplets without PL coating were hydrolyzed slower in preterm infants than large HM milk lipid droplets with a native membrane [42]. This suggests that 1) size and surface composition play a differential role in digestion kinetics and 2) these are differentially handled in early childhood and adulthood. It has been shown in rats that these physical properties also modulate plasma triglyceride appearance [43] and ß-oxidation

rate [44]. Taken together, these studies indicate that differences in lipid digestion and absorption could influence utilization of these lipids for either ß-oxidation or lipogenesis. Whether this is also true for the Concept IMF in the present study and if this affected early growth rate remains to be investigated. Unfortunately, the experimental design did not allow recording of preweaning food intake and body weight gain per individual pup which limits our understanding concerning direct effects of the Concept IMF on early growth. Future studies are required to confirm our hypothesis and acquire more insight on modulation of early growth trajectories by physical properties of lipids in infant nutrition.

The observed adult phenotype appeared to be dependent on age and/or diet challenge: At PN126, FM gain reduced in both challenged groups, as did the difference between initially divergent FM gain in Control and Concept IMF group. The results do not confirm whether the Concept IMF programs towards delayed ("slower") fat accumulation or rather towards a sustained reduced fat accumulation during aging. The WSD challenge was very mild compared to obesogenic models using 45-60 energy percent (En%) fat diets [45, 46] to model for mild obesogenic dietary patterns of many western and developing countries. However, the WSD was even relatively moderate compared to a diet resembling human western dietary fat intake [47] and was therefore insufficient to sustain an adverse metabolic phenotype compared to the non-challenged Reference group. To be able to conclude that the Concept IMF either delays, reduces or prevents excessive fat accumulation later in life, future studies should include a more severe obesogenic diet.

Since food intake during adulthood was similar between groups, the reduced FM in Concept IMF mice cannot be explained by a difference in energy intake. The moderately higher LBM in Concept IMF mice at PN42 and after WSD at PN126 indicates a different utilization of nutrients compared to Control IMF mice. This shift may direct towards development of LBM rather than FM, ultimately resulting in a different growth trajectory.

The improved adult body composition in the Concept IMF group was accompanied by an improved metabolic profile, as shown by lower fasting plasma TG, TC and glucose concentrations compared to the Control IMF group. Since systemic adipokine levels were not significantly perturbed by the moderate WSD challenge compared to normal rodent chow, the limited improvement in adult adipokine profile due to Concept IMF was to be expected. However, lower plasma leptin and resistin in the Concept IMF group suggest an

improved metabolic profile compared to Control IMF since leptin is correlated with FM, resistin is associated with insulin resistance and both are increased in obese adolescent subjects [48].

In conclusion, the present study underlines that lipid structure, e.g. large, PL-coated lipid droplets, in postnatal diet reduced fat accumulation and improved metabolic profile in mice during adolescence and adulthood. Our data suggest that protective effects of breastfeeding may be partly explained by the physical properties of lipids in HM. Taken together, it is becoming apparent that not only dietary FA composition (e.g. essential FA and LCP content), but also physical properties of dietary lipids contribute to dietary lipid quality and play a role in programming of adult metabolic health. Future studies should investigate how distinct features of lipid quality, for instance lipid composition and physical lipid structure, interact.

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Chapter 6

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Effect of dietary lipid structure in early postnatal life on mouse adipose tissue development and function in adulthood

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Abstract

Obese individuals have more (hyperplastic) and larger (hypertrophic) adipocytes in their white adipose tissue (WAT) than normal weight individuals. The difference in cell number emerges early in childhood, suggesting that this is a critical period for susceptibility to obesity. Breastfeeding has been shown to be protective against obesity and we have previously shown in mice that the physical structure of lipids in human milk may contribute to this protective effect. In the present study, we investigate how differences in physical structure of lipids in the early diet may modulate adipose tissue development. Male mice were fed a diet containing control infant milk formula (Control IMF) or Nuturis® (Concept IMF with large phospholipid-coated lipid droplets) from postnatal day (PN)16 to 42. Subsequently, mice were challenged with a moderate Western style diet (WSD) until PN98 and body composition was monitored by dual x-ray absorptiometry. Epididymal WAT was analysed for adipocyte size, number and gene expression of metabolic transcription factors. Early feeding with Concept IMF reduced fat accumulation during the WSD challenge by 30% compared to Control IMF. It reduced adipocyte size without affecting adipocyte number in adult mice. The Concept IMF decreased WAT expression of PPARγ, C/EBP and RXRα in adulthood, key regulators of metabolic activity. In conclusion, Concept IMF in early life reduced susceptibility to obesity in adult life, by preventing adipocyte hypertrophia upon adult dietary challenge without affecting adipogenesis. These data emphasize the importance of physical properties of dietary lipids in early life on obesity risk later in life.

Introduction

Development of human white adipose tissue (WAT) starts in the third trimester of gestation and continues in postnatal life through proliferation of pre-adipocytes and subsequent differentiation resulting in mature adipocytes storing energy in the form of lipids [1]. Adipocyte number increases throughout childhood and adolescence to plateau and remain constant in adulthood [2, 3]. Surprisingly, adult cell turnover is similar in obese and lean subjects although adult obese subjects have more adipocytes compared to lean subjects. Interestingly, these differences in adipocyte number already emerge early in childhood suggesting that this may be a critical period for WAT development and obesity risk [2, 3].

Comparable to human ontogeny, WAT development in rodents is initially driven by hyperplastic growth (increase in number) and subsequent hypertrophic growth (increase in cell size). When fed standard chow, total cell number is established in rodents between 9-18 weeks of age [4-6], whereas adipocyte size can increase until senescence [7]. Additionally, adipocyte size and number are higher in obese compared to lean rats [8] and hyperplastic and hypertrophic growth dynamics differ according to fat depot, gender and diet [9, 10].

Both hypertrophic and hyperplastic adipocyte growth are regulated by the concerted actions of several transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ), retinoid X receptor α (RXRα), and the CCAAT/enhancer binding protein (C/EBP) family members [11, 12]. *In vitro* studies have shown that PPARγ -deficient embryonic stem cells are unable to differentiate into adipocytes [13]. Additionally, overexpression of PPARγ in mature 3T3-L1 adipocytes reduces lipolysis, increases cell size and triglyceride (TG) content compared to control adipocytes. In contrast, a dominant negative PPARγ mutant has the opposite effect of increasing lipolysis and reducing FA uptake [14]. Data from human and mouse genetic studies showed that deletion of the WAT specific PPARγ2 isomer reduces adipogenesis and impairs insulin sensitivity. Thus, PPARγ is clearly a key regulator to maintain lipid metabolic homeostasis. Many polyunsaturated fatty acids (PUFA) and lipid-derived mediators can act as endogenous PPARγ ligands [15]. This enables sensing of nutritional signals that can be translated into a metabolic response to maintain homeostasis.

Experimental studies have provided compelling evidence for sustained effects of early life nutrition on adipose tissue development and function. For instance,

both maternal obesity and intra-uterine growth retardation (IUGR) induced by a 50% food-restricted diet in rats resulted in up-regulation of PPARγ, RXRα and C/EBP family members in the offspring directly after birth, which was associated with increased adiposity as adults [16, 17]. A clinical study investigating the obesogenic effect of a specific human PPARγ polymorphism showed that breastfeeding was correlated to a lower body mass index in adolescents carrying this polymorphism, suggesting that protective effects of breastfeeding on the development of obesity are mediated, at least in part, via PPARγ [18].

Protective effects of breastfeeding on obesity and metabolic health later in life are probably multi-factorial [19]. One specific aspect is the fact that human milk contains large lipid droplets with an average mode diameter of 4 µm surrounded by a native biological membrane composed of phospholipids (PL), proteins, enzymes, cholesterol and glycoproteins [20]. In contrast, infant milk formula (IMF) contains small lipid droplets with an average mode diameter of 0.3 - 0.5 µm lacking such a biological membrane coating. We have previously shown that this physical lipid structure may play a role in programming of obesity risk later in life [21]. We developed a concept IMF in which the lipid droplets had physical properties similar to those in HM, i.e. larger in size and coated with PL [Nuturis®] and investigated whether postnatal exposure to this Concept IMF protects against excessive fat accumulation in adulthood. Although adult body weight was comparable between Control and Concept IMF fed animals challenged by a mild Western Style Diet (WSD) from adolescence, adult fat mass was 28% lower in the animals exposed to the Concept IMF in early life. This was accompanied by an improved metabolic profile since adult fasting plasma lipids as well as plasma glucose, resistin and leptin levels were lower in the Concept IMF group. Taken together, these data suggest that the sustained effects on adult fat mass were mediated through differences in WAT development. We hypothesized that exposure of preweaning mice to the Concept IMF with large phospholipid coated lipid droplets alters WAT development by limiting hyperplastic and hypertrophic growth affecting the metabolic functions of WAT and ultimately leading to reduced adult body fat accumulation.

In the present study, we investigated morphological WAT characteristics and gene expression of transcription factors which play a key role in adipocyte differentiation and lipogenesis to gain more insight in the mechanism underlying programming effects of the Concept IMF on adult body composition.

Methods

Animals and study design. All experimental procedures were approved by the Animal Experimental Committee DEC-Consult, Bilthoven, The Netherlands and complied with the principles of laboratory animal care. Mice were housed at Wageningen University and Research Centre (CKP, WUR, Wageningen, The Netherlands) on a 12/12h light/dark cycle (light on 06:00h=Zeitgeber time (ZT) 0h) in a temperature- and humidity-controlled room (21±2°C and 50±5%, respectively). Food and water were available *ad libitum* during the entire experimental protocol. Body weight (BW) was measured per litter before weaning and individually after weaning twice a week. Male and female C57Bl/6j mice were time-mated. Dams were assigned to AIN93G diet during pregnancy and lactation. After birth, pups were randomly cross-fostered, i.e. all pups from one litter were assigned to different dams, and litters were culled to four male and two female pups on postnatal day (PN) 2. Litters were randomly assigned to one of two experimental diets on PN16. After weaning at PN21, female pups were killed; male pups were housed in pairs and continued their respective diets until PN42, followed by a western style diet (WSD). Food intake was measured weekly per cage between PN42 and 98.

To address the effects of the WSD challenge in adolescence and adulthood, we included a separate non-challenged reference group (Reference), which was maintained on Control IMF until PN42 and then changed to standard semi-synthetic rodent chow until dissection on PN98 (Figure 1). BW, fat mass (FM) and lean body mass (LBM) were measured on PN42, 70 and 98 by DEXA (PIXImus, GE Lunar, Madison, WI, USA) under general anaesthesia (isoflurane/N_2O/O_2). Relative fat mass (%FM) was calculated as percentage fat mass of total body weight.

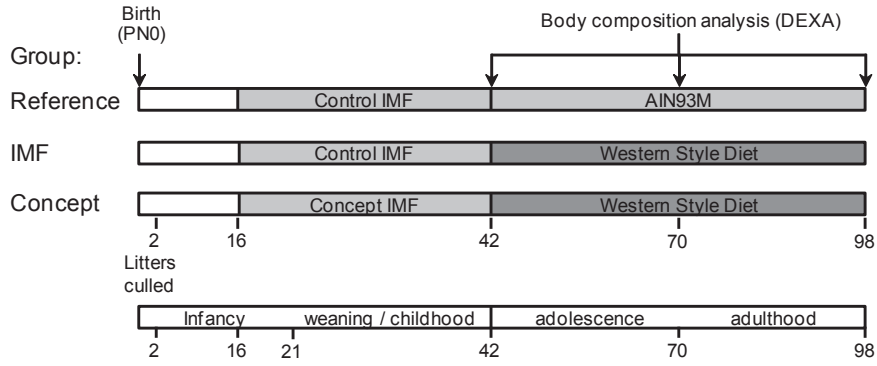

FIGURE 1 Study design

IMF production and analyses. As basis for Control IMF rodent diet, an IMF powder was produced according to standard stage 1 IMF recipe (for infants between 0 to 6 months) and processing procedure (Danone Research, Wageningen, The Netherlands). The Concept IMF powder which serves as basis for the Concept IMF rodent diet was generated by adding 0.5 g/L phospholipids (PL) of dairy origin (Fonterra Co-operative Group Limited, Auckland, New Zealand; Table 1) and applying an altered processing procedure to obtain PL coated lipid droplets larger than those in standard IMF. Briefly, the polar PL were added to the aqueous phase and blended with the lipid phase containing the neutral vegetable lipids to generate a PL coating around the lipid droplets. Homogenization pressure during processing was adjusted to secure a homogeneous mixture of ingredients but also to retain a large lipid droplet size [22]. Initial differences in FA composition between diets as a result of the dairy origin of the PL source that were not corrected for in the previous study [22], were compensated for by minor adjustments to the composition of the vegetable oil blend. Both IMF recipes contain 0.35 g arachidonic acid and 0.2 g docosahexaenoic acid /100 g total fatty acids.

Lipid droplet size distribution of the IMF powders was measured using a Mastersizer laser light-scattering analyser with a refractive index ratio of 1.46 (Mastersizer 2000, Hydro 2000G, Malvern Instruments Limited, Worcestershire, UK). PL in the coated lipids were visualized by epifluorescent microscopy. Solubilised IMF was incubated with Annexin-V Alexa Fluor® 488 (Invitrogen molecular probes, Eugene, OR, USA) and Nile Red (Sigma-Aldrich, St. Louis, MO) according to manufacturers protocol staining PL and TGs, respectively, for labeling of the fat core and PL coating in the non-fixed lipid droplets. Analyses

were performed with a fluorescent microscope (Zeiss Axioplan 2, Carl Zeiss, Weesp, The Netherlands) with a (10)x63 oil immersion objective using FITC and TRITC optical filters.

Experimental diets. All experimental diets were semi-synthetic (Research Diet Services, Wijk bij Duurstede, The Netherlands; Table 1) and had a macro- and micronutrient composition according to the American Institute of Nutrition formulation of AIN93G purified diets for laboratory rodents [23]. The postnatal IMF-based rodent diets contained 28.3 w/w% Control or Concept IMF powder, respectively, and were complemented with protein and carbohydrates to meet rodent nutrient requirements. These diets were not pelletized but presented to the mice as dough (powder mixed with water) in order to preserve the lipid structure. The moderate WSD consisted of 10 w/w% fat (5 w/w% lard and 0.1 w/w% cholesterol).

Blood Sampling and Dissection. Blood was sampled by cheek puncture under anesthesia (isoflurane/N_2O/O_2) on PN42 after 4 hours of fasting during the light phase [24]. Plasma was pooled per 3 animals per group to obtain sufficient samples for analyses. At dissection (PN98), mice were terminally anesthetised (isoflurane/N_2O/O_2) after 4 hours of fasting. DEXA scans were performed and blood collected in K_3EDTA via cardiac puncture. Liver, pancreas, *m. Tibialis*, brain and WAT depots [epididymal (EPI), retroperitoneal (RP), perirenal (PR) and inguinal (ING)] were collected and weighed. Plasma was obtained by centrifugation at 1350*g* for 12 min at 4°C and stored at -80°C.

Plasma Analyses. Total cholesterol (tC), HDL-C, LDL-C, VLDL-C and TG were analysed colorimetrically after enzymatic conversion (Reinier de Graaf Laboratory, Delft, The Netherlands).

Plasma glucose (GOD-PAP method, Roche diagnostics, Almere, The Netherlands) was measured colorimetrically in PN98 plasma samples and analysed with a microplate imaging system (Bio-Rad Laboratories Inc.®, Hercules, CA, USA). Plasma insulin was measured by means of enzyme-linked immuno-sorbent assay (ELISA; DRG, Marburg, Germany) according to the manufacturer's protocol. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin (glu(mmol/L) * ins(pmol/L)/22.5) as an indirect measure of insulin sensitivity. Plasma volumes were insufficient to perform glucose and insulin analyses on PN42 samples.

TABLE 1 Composition of the experimental diets

		IMF			
		Control	Concept	WSD	AIN93M
Carbohydrates	(g/kg)	645	645	600	720
Sugars	(g/kg)	235	235	150	100
Dextrose	(g/kg)	5	5	150	-
Lactose	(g/kg)	145	145	-	-
Sucrose	(g/kg)	85	85	-	100
Polysaccharides	(g/kg)	410	410	450	620
Maltodextrine	(g/kg)	50	50	250	155
Starch	(g/kg)	360	360	200	465
Protein	(g/kg)	179	179	179	126
Soy	(g/kg)	150	150	179	126
Whey	(g/kg)	17	17	-	-
Casein	(g/kg)	12	12	-	-
Fat	(g/kg)	70	70	100	40
Oil blend*	(g/kg)	69.1	64.4	50	-
Milk fat	(g/kg)	0.9	5.6	-	-
Lard	(g/kg)	-	-	50	-
Soy oil	(g/kg)	-	-	-	40
Saturated Fatty Acids	SFA (g/kg)	28.7	29.7	41.9	5.9
Mono Unsaturated Fatty Acids	MUFA (g/kg)	26.5	26.1	42.3	10.3
Poly Unsaturated Fatty Acids	PUFA (g/kg)	11.5	11.0	13.2	23.5
Linoleic acid (C18:2 n-6)	LA (g/kg)	9.8	9.3	11.9	20.4
α-linolenic acid (C18:3 n-3)	ALA (g/kg)	1.8	1.7	1.3	2.7
Arachidonic Acid (C20:4 n-6)	ARA (g/kg)	0.25	0.25	-	-
Eicosapentaenoic Acid (C20:5 n-3)	EPA (g/kg)	0.03	0.04	-	-
Docosahexaenoic Acid (C22:6 n-3)	DHA (g/kg)	0.14	0.14	-	-
LA/ALA		5.4	5.3	9.5	7.5
Total n-6/n-3		5.1	5.0	9.5	7.5
Phospholipids**	(g/kg)	0.1	1.1	-	-
Cholesterol	(mg/kg)	4.7	20.4	1000	-
Cellulose (Vitacel L 600-20)	(g/kg)	50	50	50	50
mineral mix	(g/kg)	35	35	35	35
vitamin mix	(g/kg)	10	10	10	10
choline bitartrate	(g/kg)	2.5	2.5	2.5	2.5
L-cystein	(g/kg)	3	3	3	3
tert-butylhydroquinone	(g/kg)	0.014	0.014	0.014	0.014

*Mixture of oils, among others canola, sunflower, fish, coconut and palm oil; **Phospholipids derived from bovine milk

Cytological and Biochemical Analyses of Adipose Tissue. Cell size distribution according to the optical method of Di Girolamo *et al (2001)* [25] were determined in fresh ING and EPI WAT depots respectively representing subcutaneous and abdominal fat. Depots were cut into 1 mm large pieces and incubated in gassed (95% O_2 / 5% CO_2) Krebs Ringer bicarbonate buffer (KRB, pH 7.4, 2 mg/ml collagenase type II (Gibco for Invitrogen, California, USA) at 37°C. The digested tissue was filtered first through a 250 µm cell strainer, washed three times with KRB and filtered again through a 40 µm cell strainer.

Images of isolated adipocytes were captured using an Axioplan 2 Zeiss microscope (Carl Zeiss, Weesp, The Netherlands) and a Sony DXC-950P videocamera (Sony, Badhoevedorp, The Netherlands). Six representative sections per slide and six slides per depot per mouse were used to obtain images of at least 300 cells for assessment of mean adipocyte volume and size distribution per animal using analySIS software (Soft imaging system, Münster, Germany).

The remaining WAT depots were snap frozen and stored at -80°C for lipid content measurement. Lipid extraction was performed according to Folch *et al* (1957) [26] using dichloromethane: methanol (2:1, v/v).

WAT cellularity was calculated by dividing the fat pad lipid content by mean adipocyte weight. The mean adipocyte weight was calculated by multiplying the mean adipocyte volume by lipid density, which was derived from triolein density $\rho = 0{,}915 g/ml$ [25].

Gene expression analyses in EPI WAT depot. Total RNA was isolated from epididimal WAT using the RNeasy mini kit (Qiagen, Germantown, USA). cDNA was prepared using the iScript cDNA synthesis kit (Bio Rad, Veenendaal, the Netherlands) and quantitative real-time PCR (qPCR) analysis performed using the CFX96 real-time PCR detection system (Bio Rad, Veenendaal, the Netherlands) with iQ SYBR green supermix (Biorad, Veenendaal, the Netherlands). Validated qPCR primers (Table 2) were obtained for *leptin* from SABiosciences (Qiagen, Germantown, USA), for *PPARα*, *CEBPα*, *CEBPβ*, *CEBPδ*, *RXRα*, *Sterol Regulatory Element-Binding Protein 1c* (*SREBP1C)* and *RPS13* from Isogen Life Science (De Meern, the Netherlands). mRNA-expression was calculated with CFX Manager software (version 1.6) and corrected for the expression of the housekeeping gene *RPS13* [27].

TABLE 2 qPCR primers

Amplified region	Forward primer	Reverse primer	Annealing temp (°C)
PPARγ	'5-AGATTAATAGTTTGACGGGGTTT-3'	'5-ACCCGTATCCCCTCTAATCGA-3'	62
CEBPd	'5-AGAGCGCCATCGACTTCAGC-3'	'5-CCAAGCTCACCACTGTCTGC-3'	63
CEBPb	'5-TACCCAGGACCCATTGGATA-3'	'5-TTCACTTGGCCACTCTTCCT-3'	62
CEBPa	'5-TGTTGGAGTTGACCAGTGAC-3'	'5-ATCCAGCGACCCTAAACCAT-3'	60
RXRa	'5-GATGGCCTGTGTGGATCTTT-3'	'5-AACCAGCAACCAGAACAAGC-3'	60
SREBP1c	'5-CAAGTGCTGCAGGAAACTGA-3'	'5-CATGGCCTTGTCAATGGAAC-3'	60
Leptin	SAbiosciences®		63

Statistical Analysis. Statistical analyses were performed using SPSS 15.0.1 software (SPSS Benelux, Gorinchem, The Netherlands). Variables were checked for Gaussian distribution with the Shapiro-Wilkes test. Levene's test for equality of variance was used to estimate the probability that treatment groups had different variances. Direct and programming effects of any experimental diet (Control IMF, Concept IMF, Reference) on development of body composition were analyzed using a Repeated-Measures analysis of variance (ANOVA). *Post hoc* analyses were performed on significant Diet*Time interactions by Univariate ANOVA of PN42, 70 and 98, separately. Repeated measures ANOVA was performed to analyse diet effects on frequency distribution of adipocyte size at PN98 and a *post hoc* analysis was performed when significant Diet*Time interactions were confirmed. Effects of experimental diets on WAT weight, organ weight, plasma parameters, average adipocyte number and gene expression at PN98 were analysed by means of Univariate ANOVA.
Data are presented as mean ± SEM unless otherwise indicated. Differences were considered significant at $p < 0.05$.

Results

IMF-based diets. The adjusted IMF processing and addition of 0.5 g/L PL from dairy origin resulted in a Concept IMF powder with PL-coated (Figure 2) lipid droplets larger than those in Control IMF (Table 3).

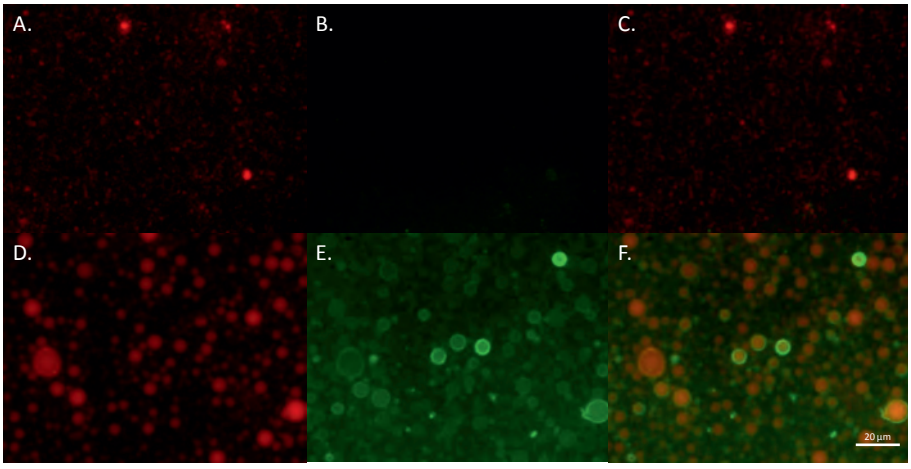

FIGURE 2 Images of conventional epifluorescent microscopy with Nile Red stained fat core (A & D), Annexin-V Alexa Fluor® 488 stained PL (B & E) and composed image of both (C & F) of Control and Concept IMF, respectively.

TABLE 3 Lipid droplet size characteristics from Control and Concept IMF derived from particle size distribution analysis.

	Control IMF	Concept IMF
d (0.1)[a], µm	0.183	0.407
d (0.5)[a], µm	0.349	4.951
d (0.9)[a], µm	0.855	11.67
d(1.0)[a], µm	1.633	23.24
D [3. 2][b], µm	0.285	1.337
D [4. 3][c], µm	0.468	6.000
< 1 µm[d], %	94.34	12.59
2 - 12 µm[e], %	0.000	70.30
Mode[f], µm	0.439	6.500

IMF, Infant milk formula, [a] 10, 50, 90 and 100% particle volume percentiles, [b] Surface weighted average diameter, [c] Volume weighted average diameter, [d] Sum volume of particles < 1.000 µm (%total volume of all particles), [e] Sum volume of particles 2-12 µm (%total volume of all particles), [f] Peak diameter of particle size distribution

Direct effect of Control and Concept IMF.

BW gain in Control and Concept IMF fed mice were similar from PN2 to 42 ($F_{(2, 31)} = 0.758$, $p = 0.477$; data not shown). Additionally, there was no direct effect of the diet during early development on body composition, since BW, LBM, FM and %FM were comparable (Figure 3) at PN42. Plasma TG, TC, HDL-C, LDL-C and VLDL-C concentrations were similar in all groups (Table 5).

Programming effect of Concept IMF during adult WSD challenge.

Body composition and food intake. BW gain during WSD challenge was significantly lower in Concept IMF compared to Control IMF mice ($p = 0.028$; Figure 3). The BW difference was exclusively due to a lower FM gain in Concept IMF compared to Control IMF mice, since LBM gain from PN42 to 98 was similar in both groups. Early postnatal Concept IMF exposure reduced FM gain due to the WSD by 30% and %FM gain by 20% compared to Control IMF. The %FM in the Concept IMF mice was similar to non-challenged Reference mice ($p = 0.234$) at PN70 and showed intermediate values at PN98 ($p = 0.001$ and $p = 0.012$) for the Control and and the non-challenged Reference mice, respectively; Figure 3). Weights of WAT depots followed a similar pattern as total FM and %FM, with 26 to 34% lower WAT depot weights in Concept IMF compared to Control IMF mice. Average liver weight of Concept IMF mice was intermediate between the liver weight of Control IMF and Reference mice (Table 4). Food intake was similar in both groups (12.69 ± 0.3 versus 12.16 ± 0.2 kcal/day/mouse for Control and Concept IMF group, respectively).

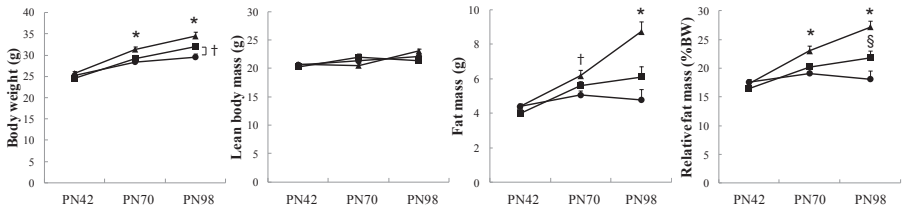

FIGURE 3 Development of body composition[§] during WSD challenge of mice fed early Control IMF (▲; n=11) or Concept IMF (■; n=11) and Reference group (● ; n=12) fed AIN-93M from PN42 to 98. *$p < 0.05$ compared to Reference and Concept IMF group, [†]$p < 0.05$ compared to Reference group; [§] $p < 0.05$ compared to Reference and Control IMF group.

TABLE 4 Average weight of WAT depots and organs at PN98 during WSD challenge of mice fed Control IMF or Concept IMF and Reference group fed AIN-93M from PN42 to 981

		Reference		Control IMF		Concept IMF	
		MEAN	SEM	MEAN	SEM	MEAN	SEM
WAT	Epididymal (mg)	516.8	39.9	1138.0†	99.7	827.2*†	106.5
	Perirenal (mg)	26.2	2.7	67.4†	8.3	43.8*†	5.5
	Retroperitoneal (mg)	137.5	10.4	299.8†	26.3	215.2*†	29.5
	Inguinal (mg)	374.8	32.0	542.8†	40.3	401.7*	28.4
Liver (g)		1.41	0.0	1.69†	0.1	1.57	0.1
Pancreas (mg)		159.3	8.0	167.5	7.8	172.1	9.5
M. Tibialis (mg)		50.4	2.3	49.2	0.8	50.9	0.8
Brain (mg)		425.8	7.6	413.0	7.1	422.2	6.0

WAT, white adipose tissue; WSD, western style diet; IMF, infant milk formula.
[1]n=11-12. †$p < 0.05$ compared to Reference group; *$p < 0.05$ compared to Control IMF group

Adipocyte size and number. We aimed to address whether the differences in FM were related to a decrease in adipocyte cell number, adipocyte cell size, or both in fat depots representing subcutaneous and abdominal fat [9,10]. The data on cell number (Figure 4) showed that this parameter was not affected by early dietary exposure; there was no difference in adipocyte number in EPI and ING WAT depot between Control IMF and Concept IMF group.

The early diet did affect adipocyte size. Cell size distribution curves (Figure 4) illustrate that animals fed Concept IMF prior to WSD had a significantly smaller number of large adipocytes in the ING depot compared to Control IMF mice ($F_{(14, 266)} = 3.199$, $p = 0.002$). This difference was mainly found in the cell size category between 80 and 150 µm diameter. Average ING adipocyte volume was 25% lower in Concept IMF compared to Control IMF group ($t_{(18)} = 2.016$, $p < 0.05$). The average EPI adipocyte volume at the end of WSD challenge was 21% lower in mice fed Concept IMF compared to Control IM mice, but this difference did not reach significance.

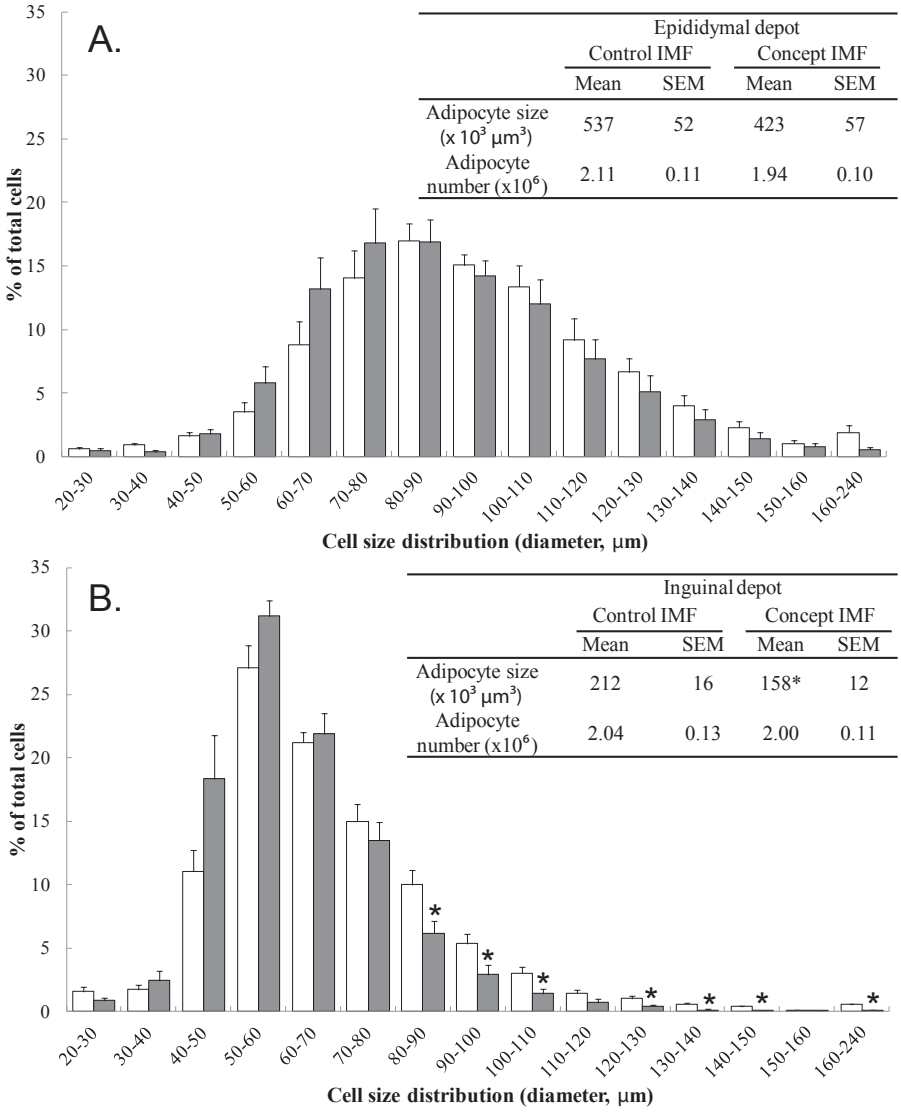

FIGURE 4 Frequency distribution of adipocyte cell size, average cell size and number of epididymal (A) and inguinal (B) WAT depot at PN98 in Control IMF (white bar) and Concept IMF group (black bar); n=9-12/group. *p < 0.05 compared to Control IMF group.

As stated earlier, hyperplastic and hypertrophic growth of WAT is regulated by transcription factors, including PPARγ, RXRα, CEBPs and SREBP1c. The expression of PPARγ ($F_{(1, 7)}$ = 4.701, p < 0.01), RXRα ($F_{(1, 7)}$ = 1.919, p < 0.05) and CEBPα ($F_{(1, 3)}$ = 15.90, p < 0.001) were all decreased in EPI WAT depots of Concept IMF

compared to Control IMF mice. No differences in expression of CEBPß, CEBPδ or SREBP1c were observed between both IMF groups. The expression of leptin was significantly lower ($F_{(1, 7)} = 2.077$, $p < 0.05$) in Concept IMF mice compared to Control IMF mice (Figure 5).

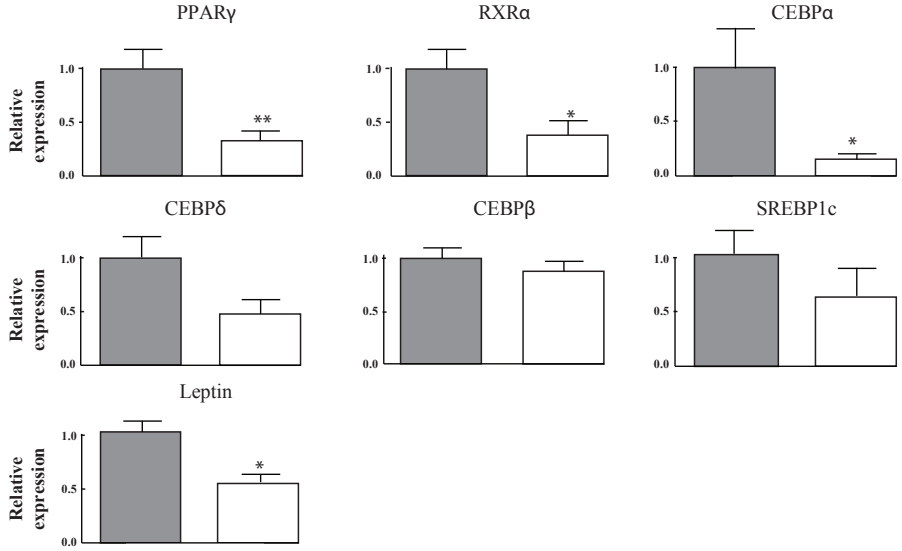

FIGURE 5 Relative mRNA expression in epididymal WAT depot in Control IMF (grey bar) and Concept IMF group (white bar); n=12/group. *p < 0.05 compared to Control IMF group.

Plasma metabolic profile. Although both IMF groups had higher concentrations of TC, HDL-C, LDL-C compared to the non-challenged Reference group ($p < 0.05$), Concept IMF mice had markedly lower concentrations compared to Control IMF mice ($p < 0.05$). Experimental diets did not significantly affect any of the analyzed parameters for glucose homeostasis (Table 5).

Effect of WSD challenge during adolescence and adulthood.

To confirm that the mild WSD indeed represented a diet challenge which affects adult phenotype, we compared the Control IMF group exposed to the WSD in adulthood to a non-challenged Reference group which was switched to a standard rodent chow at PN42.

WSD intake enhanced BW gain (17%) in Control IMF mice compared to the non-challenged Reference mice (Figure 3; $p = 0.002$) from PN42 to 98. LBM gain was similar in both groups ($F_{(2, 31)} = 0.056$, $p = 0.956$), but the WSD challenge increased

FM by 80% and %FM by 50%. Weights of *m. Tibialis*, pancreas and brain were unaffected (Table 4), whereas average liver weight of Control IMF mice was higher compared to Reference mice ($F_{(2, 31)} = 5.656$, $p = 0.008$).

The WSD challenge resulted in more large adipocytes of the Control IMF compared to the Reference group. Average EPI adipocyte volume was 94% increased as a result of the 56 days of WSD challenge, whereas average ING adipocyte volume appeared to be only 17% higher following the WSD challenge. This difference did not reach significance (data not shown). Control IMF and Reference group had comparable adipocyte numbers in both the EPI and ING depots (data not shown).

Fasting plasma TC ($F_{(2, 27)} = 17.352$, $p < 0.001$), HDL-C ($F_{(2\ 27)} = 19.686$, $p = 0.049$) and LDL-C ($F_{(2, 27)} = 18.424$, $p < 0.001$) concentrations were increased in WSD challenged Control IMF animals compared to the non-challenged Reference group. Fasting plasma TG, glucose, insulin and HOMA-IR concentrations were not affected at PN98 due to WSD; concentrations of Control IMF and Reference mice were not significantly different (Table 5).

TABLE 5 Average fasting plasma parameters at PN98 during WSD challenge of mice fed Control or Concept IMF and Reference group fed AIN-93M from PN42 to 98[1]

		Reference		Control IMF		Concept IMF Mean	SEM
		MEAN	SEM	MEAN	SEM	MEAN	SEM
PN42	TG (mmol/L)	1.66	0.1	1.44	0.1	1.85	0.1
	TC (mmol/L)	3.48	0.1	3.58	0.2	3.18	0.1
	HDL-C (mmol/L)	1.84	0.0	1.88	0.2	1.63	0.1
	LDL-C (mmol/L)	0.89	0.1	1.04	0.1	0.71	0.1
	VLDL-C (mmol/L)	0.76	0.1	0.66	0.0	0.84	0.1
PN98	TG (mmol/L)	0.97	0.1	1.00	0.1	0.82	0.0
	TC (mmol/L)	2.98	0.2	4.40†	0.2	3.79*†	0.1
	HDL-C (mmol/L)	1.76	0.1	2.63†	0.1	2.32*†	0.1
	LDL-C (mmol/L)	0.77	0.1	1.31†	0.1	1.09*†	0.1
	VLDL-C (mmol/L)	0.44	0.1	0.45	0.0	0.37	0.0
	Glucose (mmol/L)	13.7	1.1	12.0	1.0	13.2	0.7
	Insulin (pmol/L)	90.6	24.8	109.4	29.9	70.1	7.4
	HOMA-IR((mmol/L*pmol/L)/22.5)	42.0	12.6	56.8	14.3	42.0	5.8

WSD, western style diet; IMF, infant milk formula ;TG, Triacylglycerols; TC, Total Cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; VLDL-C, very low density lipoprotein cholesterol. [1]n=2-4/group at PN42. n=8-11/group at PN98. †$p < 0.05$ compared to Reference group; *$p < 0.05$ compared to Control IMF group.

Discussion

The present study demonstrated that the physical structure of dietary lipids programs body composition development of pre-weaning mice towards reduced body fat accumulation in adulthood. These findings are in line with our previous study [22] despite differences in FA composition, indicating that the physical lipid structure indeed is the main determinant of the programming effect. Programming of adult fat mass by the Concept IMF diet was not mediated by a reduction in the number of adipocytes, but rather by reduction of adipocyte size, indicating reduced lipid storage. The phenotype coincided with a reduced expression of transcription factors that promote lipogenesis in WAT.

Reduced adult fat mass and lipogenic gene expression

Development of human WAT starts in the third trimester of gestation and continues in postnatal life through proliferation of pre-adipocytes and subsequent differentiation resulting in mature adipocytes storing energy in the form of lipids [1]. The amount of total body fat mass is determined by adipocyte size and by the number of differentiated adipocytes in WAT [2, 28]. Feeding Concept IMF diet during a relatively short period in early life partially prevented excess body fat accumulation upon a WSD challenge in later life. Concept IMF mice had a reduced storage of TG compared to Control IMF mice. The reduced gene expression of leptin in Concept IMF compared to Control IMF mice reflects both reduced FM and reduced adipocyte size [29]. The reduced lipid accumulation upon WSD challenge in WAT of adult mice in the Concept IMF group was associated with reduced PPARγ and RxRα mRNA expression in WAT, suggesting reduced fatty acid uptake and/or *de novo* lipogenesis. Expression of another key regulator of (de novo) fatty acid and triacylglycerol synthesis, SREBP1c, was not affected by postnatal diet. Possibly, the Concept IMF did not have a sustained impact on WAT SREBP1c expression. Alternatively, SREBP-1c expression is upregulated by insulin, resulting in increased FA synthesis and reduced FA oxidation after feeding [30]. In this study, SREBP1c expression was measured after 4 hours of fasting which may have blunted possible differences between groups in fed state. [31]. Additionally, as SREBP-1c is expressed in both WAT and liver, the Concept IMF may have exerted organ [32] and WAT depot specific [33] effects. Indeed, it is suggested that SREBP-1c in WAT regulates genes involved in cholesterol metabolism rather than genes involved in fatty acid and TG synthesis [34].

Reduced adult fat mass: reduced adipocyte size, but not adipocyte number

Concept IMF mice had ~30% less total FM after the WSD challenge than Control IMF mice, which was reflected in all four WAT depots measured. However, programming of adipocyte size by the Concept IMF appeared to be more pronounced in subcutaneous ING than abdominal EPI depots, despite the comparable depot weight reduction. This difference could be explained by the major difference in EPI size distribution in the size category with the largest cells (150- 240 µm). These cells represent a small portion of the total cell population but store a relatively large amount of lipids compared to the smaller adipocytes. Thus, large adipocytes contribute significantly to the average adipocyte volume and storage capacity. It is known that there are WAT depot-specific differences in responsiveness of adipocytes to nutrients, growth patterns and metabolic and endocrine activity [35-39]. For instance, WAT expansion in aging rats was associated with hypertrophic growth in visceral depots and hyperplastic growth in subcutaneous depots [35]. Additionally, adipocyte size, number and gene expression have shown to differ considerably between WAT depots in adult rodents [40, 41] which is in accordance with findings from human studies [42-44]. Several cohort studies have shown that fetal and postnatal growth velocity differentially affects human body fat distribution with specifically rapid postnatal weight gain strongly associated with visceral adiposity [45-48]. This suggests that the critical window for WAT development may be different for visceral and subcutaneous depots. Indeed, data from a Dutch infant cohort demonstrated that the amount of visceral body fat increased between 12 to 24 months of age, in contrast to the amount of subcutaneous fat which remained constant over this period [49]. These age dependant growth differences might also appear in rodents as Digirolamo and colleagues [10] demonstrated distinct adipose tissue growth patterns among fat depots at different stages of life in rats. Additionally, both early life over-[50] and undernutrition[51] can modulate these differential depot growth patterns. Unfortunately, we were unable to investigate adipocyte size distribution and number in visceral depots in other than EPI WAT due to the small fat depot size, providing insufficient material for analysis. Therefore, the present study could not provide conclusive evidence for postnatal nutritional programming by Concept IMF of adipocyte number. One could hypothesize that the nutritional intervention took place after a critical window for adipogenesis. Earlier dietary exposure to exclude this possibility would require an artificially fed animal model. However, early life handling is known to induce

stress as a confounding factor since it influences later life body composition and fat distribution [52, 53]. Alternatively, the absence of a sustained effect on adipocyte number could be related to the fact that both IMF containing early diets meet the requirements for normal growth and development. Therefore, there would be no need for the animals to utilize the plasticity of the WAT to increase adipocyte number in response to this nutritional stimulus. It has been hypothesized by Spalding and colleagues that increased adipocyte number in early life is a major risk factor for adult obesity [3]. However, it is still unclear whether this is a causal relationship; it is unknown whether increased adipocyte number itself predisposes to adult obesity, or if both increased adipocyte number and adult obesity are caused by for instance excessive energy intake during childhood through a different mechanism altogether. In accordance with this hypothesis, early postnatal overnutrition in rats has been found to specifically increase adipocyte number and thereby increase adult fat mass but did not affect adipocyte size [54]. Additionally, hyperplasia and the cell turnover capacity are positively correlated with insulin sensitivity independent of BW, whereas hypertrophy is negatively correlated with insulin sensitivity [55]. Our present data clearly indicate that early diet can induce programming activity that is independent of hyperplastic growth, but seems to affect the capacity of adipocytes to enlarge upon a WSD challenge.

As stated previously, critical periods for hyperplastic growth of WAT encompass early postnatal life and adolescence [2, 56]; Cell number remains constant in healthy non-obese adults [3]. However, in case of sustained excessive energy intake in obesity, adult WAT is still capable of adjusting by both hypertrophic and hyperplastic growth [57]. During the moderate WSD exposure in the present study, adipocyte size increased significantly compared to standard chow, but was not such a severe challenge as to promote hyperplasia in adolescence and adulthood. We cannot exclude that hyperplasia would have occurred if a more severe dietary challenge was performed. Thus, at this time we cannot rule out potential programming effects of postnatal diet on hyperplastic WAT growth.

Programming of adult fat mass and metabolic homeostasis

We determined to what extent the observed differences in adult body composition and fat mass corresponded with other parameters of lipid homeostasis, such as plasma lipids. Plasma TC, HDL-C and LDL-C concentrations were reduced in Concept IMF mice compared to Control IMF mice. Liver weight

of Control IMF mice was higher than that of non-challenged Reference mice, whereas liver weight of Concept IMF mice did not differ significantly from both groups. This may suggest an improved metabolic profile and perhaps the absence of ectopic lipid accumulation due to the Concept IMF. Since plasma TG, glucose and insulin concentrations were not significantly perturbed by the moderate WSD challenge compared to normal rodent chow, the limited improvement in adult glucose homeostasis and TG concentrations of Concept IMF fed animals was to be expected.

Effect of milk lipid physical structure: potential mechanisms

Previous studies comparing digestion of raw cow's milk with processed milk with different milk lipid droplet size and surface composition, showed differences in both gastric emptying, lipid digestion and ß-oxidation rate [58-60]. The results suggest differences in utilization of these lipids for either ß-oxidation or lipogenesis. In turn, this could underlie the early programming of the lipid metabolism resulting in differences in metabolic capacity of adipocytes.

The sustained effects of the Concept IMF on body composition and metabolic profile cannot simply be explained by reduced food intake. Although individual food intake during the early diet could not be measured since it was provided as dough to the entire litters until weaning, BW and body composition were similar between groups at PN42. Moreover, daily food intake was comparable between groups during the WSD challenge. Possibly, Concept IMF-fed mice utilize energy from the WSD different by storing less energy as lipids in adipose tissue suggesting a higher basal metabolic rate. However, this information is currently not available. Future studies gaining more insight on energy expenditure are pivotal. Also, further studies to address which specific aspects of the Concept IMF, i.e. fat droplet size, PL addition and/or coating, may contribute to the observed long term effects, are ongoing.

Conclusion

In conclusion, early exposure to the Concept IMF resulted in a diet resistant phenotype in adult life. Concept IMF prevents excessive hypertrophic growth of white adipose tissue in adulthood despite unaltered adipogenesis. Our data support the hypothesis that early Concept IMF exposure programs metabolic responses through altered gene expression of factors regulating adipose tissue lipogenesis. Our present results provide support for the emerging notion that

dietary lipid structure should be considered a key component of dietary lipid quality in early life and is a determinant in later life obesity risk.

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Conflicts of interest. A.O., D.K, L.S., M.A., S.R. and E.M.B, are employed by Danone Research and have contributed to the study design, conducted the study, performed sample and data analysis, interpreted findings and prepared the manuscript. H.J.V. is a consultant for Danone research outside the submitted work for which his institution (Department of Pediatric Gastroenterology and Hepatology, University Medical Center Groningen, The Netherlands) is compensated financially. The previous position of N.V. was partly funded by Danone research, but she has currently no affiliation with Danone Research and therefore no conflict of interest.

Authorship. A.O., D.K., M.A. and E.M.B. designed the research; A.O., L.S., N.V., and D.K. conducted the research; S.R. provided essential materials; A.O., N.V., L.S., S.R. and D.K. analyzed data, A.O. wrote the paper, H.J.V. reviewed the manuscript, E.M.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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Chapter 7

General discussion

The aim of this thesis was to determine in animal models whether relative moderate nutritional changes in early life could reduce the obesity risk in later life. Our preclinical studies, focusing specifically on potential impact of qualitative changes in the early postnatal diet, indeed indicate that moderate changes in lipid quality protects against later life obesity. The postnatal lipid quality, characterized by FA composition and physical lipid structure, can program towards reduced adult fat mass accumulation and an improved metabolic phenotype.

Early life is considered a period of developmental plasticity in which environmental signals modulate growth and development of an individual, thereby ultimately affecting adult phenotype. Appropriate "predictive adaptive responses" (PARs) generate a phenotype able to cope with challenges throughout life resulting in a low disease risk. Accumulating evidence now indicates that specific nutritional conditions during early life are contributing to the current increase in obesity incidence worldwide. Since the initial reports of Barker and colleagues showing the correlation between birth weight and coronary heart disease in 1989, this scientific field expanded rapidly. Many observational and experimental studies have investigated the contribution of the perinatal environment, including nutrition, to the etiology of obesity and non-communicable diseases (NCDs). Until our present studies, however, limited attention had been paid on opportunities to improve early life nutrition with the aim to improve adult health outcome and specifically to prevent adult obesity. Intervention studies investigating potentially beneficial nutrients that are relevant for maternal and/or infant diets and applicable in preventive public health strategies, are rare. Randomized clinical trials have not yet been performed on a large scale and there is even a paucity of preclinical studies assessing the effects of specific nutrients or dietary concepts on adult disease risk.

Programming of adult metabolic health by dietary PUFA in early life

The increased use of vegetable oils as dietary lipid source and changes in processing in the food industry has resulted in a contemporary global increase in exposure to dietary LA and a decrease in n-3 LCP over the last decades [1, 2].

The net result is a high n-6/n-3 ratio in western diets, which has been associated with the pathogenesis of cardiovascular disease, cancer, inflammatory and auto-immune diseases [3-5]. The effects of high dietary LA are thought to be mediated through several mechanisms including proinflammatory eicosanoid signaling, enhanced LDL oxidation, enhanced platelet aggregation and reduced incorporation of n-3 LCPs in membranes [6]. The latter is of importance, because reduced membrane n-3 LCP composition affects activity of membrane proteins, membrane fluidity, membrane permeability and cell signaling pathways [7]. A striking example of the potentially adverse health effects of LA comes from the Sydney Diet Heart study [5]. Adult men aged 30-59 years with a recent coronary heart event, received dietary advice and safflower oil supplements to increase dietary LA intake as replacement of saturated fatty acids from animal fats, traditional margarine and shortenings. The high intake of LA was associated with higher mortality rates from both cardiovascular and coronary heart disease, in contrast to widely held beliefs on the positive effects of the polyunsaturated fatty acids (PUFA) compared to saturated fatty acids (SFA) [5]. Ailhaud and colleagues hypothesized that specifically during early life, excessive dietary n-6 PUFA increases obesity [8-10]. This hypothesis is based on a few rat studies showing enhanced fat accumulation due to exposure to a high fat, high LA diet throughout life or even over multiple generations, compared to an isocaloric high fat, LA/ALA diet [11, 12]. However, these studies do not provide conclusive data on possible programming effects of exposure confined to early life, nor does it allow differentiation between effects attributable to n-6 PUFA or high fat. In contrast to n-6 PUFA, high intake of dietary n-3 LCPs and a low n-6/n-3 ratio reduces disease risk, presumably by inhibiting platelet aggregation, anti-inflammatory eicosanoid signaling, improving endothelial function and through hypotensive and hypotriglyceride effects [3, 13]. n-3 LCP supplementation has been effective in treatment of adult obesity and metabolic syndrome as it reduced fat mass, insulin resistance and dyslipidemia [14]. Supplementation of the n-3 essential fatty acid ALA, precursor of n-3 LCPs DHA and EPA, is investigated to a lesser extent and there is little clinical evidence of treatment effects.

Since milk FA composition reflects maternal dietary FA intake (**Chapter 2**), the exposure to the n-6/n-3 imbalance may already start early in life [9, 15]. This also holds true for formula fed infants since the FA composition of infant milk formulas (IMF) has been based on human milk (HM) of Caucasian women on a western diet [16]. As it has been hypothesized that the high dietary n-6/n-3 ratio contributes to

the current obesity incidence, changing dietary FA quality by increasing n-3 LCP and decreasing LA, reaching levels closer to ancient diets [16, 17], might decrease obesity risk. Indeed, our studies have shown that early postnatal exposure of mice to 5% (of total fatty acid content) n-3 LCP or to a 50% reduction in dietary LA, both decreasing the n-6/n-3 ratio, protected against excessive fat accumulation in response to a mild western style diet challenge in adulthood. These two manipulations in early life PUFA composition prevented fat accumulation to a comparable extent: 27% (LA reduction) and 30% (n-3 LCP increase), respectively. Since a 5% reduction in body fat percentage already improves cardio-metabolic risk profile in obese children [18] and adults [19], the presently observed decrease due to lowering of the dietary n-6/n-3 ratio in early life is substantial and probably clinically relevant. Accordingly, the diet-induced decrease in adult fat mass was associated with improved metabolic profile in adulthood in our studies.

Although our mouse studies demonstrated that either increased postnatal n-3 LCP (**Chapter 3**) or reduced LA (**Chapter 4**) reduced adult fat accumulation during an adult western diet challenge, the mechanisms by which these diets decreased adult fat mass may be different. For effects of the two dietary manipulations differed with respect to adult adipocyte size and number. On the one hand, supplementation of a 10 %wt fat diet with n-3 LCP resulted in reduced fat mass and smaller adipocytes, compatible with an altered homeostatic "set point" of adipocyte lipid metabolism resulting in enhanced lipolysis or reduced lipogenesis. Indeed, n-3 LCPs are known to reduce lipogenesis and enhance β-oxidation *in vitro* and *in vivo* [20]. On the other hand, lowering of the relative dietary LA content increased adipocyte size and reduced adipocyte number (**Chapter 4**). These data suggest that low LA content in early life reduces preadipocyte differentiation (adipogenesis) and thereby the long term lipid storage capacity. We do realize, however, that other mechanisms cannot be excluded. The reduced fat accumulation in mice fed low LA in early life, despite enhanced adult food intake, does suggest programming effects on adult energy partitioning and feed efficiency. In accordance with this suggestion, a fat balance study in mice showed that dietary LA tended towards an enhanced energy storage in WAT and towards a lower energy expenditure compared to saturated fatty acids, n-3 PUFA or conjugated LA [21]. Additionally, altered hypothalamic regulation of energy homeostasis could also play a role, considering the lipid sensing ability of the hypothalamus [22, 23]. Indeed, other studies from our group have shown that both reducing LA and increasing

n-3 LCP reduced the outgrowth of orexogenic and anorexogenic neuronal projections in the developing hypothalamus with potential effects on central regulation of satiety and energy [24].

Established health benefits of dietary LCPs during pregnancy and infancy have concentrated on brain and immune development in term infants [25] and, to a lesser extent, on growth in preterm infants [26]. Only a limited number of human and animal studies have investigated effects of early pre- and postnatal n-3 LCP on the development of body composition in later life. Also, the few available data have been inconsistent, possibly due to differences in dose, timing and duration of n-3 LCP supplementation and/or use of BMI as indirect growth variable rather than actual body composition measurements [27-30]. A recent systemic review by Muhlhausler and colleagues [29] identified only four rodent studies investigating the effects of ALA or n-3 LCP on adipose tissue development [12, 31-33], of which only one study with an intervention confined to the perinatal period [32]. Continuous ALA or n-3 LCP intervention from early life until adolescence or adulthood reduced fat mass and adipocyte size in two out of three studies [12, 33]. ALA supplementation during gestation and lactation reduced the weight of the inguinal fat depots and adipocyte size at weaning. These findings are in accordance with findings from adult obesity and high fat diet (HFD) models [34-36] but do not allow any conclusions on programming effects of ALA or n-3 LCPs. Supplementation of n-3 LCPs during gestation and lactation moderately increased fat mass in 6-week-old rat offspring compared to controls, despite an improved metabolic phenotype, reflected by reduced plasma non-esterified fatty acids at weaning and reduced plasma glucose at 6 weeks [37]. Similarly, inconsistent data were obtained in the few randomized clinical trials that have been performed, showing increased, reduced or no effect of n-3 LCP supplementation during pregnancy and/or lactation on later life body mass index and fat mass [28].

Our data suggest that the balance of n-6 and n-3 EFA and LCPs is of importance for the observed programming effects on adult obesity development in mice and rats. The net balance of n-6 and n-3 PUFA in tissues is determined by dietary intake of LA and ALA as well as their respective LCPs, because LA and ALA depend on the same set of elongases and desaturases for conversion to their respective LCPs, and because dietary LCPs inhibit endogenous LCP synthesis. For instance, supplementation of ALA to a high LA diet may have very limited effects on n-3 LCP status and metabolic health, because LA inhibits both n-3 LCP synthesis

from ALA and incorporation in biological membranes [15]. Besides LA and ALA competing for enzymes synthesizing their respective n-6 and n-3 LCPs, n-6 and n-3 LCPs also compete for the same set of enzymes for eicosanoid synthesis, cyclooxygenase (COX) and lipoxygenase (LOX). The balance between n-6 and n-3 LCPs therefore determines the relative abundance of pro-inflammatory and anti-inflammatory eicosanoids [14]. Apart from the n-6 / n-3 balance, absolute amounts of LA and ALA are important as well, based on the findings of Goyens *et al* (2006) who demonstrated that a high amount of LA inhibited ALA conversion to EPA irrespective of the relative dietary LA/ALA ratio in healthy human subjects [38]. In addition, a study in which adult rats were exposed to a fixed amount of LA with increasing ALA concentrations demonstrated that LA was an independent predictor for adipocyte SREPB1c expression [39].

Besides absolute n-3 and n-6 content and n-6/n-3 ratio, the total dietary fat content modulates the PUFA effects. N-3 LCP concentrations ranging from 0 to 1.5 %wt combined with fat content ranging from 5 to 20 %wt of total diet under a constant LA concentration result in differential patterns of ARA derived eicosanoids in mice, including series 4 and 5 leukotrienes, prostaglandin E2 (PGE2) and prostacyclin (PGI2) [40]. A high n-6, low n-3 LCP, high fat diet throughout life gives rise to hyperplastic and hypertrophic expansion of mouse WAT, attributed to the strong adipogenic effects of ARA through its PGI2 metabolite and increased lipid accumulation per adipocyte due to lipogenic effects of high absolute amounts of both n-6 PUFA and saturated fatty acids [12]. Surprisingly, adult rat offspring exposed to a high n-6, low fat diet during gestation and lactation, had lower BW, blood pressure, serum TG and insulin compared to offspring exposed to a more balanced n-6/n-3 diet [41]. Taken together, these data suggest that adipogenic, lipogenic and proinflammatory effects of ARA and its eicosanoid metabolites are modulated by total fat content and n-3 LCP abundance [9, 39, 40].

In addition to fat content and n-6/n-3 balance, also macronutrient composition may modulate adipogenic effects of ARA during early development. Massiera and colleagues [12] found enhanced fat mass accumulation, increased adipocyte size but reduced adipocyte number in rats fed a high fat, high n-6 diet compared to an high fat, n-6/n-3 diet. These data are in contrast to findings described in the same paper showing that n-6 ARA promoted adipogenesis *in vitro*, i.e. enhancing adipocyte number through stimulation of preadipocyte differentiation. However, *in vitro* studies with 3T3 pre-adipocytes have shown

that both n-3 and n-6 PUFAs have the capacity to stimulate PPARγ expression and thus adipocyte differentiation [42].The pro- or anti-adipogenic effects of n-6 PUFA appear to depend on intracellular cAMP levels and cyclooxygenase expression which in turn are determined by protein and carbohydrate content of the diet. The combination of high LA with high sucrose (43%wt) was pro-adipogenic, whereas the combination of high LA and high protein (50 %wt) was anti-adipogenic in adult mice [43].

Taken together, these data indicate that (programming) effects of early life PUFAs depend on overall dietary EFA and LCP content and ratio, total dietary fat content and overall macronutrient composition, which may explain the ambiguous data from the few animal and human intervention studies that have investigated metabolic programming by dietary lipids.

To what extent can our present preclinical data be extrapolated towards the human situation? No consistent data concerning early life n-3 LCP programming of later life body composition could be retrieved from the six randomized clinical trials reviewed by Hauner and colleagues [28]. This observation does not seem to support the extrapolation of the present findings. However, three out of the six intervention studies did not include body composition measurements and merely recorded BMI. Only two studies incorporated skin fold thickness and one study measured waist circumference as a proxy for body composition. The study populations encompassed between 90 and 270 subjects aged between 1 and 19 years [28]. The low number of subjects, young age of the subjects and the lack of precise techniques to measure body composition probably limits the power of these studies. Relatively small study populations with generally healthy subjects at a relatively young age may be too resilient to current environmental challenges to have an increased disease risk and reveal any (in) adequate nutritional programming. If the later life environment is relatively healthy and does not challenge the system, for instance by means of high fat feeding and/or sedentary lifestyle, latent (in)appropriate "PARs" may not (yet) become manifest. In case of an adult obesogenic environment, however, high n-3 LCPs in early life may prove to reduce obesity and NCD risk similar to our mouse study (**Chapter 3**). Indeed, experimental studies show that an obesogenic adult environment is often required for an adverse phenotype originating from perinatal malnutrition to appear [44-46]. An alternative explanation for the inconclusive clinical data could be that protective effects of early life n-3 LCPs may only manifest if preceded by a suboptimal fetal environment. Clinical

studies investigating the role of n-3 LCPs in growth and brain development do support this notion: n-3 LCP supplementation is consistently effective in preterm or small-for-gestational-age infants whereas effects in healthy term infants are less evident [26, 47]. In addition, the few n-3 LCP programming studies performed in animal models [29] have investigated the capacity of n-3 LCP to ameliorate unfavorable metabolic outcome due to an adverse perinatal environment, i.e. fetal dexamethasone exposure or neonatal overfeeding [33, 48].

N-3 LCPs mediate some of their protective effects by reducing (n-6 PUFA mediated) enhanced adipogenesis, lipogenesis and inflammation. One might hypothesize that n-3 LCP supplementation may therefore only be effective when the offspring is at risk due to exposure to (chronic) inflammation or energy excess during gestation, e.g. due to maternal stress, obesity or high fat, high sugar feeding.

Long term follow up of subjects from the n-3 LCP randomized clinical trials, including imaging techniques to measure body composition, is required to confirm whether or not n-3 LCP supplementation during pregnancy and/or lactation programs adult metabolic health.

In summary, few (pre)clinical studies have investigated the role of dietary n-6/n-3 balance during early postnatal life alone. Even fewer studies have investigated the effect of moderate changes in PUFA composition combined with a standard rodent macronutrient composition, i.e. without concurrent high fat, or a fetal challenge. We found reduced fat accumulation in adult mice on a western style diet due to early postnatal n-3 LCP supplementation (**Chapter 3**) or n-6 PUFA (LA) reduction (**Chapter 4**). Preclinical studies investigating underlying mechanisms indicate that PUFA modulate organ development during the early postnatal period, including WAT (this thesis) and brain [49, 50]. It is unclear to what extent DNA methylation plays a role in these processes although n-6 and n-3 LCPs have been demonstrated to affect gene expression through modulation of DNA methylation in a limited number of *in vitro* and *in vivo* studies [51].

Programming of adult metabolic health by physical lipid structure in early life

Increased weight gain in the first year of life has been associated with increased obesity risk [52]. Accordingly, the reduced obesity risk in BF infants has often been attributed to their lower growth velocity in the first year of life, but the

available data are inconsistent [53-55]. Many factors associated with infant feeding have been suggested to contribute to obesity risk, including volume and intake patterns, timing of weaning, energy density, protein content, n-6/n-3 PUFA content and bioactive compounds such as leptin, ghrelin and adiponectin [56, 57]. It should be realized, however, that the role of these factors in obesity risk has often been extrapolated from their effects on early growth trajectories rather than clearly demonstrated through direct association with (childhood) obesity or through intervention studies [56, 57].

The contribution of HM dietary lipids has not been investigated in great depth apart from observational and intervention studies focusing on correlations between milk ARA and DHA content and body composition in (early) childhood. These studies have provided conflicting data on association between HM DHA and/or ARA and childhood adiposity [28, 58-60]. We now have shown in preclinical studies that, apart from FA composition, physical lipid structure may be a key feature of early life dietary lipid quality. Exposure of preweaning mice to an infant formula with a physical lipid structure closer to that of HM, i.e. increased lipid droplet size and a phospholipid (PL) coating (Nuturis®; Concept IMF), protected against excessive fat mass accumulation in adolescence and adulthood (**Chapter 5**). The effect size of programming on body composition was comparable to those resulting from the adjustments in early life PUFA composition (**Chapters 3 and 4**), namely a 30% lower adult fat mass after feeding Concept IMF compared to Control IMF.

The underlying mechanism of this so called 'lipid matrix' effect remains elusive to some extent. The data on WAT morphology (**Chapter 6**) showed that the altered physical lipid structure did not affect adipogenesis. Two explanations could apply. Either the intervention started after the early critical window for adipogenesis in mice, or the critical window was still open but the lipid structure did not affect adipogenesis. The former possibility seems unlikely because adipocyte number can increase until at least young adulthood in rodents [61-63]. The latter explanation appears more plausible: The early diets used in our studies meet the requirements for normal rodent growth and development, and there seems to be no need to utilize the plasticity of the WAT to increase adipocyte number in response to this nutritional stimulus. In contrast, early postnatal overnutrition in rats has been found to increase adult fat mass specifically by increasing adipocyte number rather than size [64].

Postnatal exposure to the altered physical lipid structure did have sustained effects on adipocyte size, indicating programming towards reduced lipid

storage. This notion was supported by reduced expression of lipogenic transcription factors in adulthood. Physical lipid structure could, one way or another, have modulated the homeostatic set point of adipocyte lipid metabolism, thereby limiting lipid storage. Alternatively, as suggested for n-6 and n-3 PUFAs, lipid structure may have programmed adult energy partitioning and feed efficiency towards enhanced energy expenditure for instance due to higher basal metabolic rate or enhanced heat production. Consequently, this energy partitioning would favor reduced energy storage, resulting in lower fat mass despite similar food intake. Indeed, Michalski and colleagues have shown that physical properties of dietary lipids, including lipid droplet size and surface composition, modulate acute absorption and digestion kinetics as well as metabolic fate of lipids [65]. For instance, small lipid droplets are hydrolyzed faster, but also delay gastric emptying compared to larger lipid droplets in healthy adults. In contrast, large lipid droplets with a native milk fat globule membrane (MFGM) on the lipid/water interface are hydrolyzed faster than small droplets with proteins on the interface in preterm infants. In adult rats, large MFGM coated lipid droplets decreased plasma triglyceride appearance and increased β-oxidation compared to small droplets with proteins at the interface [65]. Taken together, these data indicate that differences in lipid digestion and absorption may preferentially target these lipids towards either β-oxidation or storage in WAT. However, data on *in vivo* lipid digestion and absorption kinetics are scarce. Apart from the studies mentioned above little is known about the effect of physical lipid structure on utilization of dietary lipids. Additionally, whether effects found in human adults and adult rodents are applicable to infants is presently unknown since absorption and digestion kinetics differ between infants and adults due to immaturity of the infant digestive system [66, 67]. Also, whether potential differences in absorption and digestion kinetics contribute to the programming effects observed in our mouse studies remains elusive. One might hypothesize that differential postprandial FA kinetics and bioavailability due to physical lipid structure modulates the structural development of metabolic organs during the postnatal period of plasticity, thereby affecting later life metabolic function.

We recently found in adult mice which had been fed the diet with the large PL coated lipid droplets postnatally changes in mitochondrial function and genes regulating thermogenesis compatible with enhanced energy expenditure (personal communication). Future studies dedicated to measurement of energy

balance, nutrient partitioning and feed efficiency are pivotal to determine the potential value of these initial findings as a potential underlying mechanism for the observed protective effects on adult body composition and metabolic phenotype.

Critical window: Of Mice and Men

Our studies (**Chapter 3-6**) show that early postnatal life represents a critical window of developmental plasticity in which relatively moderate changes in dietary lipid quality have sustained effects on adult body composition and metabolic profile in mice. In rats (**Chapter 4**), the low LA diet did not affect adult fat mass or body composition, but it did decrease adipocyte number, increase adipocyte size and improved glucose tolerance in adulthood. When extrapolating these findings to human development one needs to take into account that rodents are born relatively immature compared to humans. Humans are born with a mature hypothalamus-pituitary-adrenal (HPA) axis, important in regulation of adipose tissue development, which starts in the third trimester of gestation [(68)]. WAT development starts after birth in rodents and coincides with maturation of the HPA axis in the first two weeks of life [(69)]. Based on these species comparisons, it seems reasonable to assume that the PUFA interventions in our rodent studies starting at postnatal day (PN) 2 can correspond to a nutritional intervention during late gestation in humans continuing into early postnatal life as we extended the diet intervention beyond weaning of the pups. Effectiveness of moderate FA composition changes (**Chapter 3 and 4**) for humans may therefore not only depend on postnatal but also on fetal nutritional environment. Alternatively, a diet intervention starting at PN2 in mice may also represent a postnatal diet in infants born (very) preterm. DHA supplementation has been more effective in improving brain development in preterm infants compared to term infants presumably because it corresponds with the increased requirement for DHA deposition in the brain during the last trimester of pregnancy [(26)]. N-3 and n-6 LCP supplementation moderately enhances growth in preterm infants whereas LCP effects on growth and cognitive function in term infants are less evident [(26, 47)].

In other words, manipulations in n-6 PUFA and n-3 LCP composition in our mouse studies could be extrapolated to adjustment in dietary FA composition

from the third trimester in pregnancy to the early postnatal period (infancy and early childhood) for term infants and the early postnatal period for preterm infants. According to this line of reasoning and assuming extrapolation of the present results to the human situation, dietary PUFA intake by women during (late) pregnancy could be considered important in programming of adult life body composition and metabolic health of their offspring and maybe deserves more attention from a scientific and public health perspective. Effectiveness of reduced LA or enhanced n-3 LCP on body composition in human adults might by enhanced when provided throughout pregnancy and lactation. We have several arguments to support this notion: Firstly, it could then beneficially modulate fetal WAT development in the last trimester. Secondly, it would also increase the n-3 LCP and decrease n-6 LCP content in human milk significantly since only one-third of the milk PUFA content is determined by dietary PUFA during lactation (**Chapter 2**) and the rest is derived from maternal fat depots.

The extrapolation of critical window from PN2 in mice to third trimester of pregnancy in human does not apply to dietary medium chain fatty acids (C8:0-C10:0; MCFA), because our mouse study showed that the relative dietary MCFA content is not translated to milk MCFA (**Chapter 2**). Thus, the mouse pups from dams fed a MCFA-enriched diet were only exposed to increased amounts of MCFA when they started to eat from the maternal diet, i.e. from PN16 onwards. Despite this late onset of dietary intervention, exposure of mice to 20 wt/wt% (of total FA) MCFA from PN16 to 42 resulted in a 28% reduced fat accumulation on an adult western style diet (personal communications). Our studies with altered lipid structure (**Chapter 5 and 6**), like the MCFA intervention, started at PN16, which more likely corresponds with early postnatal life in term infants considering maturation of the HPA axis and ontogeny of WAT.

The critical window for hyperplastic and hypertrophic WAT development in humans is not well defined, which makes extrapolation of our preclinical findings on adipocyte size and number difficult. It has been suggested that human preadipocytes have highest proliferation and differentiation capacity at the 1st year of age [28], suggesting that the critical period for adipogenesis is still open during infancy and that nutritional signals could influence adipocyte number in this period. However, our mouse study did not show effects on adipocyte number, rendering it less likely that postnatal exposure to large PL-coated milk lipid droplets affects adipocyte number in healthy term infants. We feel that this hypothesis deserves to be tested in a randomized clinical trial, but including the

analysis of adipose tissue of healthy neonates may be difficult. Whether preterm infants or infants exposed to an adverse (nutritional) environment in fetal life would have a similar response to the lipid structure is also unknown and merits further attention.

HM is the preferred nutrition for infants and the large PL-coated droplets in HM may exert sustained beneficial effects on later life body composition and metabolic profile. In fact, especially breastfeeding *duration* has been associated with protective effects on (childhood) obesity. One could hypothesize that, if these protective effects was (partially) mediated by the lipid structure, prolonged exposure to this lipid structure would be beneficial. In our mouse model, we actually extended exposure to the large, PL-coated droplets (present in mouse milk) beyond lactation into the early weaning period by the diet containing infant formula with the large PL-coated lipid droplets (**Chapter 5 and 6**). Ideally, one would prolong breastfeeding but if this is not feasible, infant formula with adjusted lipid structure might provide a better alternative than an infant formula with small lipid droplets without PL coating.

The impact of early nutritional interventions depends on the timing of critical windows in development and on whether or not (adverse) programming at a certain stage of life could be ameliorated through intervention in a later critical window. Unfortunately, these critical windows are not very accurately defined. Pregnancy is well established as a period of developmental plasticity and the importance of infancy is increasingly acknowledged from either studies investigating the role of early postnatal growth velocity [52] or the protective effects of breastfeeding [70]. In contrast, although weaning and adolescence have been suggested as critical periods of development [71, 72], very few experimental studies are available to support the relevance of these later time windows specifically [73-76].

Apart of the timing of a critical developmental window, also the duration of this period is of importance. Until which age can nutrition be effective in (de-) programming adult health? Does the window close in late infancy or does early childhood also provide an opportunity for adult health? Our postnatal interventions continued until PN42, thus including the early postweaning period representing infancy and childhood in humans, but excluding the onset of puberty. Mice mature relatively fast: puberty in males starts around PN46, and reproductive capacity is reached between PN 55-60. In fact, adolescence can also be considered a critical developmental period [77, 78] characterized by fast

growth (induced by the changed concentrations of sex hormones and growth hormone), changes in insulin sensitivity, increased adiposity through increased adipocyte size and number, and a high proliferation and differentiation capacity of preadipocytes [28, 79]. Apparently, the programming effects we observed were rather robust against these pubertal events. For, in our mouse studies, the effects of postnatal PUFA composition sustained, despite the western diet challenge during adolescence.

The short duration of infancy and childhood in mice renders it is a less suitable model to investigate the role of infant nutrition on postnatal growth trajectories longitudinally. On the other hand, the short lifespan makes it a valuable and well established tool to investigate nutritional programming effects on adult health. Future studies, perhaps in other model animals, are required to define critical developmental periods in more detail, specifically weaning (early childhood) and (pre)adolescence.

Health implications and future directions

Our data demonstrate that quality of nutrition during infancy, more specifically different aspects of lipid quality, such as FA composition and physical lipid structure, are important determinants for adult life metabolic health and disease risk. Since human milk or formula is the only source of food during early infancy, defining appropriate milk lipid composition and matrix is crucial for early development and adult health. Preterm infants are, in addition to enteral human milk or preterm formula, often exposed to parenteral nutrition. These vulnerable infants have very specific needs due to their immaturity at birth, rendering appropriate dietary lipids even more important. Although human milk lipids have been investigated intensively [80-83], many aspects have remained relatively unclear, such as the role of specific maternal factors and their interactions. For example, how does maternal body composition and metabolic phenotype impact HM (lipid) composition? What is the window of opportunity to improve HM milk composition by improving maternal nutrition? During which critical period is nutritional intervention most effective for which adult phenotype?

Our present results clearly indicate that maternal dietary PUFA content during lactation is readily translated to PUFA content in milk (**Chapter 2**). Similarly,

dietary PUFA have been retrieved from human milk within 6 hours after intake in lactating women [84, 85]. The global increased LA intake and decreased n-3 LCP intake is reflected in mature milk of women from Europa, Australia and Northern America [9, 15, 86]. In other words, upon changing the maternal diet towards more LA content during the last decades [1, 2], maternal HM lipid composition has changed accordingly, potentially contributing to obesity risk of their offspring. N-3 LCP recommendations during pregnancy, but especially during lactation, are based on infant LCP status and safety rather than on (long-term) health outcomes due to lack of scientific proof in healthy term infants. Based on our present results it is tempting to speculate that specific subpopulations of infants at risk for obesity and NCD due to an adverse fetal environment attributable to for instance maternal obesity, metabolic syndrome, T2D or GDM [87, 88], benefit from increased n-3 LCP intake due to its anti-inflammatory properties and the ability to counter detrimental effects of n-6 PUFAs. Generally, however, rather than increasing n-3 LCP intake, reducing LA intake might ultimately prove to be more effective due to its strong adipogenic and lipogenic characteristics, its strong inhibition on n-3 PUFA conversion and thus detrimental effects on n-3 LCP status. In fact, Hibbeln and colleagues [4] argue that n-3 LCP recommendations could even be reduced to a tenth of current recommendations by reducing n-6 PUFA intake. Increasing tissue n-3 LCP content by supplementing dietary n-3 LCPs on a background diet with high LA is difficult, because LA inhibits incorporation of DHA and EPA in biological membranes. Additionally, n-6 and n-3 PUFAs compete for the same set of enzymes for eicosanoid synthesis. Lowering LA would reduce the pro-inflammatory eicosanoids originating from the n-6 pathway and therefore less n-3 LCPs might be required to rebalance through synthesis of anti-inflammatory eicosanoids [4].

HM milk remains in many aspects superior to IMF due to reasons discussed in previous sections of this thesis. It is now clear that dietary lipid structure can be added to the factors contributing to the potential long term health benefits of breastfeeding. Human milk substitutes, i.e. IMFs, are available if infants cannot be breastfed. It is impossible to exactly mimic all the characteristics of human milk and many of the behavioral factors implicated in breastfeeding, However, our present studies suggest that we can further improve IMF lipid quality by adjusting FA composition as well as lipid structure. We do realize that these preclinical findings should now be tested in randomized clinical trials in infants to justify changes in IMF lipid composition.

As stated previously, current recommendations for EFA and LCPs in IMF are based on content in human milk of Caucasian women, data on infant PUFA status and data on functional outcome such as growth and visual acuity [89]. With the concerns of scientists and health care professionals about the imbalance between dietary n-6 and n-3 intake, recommendations based on contemporary HM originating from Caucasian women on a typical Western diet merits re-evaluation. Kuipers and others suggest IMF recommendations should be based on HM FA composition originating from ancient paleolithic diets rather than from our contemporary western diets [16]. This ancient, paleolithic diet would entail a high n-3 PUFA (ALA), high n-3 LCPUFA (DHA and EPA), high n-6 LCP (ARA) content and a low ARA/DHA ratio compared to our contemporary western diets [16], which might protect against proinflammatory eicosanoid signaling, enhanced LDL oxidation, enhanced platelet aggregation and reduced incorporation of n-3-LCPs in membranes associated with high dietary n-6 PUFA intake and many chronic diseases [3, 6].

Our present studies showed long term beneficial effects of PUFA composition and physical lipid structure in an animal model. Future preclinical studies should at least investigate interaction between individual lipid compounds, because effects of individual compounds can be either enhanced or abrogated when combined. For instance, high n-3 LCP supplementation may be most effective under a low LA background [4] as discussed before. High LA inhibits ALA conversion to EPA irrespective of LA/ALA ratio [38] and dietary ALA inhibits conversion of ALA to DHA [15]. Additionally, PUFAs, including LA, are essential for growth and normal physiological function, and may only be detrimental in combination with high fat [9] and/or high carbohydrate (sugar) [43] diet. In summary, both the ratio and the absolute amounts of dietary EFA and LCP should be taken into account for a balanced n-6/n-3 status. Combining an optimized PUFA content and ratio with the altered lipid structure may be a promising strategy to improve lipid quality of infant nutrition.

Preclinical studies for further substantiation of the underlying mechanisms should establish whether energy expenditure and partitioning of nutrients is programmed differently due to our postnatal nutritional interventions. These studies should include measurements concerning central regulation of energy homeostasis (metabolic rate and food intake) and mitochondrial function. Both mechanisms were considered to play a role in nutritional programming [49, 50, 90-92].

Additionally, a more detailed analysis of lipid homeostasis, insulin sensitivity, inflammation and (other) established risk factors for NCDs is required to determine whether the observed changes in body composition, i.e. reduced fat mass, actually attenuated (cardio)metabolic disease risk.

The role of hyperplastic and hypertrophic growth of WAT in adult WAT function and disease risk also merits further investigation. Specific nutrients and nutritional concepts driving WAT development, critical windows for WAT development and features of appropriate or "healthy" WAT development are at present largely unknown. For instance, Spalding and colleagues correlated high adipocyte number, which already emerged in early childhood, with obesity [93]. Whether this correlation is based on a causal relationship, is not known. We have shown that WAT function can be beneficially programmed by dietary lipid structure without affecting adipocyte number. We have also shown that low LA programs towards fewer but relatively larger adipocytes which was accompanied by reduced fat mass and improved phenotype, whereas large adipocytes in WAT are usually associated with an adverse metabolic phenotype and increased disease risk [94, 95]. Hence, how adipocyte size and number affect (later life) WAT functionality and disease risk remains to be determined.

Randomized clinical trials are indispensible to generate solid data concerning safety and efficacy in infants and to test whether the present promising results ultimately allow future implementation of new IMF concepts in order to improve long-term health. These changes in dietary lipids during infancy may especially benefit infants "at risk", including small-for-gestational age infants that may have experienced fetal undernutrition, preterm infants which have specific nutritional needs due to their immaturity at birth and infants from women with GDM or obesity which are potentially exposed to fetal overnutrition. The first randomized clinical trial (VENUS) investigating the role of dietary lipid structure in infant formula in early growth of healthy term infants has started.

Conclusion

We aimed to determine whether later life obesity risk could be altered by means of relative moderate nutritional changes in early life in preclinical studies. Our results show that manipulation of the PUFA composition during infancy and childhood, either by decreasing relative amounts of dietary LA or by increasing n-3 LCP, protects against excessive fat accumulation in a mild obesogenic adolescent and adult environment. Next to these compositional effects, also the physical lipid structure, i.e. large, PL-coated lipid droplets, reduced fat accumulation and improved metabolic profile in mice during adolescence and adulthood. These data emphasize the importance of the early postnatal period and of nutrition in this period in programming later life metabolic health. Moreover, the critical window may extend to later in postnatal life than previously thought since our intervention with the physical lipid structure started after lactation. Reported protective effects of breastfeeding may be partly explained by the physical properties of lipids in HM and not by differences in FA composition alone. If the present findings in rodents can be confirmed in humans, novel strategies can be developed to adapt infant nutrition by changing dietary lipid quality and potentially extending duration of exposure, for (targeted) beneficial manipulation of long term (adult) health.

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Chapter 8

Summary

The high, worldwide prevalence of obesity poses a global economic and health burden due to high medical costs, loss of productivity and loss of health-related quality of life. Obesity increasingly emerges at an early age. This is particularly alarming since early onset obesity is associated with adult obesity, severe and early onset metabolic disease and low therapeutic effectiveness. Therefore, early prevention is crucial.

Besides established contemporary lifestyle factors and genetic disposition, accumulating evidence shows that the early life environment, especially nutrition, is a determinant of later life obesity and metabolic disease risk. The Developmental Origins of Adult Health and Disease (DOHaD) concept was originally put forward by David Barker and colleagues as the "fetal origins of adult health" hypothesis. Their notion originated from retrospective birth cohort studies which demonstrated the association between impaired fetal growth and increased mortality due to ischemic heart disease at middle age. Since then, many epidemiological and experimental studies have demonstrated that nutritional disturbances during critical periods in early life development predispose to increased risk on obesity and (cardio)metabolic diseases later in life. The precise mechanism underlying this "programming" of later life health is still largely unclear, although several mechanisms have been proposed. These mechanisms include irreversible changes in organ and tissue structure, epigenetic regulation of gene expression, altered set-point of homeostatic neuroendocrine systems and changes in mitochondrial function. Adult phenotype and underlying mechanisms can vary depending on the severity of the early life nutritional insult, the type of nutrients, duration and timing of the nutritional signal, and the organ systems implicated. Most early studies concerning nutritional programming focused exclusively on the fetal environment. However, later studies indicated that the early postnatal period is an important and independent critical developmental period and a determinant of disease risk in later life. For instance, observational studies have shown that breastfeeding (duration) is associated with a moderately reduced risk on later life obesity and metabolic disease. Many factors implicated in breastfeeding might play a role in this long term protective effect, including feeding behaviour and milk composition.

In contrast to the potentially detrimental impact of early life nutritional insults, relatively little is known about nutritional interventions that *improve* metabolic health and could therefore play a role in countering the global obesity epidemic by early prevention. The overall aim of this thesis was to investigate whether moderate changes in fatty acid (FA) composition and physical lipid structure of the early postnatal diet could protect against later life obesity in a murine model for nutritional programming.

In order to determine effects of early postnatal dietary FA composition on later life metabolic health in mice, we aimed to manipulate the dietary FA composition of mouse pups during lactation without inducing stress. In **chapter 2** we determined whether and to what extent manipulation of the dietary FA composition of lactating dams was translated into the milk FA composition, and thus, whether it affected the dietary FA intake of the mouse pups. The results showed that the medium chain fatty acid (MCFA) content in milk was essentially independent from maternal dietary MCFA intake. In contrast, reduction of dietary linoleic acid (LA), a n-6 polyunsaturated fatty acid (PUFA), reduced milk LA content. Similarly, the maternal dietary n-3 long chain polyunsaturated fatty acid (LCP) intake strongly influenced milk n-3 LCP content. From these data, we concluded that manipulation of the diet of lactating mice can strongly and rapidly affect breast milk FA composition, in particular of n-6 PUFA and n-3 LCP. Targeted manipulation of dietary FA composition of milk thus enabled the modulation of nutrition in early postnatal life of mouse pups, without artificial feeding techniques.

Changes in food processing, sourcing of dietary lipids and dietary intake patterns have resulted in a contemporary global increase in exposure to dietary LA and a decrease in n-3 LCP over the last decades. The increase in n-6 / n-3 ratio in contemporary diets has been suggested to contribute to the pathogenesis of (cardio)metabolic disease. If this hypothesis were true, a reduction of the dietary n-6 / n-3 ratio would then be expected to have counteractive effects, i.e. prevent obesity and (cardio)metabolic diseases. **Chapter 3** and **4** address the metabolic programming effects of a reduced n-6/n-3 ratio, by either an increased postnatal n-3 LCP or a reduced LA, respectively, during lactation and early weaning. The results showed that both increasing n-3 LCP and reducing LA prevented fat accumulation in mice challenged by a western style diet in adolescence and (young) adulthood. This was accompanied by an improved metabolic profile. The mechanisms by which these diets decreased adult

fat mass accumulation may be different as the two dietary manipulations differentially affected adipocyte size and number. Postnatal supplementation with n-3 LCP resulted in smaller adipocytes suggesting an altered homeostatic "set point" of adipocyte lipid metabolism. In contrast, a low dietary LA content in early postnatal life increased adipocyte size and reduced adipocyte number in adult life. These data indicate that low dietary LA content in early life reduces preadipocyte differentiation (adipogenesis) and thereby the long term lipid storage capacity.

In **Chapter 5** and **6** we describe the metabolic programming effects of a specific feature of dietary lipids differing between human milk (HM) and infant milk formula (IMF), namely the physical lipid structure. HM contains large lipid globules (average mode diameter of 4 µm) and is surrounded by a biological phospholipid (PL) membrane whereas IMF lipid globules have a diameter of approximately 0.3-1 µm and consist of a core of triglycerides with milk proteins adhering to the globule surface. Absorption and digestion kinetics of HM and IMF lipids could differ according to their physical structure, which in turn may affect growth and body composition development with potential long term effects on adult obesity risk. The studies in **chapter 5** and **6** concern programming effects of an concept IMF with a lipid structure closer to that of HM, i.e. large lipid droplets coated with PL (Nuturis®). The data showed that mice exposed to this concept IMF in early postnatal life had a lower fat accumulation and a relative beneficial metabolic profile after being challenged by a western style diet in adulthood. The Concept IMF decreased white adipose tissue (WAT) expression of PPARγ, C/EBP and RXRα in adulthood, key regulators of metabolic activity, suggesting reduced fatty acid uptake and/or *de novo* lipogenesis. In accordance with these findings, adipocyte size was reduced in adult mice fed the Concept IMF postnatally. In contrast, adipocyte number was not affected by the early programming diet.

In conclusion, manipulation of the PUFA composition during infancy and childhood, either by decreasing relative amounts of dietary LA or by increasing n-3 LCP, protects against excessive fat accumulation in a mild obesogenic adult environment in a mouse model. Next to these compositional effects, also the physical lipid structure, i.e. large, PL-coated lipid droplets, showed programming effects in the form of reduced fat accumulation and improved metabolic profile in adult mice. Thus, reported protective effects of breastfeeding may be partly explained by these physical properties of lipids in HM and not by differences in

FA composition alone. Taken together, our data emphasize the importance of the early postnatal period and of the nutrition during this period with respect to programming of the metabolic health (risks) in later life. If the present findings in rodents can be confirmed in humans, novel strategies can be developed to adapt infant nutrition by changing dietary lipid quality and potentially extending duration of exposure, for (targeted) beneficial manipulation of long term (adult) health.

Samenvatting

Obesitas is wereldwijd een zware belasting voor de economie en volksgezondheid als gevolg van hoge medische kosten, verlies van productiviteit en verminderde kwaliteit van leven door gezondheidsproblemen. In toenemende mate komt obesitas vroeg in het leven voor, wat zorgwekkend is wegens associaties met obesitas later in het leven, metabole ziekten vroeg in het leven en zeer beperkte effectiviteit van behandeling. Preventie is daarom van groot belang.

Bekende oorzaken van obesitas zijn onder andere genetische aanleg, maar ook omgevingsfactoren, zoals te weinig lichaamsbeweging en te hoge energie inname. Daarnaast is er echter steeds meer wetenschappelijk bewijs dat (omgevings)factoren in het vroege leven, met name voeding, een rol speelt in het risico op de ontwikkeling van obesitas en metabole ziekten later in het leven. Dit fenomeen werd gedefinieerd als het "Developmental Origins of Adult Health and Disease" (DOHaD) concept. David Barker was één van de eerste onderzoekers die hier aanwijzingen voor vond. In een geboortecohort in Hertfordshire toonde hij de correlatie aan tussen een laag geboortegewicht, bij benadering een maat voor verstoorde foetale groei, en verhoogde mortaliteit als gevolg van coronaire hartziekte. Sindsdien hebben veel epidemiologische en experimentele studies aangetoond dat een suboptimale inname van nutriënten tijdens kritische periodes in het vroege leven het risico op obesitas en metabole ziekten later in het leven verhogen. Naast ondervoeding, bleken ook *over*voeding en/of specifieke tekorten aan bepaalde macro- of micronutriënten van belang te zijn.

Initiële studies waren voornamelijk gericht op het belang van de foetale periode. Latere studies toonden echter aan dat de vroege postnatale periode ook een belangrijke, onafhankelijke kritische periode van ontwikkeling is en een determinant van gezondheid en ziekte later in het leven. Zo hebben observationele studies bijvoorbeeld laten zien dat zuigelingen die borstvoeding hebben gekregen later een verlaagd risico op obesitas en metabole ziekten hebben. Vele kenmerken van borstvoeding zouden hiervoor verantwoordelijk kunnen zijn, zoals de samenstelling van de moedermelk, maar ook het voedingspatroon van borstgevoede zuigelingen en het gedrag van de moeder. Tot op heden is het grotendeels onbekend *hoe* voeding in het vroege leven een effect heeft op de gezondheid later in het leven. Desondanks zijn er aanwijzingen

voor een aantal onderliggende mechanismen die een rol kunnen spelen: irreversibele veranderingen in orgaan en weefselstructuur, epigenetische regulatie van genexpressie, een veranderde setpoint van homeostatische neuro-endocriene systemen en veranderingen in mitochondriële functie. Het volwassen fenotype en onderliggende mechanismen kunnen variëren afhankelijk van de ernst, duur en timing van het nutritionele signaal, het type nutriënt en het orgaan systeem dat beïnvloed wordt.

Tot op heden zijn voornamelijk de negatieve gevolgen van suboptimale voeding in het vroege leven veelvuldig onderzocht. Data met betrekking tot nutriënten met een potentieel beschermend effect tegen later leven obesitas zijn daarentegen nauwelijks beschikbaar. Het onderzoek beschreven in dit proefschrift heeft betrekking op effecten van vetzuursamenstelling en de fysische structuur van vetten in postnatale voeding op obesitas later in het leven. Hierbij is gebruik gemaakt van een muismodel voor nutritionele programmering.

Om bovenstaande te kunnen bestuderen was het van belang de vetzuurcompositie van de voeding van muizenpups kort na de geboorte te kunnen beïnvloeden zonder stress te induceren door hanteren en handmatig voeden van de jonge pups. Daarom hebben we in **hoofdstuk 2** onderzocht of het mogelijk is de vetzuurcompositie van de moedermelk te veranderen door de moedermuizen op diëten met verschillende vetzuurcomposities te zetten vanaf dag 2 na de geboorte van de pups. De resultaten toonden aan dat een gedeelte van de vetzuren in de voeding van de moeder vertaald werd in vetzuurcompositie van haar melk. Een dieet met verlaagd linolzuur (linoleic acid; LA), een n-6 meervoudig onverzadigd vetzuur, verlaagde ook het linolzuur in de melk. Verhogen van n-3 langketenige meervoudig onverzadigde vetzuren (n-3 long-chain poyunsaturated fatty acids; n-3 LCP) in het dieet van de moeder verhoogde ook de hoeveelheid n-3 LCP in haar melk. De vetzuren die niet uit het dieet vertaald werden in de moedermelk waren de middellange verzadigde vetzuren (medium chain fatty acid; MCFA). Uit deze data concludeerden we dat het mogelijk was om pasgeboren muizenpups bloot te stellen aan verschillende voedingsvetzuren, voornamelijk LA en n-3 LCP, via vetzuren in het dieet van de moeder, omdat deze vertaald worden in de melk tijdens lactatie.

Door ontwikkelingen in productie van voedingsmiddelen en toename van het gebruik van plantaardige oliën in voedingsmiddelen gedurende de laatste tientallen jaren, is de hoeveelheid n-6 vetzuren, met name LA, die we dagelijks

innemen, sterk toegenomen. Als gevolg hiervan worden we blootgesteld aan een hogere n-6/n3 vetzuur ratio dan voorheen, wat wordt geassocieerd met een toename van chronische ziekten. Een beperkt aantal studies suggereert dat de toename in n-6/n-3 ratio ook bijdraagt aan de hoge obesitas incidentie wereldwijd. Als deze aanname inderdaad juist is, zou verlaging van de n-6/n3 ratio in onze voeding het risico op obesitas moeten verlagen.

H**oofdstuk 3** en **4** beschrijven twee studies waarin de n-6/n-3 ratio van het vroege postnatale dieet van muizenpups is verlaagd door toevoeging van n-3 LCP of door vermindering van LA (n-6 vetzuur). Vervolgens is onderzocht wat de programmerende effecten zijn van deze dieetinterventies tijdens adolescentie en volwassenheid. De resultaten toonden aan dat beide vroege dieetinterventies resulteerden in een verminderde vet aanzet en een verbeterde metabole gezondheid in het volwassen leven. De onderliggende mechanismen achter deze programmerende effecten leken echter wel anders, omdat de vroege dieetinterventies een verschillend effect hadden op het aantal en de grootte van de vetcellen in het vetweefsel. Toevoeging van n-3 LCP aan het vroege dieet resulteerde namelijk in kleinere vetcellen in volwassenheid zonder een effect op het aantal vetcellen, terwijl verlaging van LA resulteerde in een verminderd aantal, maar wel grotere vetcellen. Dit suggereert dat n-3 LCPs een blijvende verandering teweeg hebben gebracht in de stofwisseling van de vetcellen, waardoor ze minder vet opslaan en/of meer vet afbreken en daardoor kleiner blijven. De lagere LA in het postnatale leven leek daarentegen vetcel ontwikkeling in het vroege leven te remmen, waardoor er minder vetcellen zijn. Het gevolg hiervan is dat de vetopslag capaciteit van het vetweefsel in deze muizen waarschijnlijk lager is. Samengevat, ondanks dat de mechanismen lijken te verschillen, is het netto effect in beide gevallen was een lagere vetmassa in volwassenheid.

Hoofdstuk 5 en **6** omschrijven studies met betrekking tot een ander aspect van vet in vroege voeding, namelijk de fysische vetstructuur. Moedermelk bevat grote vetdruppels die worden omgeven door een fosfolipide membraan, terwijl vetdruppels in flesvoeding (of "infant milk formula"; IMF) een factor 5 tot 20 kleiner zijn en niet omgeven worden door een fosfolipide membraan, maar door melkeiwitten. Er zijn aanwijzingen dat de snelheid ("kinetiek") van vertering en absorptie van vetten in het maagdarmkanaal afhankelijk zijn van vetdruppelgrootte en de structuur aan de oppervlakte van de vetdruppel. Op basis van dit gegeven is het goed mogelijk dat de vertering en absorptie kinetiek van vet uit moedermelk en IMF verschillen. Deze verschillen kunnen

een effect hebben op groei en metabole ontwikkeling en daarmee ook een programmerende effect hebben op metabole gezondheid later in het leven.
Om dit te onderzoeken hebben we een concept IMF ontwikkeld met een fysische vetstructuur die meer lijkt op dat van moedermelk, namelijk met grotere vetdruppels en fosfolipiden rond de vetdruppel. Dit concept is onderzocht in hetzelfde muismodel als de eerder genoemde studies met LA en n-3 LCP. Uit de data blijkt dat de fysische lipide structuur van vet in de vroege voeding inderdaad programmerende effecten heeft op lichaamssamenstelling in volwassenheid. De grote, met fosfolipiden omgeven vetdruppels in voeding in het vroege leven zorgden dat de muizen tijdens adolescentie en volwassenheid minder vet aanzetten en een betere metabole gezondheid hadden dan muizen die zijn blootgesteld aan kleine vetdruppels zonder fosfolipiden. Dit ging gepaard met lagere expressie van een aantal zogenaamde "transcriptiefactoren" in vetweefsel. Dit zijn eiwitten die de stofwisseling in het vetweefsel reguleren. De verlaagde expressie van deze transcriptiefactoren, zoals peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein (C/EBP) and retinoid X receptor α (RXRα), zijn geassocieerd met een verlaagde opname en opslag van vetten in het vetweefsel. Dit is in overeenstemming met de waarneming dat muizen blootgesteld aan het concept IMF kleinere vetcellen hadden dan de muizen die blootgesteld waren aan standaard IMF.
Uit de studies beschreven in dit proefschrift kunnen we concluderen dat het verlagen van de n-6 / n-3 vetzuur ratio in vroege voeding, door verlaging van LA of door toevoeging van n-3 LCP, beschermt tegen overmatige vetaanzet in het volwassen leven in ons muismodel. Naast de vetzuursamenstelling, blijkt de fysische vetstructuur ook van belang te zijn voor programmerende effecten op metabole gezondheid: grote, met fosfolipiden omgeven vetdruppels in vroege postnatale voeding beschermden muizen tegen overmatige vetaanzet later in het leven. Mogelijk kunnen een deel van de beschermende effecten van borstvoeding tegen obesitas later in het leven toegeschreven worden aan de fysische eigenschappen van vetten in moedermelk. Tezamen benadrukken onze data het belang van de kwaliteit van nutriënten in de vroege postnatale periode voor metabole gezondheid en het risico op obesitas later in het leven. Indien de huidige bevindingen in muizen bevestigd kunnen worden in mensen, zou dit de basis kunnen zijn voor nieuwe strategieën om obesitas in een vroeg stadium te voorkomen middels eenvoudige aanpassingen in de voeding van zuigelingen en jonge kinderen.

Frequently used abbreviations

ALA	α-linolenic acid
ARA	arachidonic acid
BW	body weight
CRL	crown-rump length
CTRL	control
DEXA	dual energy x-ray absorptiometry
DHA	docosahexaenoic acid
FA	fatty acids
FFA	free fatty acids
FM	fat mass
HM	human milk
HOMA-IR	homeostasis model assessment of insulin resistance
IMF	infant milk formula
LA	linoleic acid
LBM	lean body mass
LCP	long-chain polyunsaturated fatty acids
PL	phospholipids
PN	postnatal day
RXRα	retinoid X receptor α
SREBP1c	sterol regulatory element-binding protein 1c
TC	total cholesterol
TG	triglycerides
WAT	white adipose tissue
WSD	western style diet

Dankwoord

Eindelijk is het zover! Ik mag het dankwoord schrijven van **mijn** proefschrift! Een mooie afsluiting van vele schrijfuren. Omdat dit onderzoek is niet via het "klassieke" PhD proces tot stand is gekomen hebben veel mensen (extra) tijd en energie moeten investeren om mijn *doctor*-wens in vervulling te laten gaan. Zoals promotor Henkjan in onze gesprekken loepzuiver waarnam en zeer terecht terugkoppelde: ik heb wat moeite kort en bondig mijn punt te maken. En, zoals een collega eens opmerkte: "je schrijft zoals je praat". Dus nu weten jullie waar je aan toe bent: ik grijp deze kans mooi aan om heel veel mensen uitgebreid te bedanken. Hou je vast, het wordt een lang verhaal.

Dit dankwoord wordt overwegend gedomineerd door sterke, intelligente, gedreven, mondige, enthousiaste en betrokken vrouwen. Niet verwonderlijk gezien mijn werkomgeving. De wereld van voormalig Numico, voormalig Danone Baby Nutrition, huidig Nutricia R&D Early Life Nutrition, wordt nu eenmaal bevolkt door veel, heel veel vrouwen. Maar het is een voorrecht om te kunnen werken met en leren van deze dames. Voor 3 mannen maak ik een uitzondering. Voor de juiste balans zal ik beginnen en eindigen met de mannen…

Beste Henkjan, een groot deel van het onderzoek beschreven in dit proefschrift was al tot stand gekomen voordat jij benaderd werd om mijn promotor te zijn. Voor een professor, arts en onderzoeker met een sterke wetenschappelijke interesse en visie moet dat een wat ondankbare en weinig uitdagende taak geweest zijn. Dus in eerste plaats: Dank je voor het opnemen van deze vreemde eend in de bijt. Daarnaast duizendmaal dank voor je pragmatisme, sterke analyses, duidelijke taal en concrete uitdagingen. Ondanks de relatief korte periode heb je me vele wijsheden bijgebracht. Voor mij inmiddels de klassiekers die ik te pas en te onpas loslaat op mijn teamgenoten: "Keep-it-simpel" (moeilijk!) en "laat de lezer nooit zelf denken" (Huh? Nooit eerder over nagedacht). Tot slot, dank voor de vele vrije uren die je hebt gestoken in mijn manuscripten. Ik heb nog altijd het beeld voor me van een terecht geïrriteerde familie Verkade aan de eettafel met een koud geworden feestmaal, omdat je in je week kerstvakantie zowel op tweede kerstdag als op oudejaarsavond mijn laatste stuk moest doorwerken (sorry daarvoor). Zonder jouw inspanningen was ik zeker niet op dit punt aangekomen.

Beste Eline, als 17-jarig kuiken zat ik bij het practicum Human Biologie en Pathobiologie te stralen, omdat ik dankzij jou eindelijk begreep hoe de osmolariteit van de omgeving de turgor van een plantcel beïnvloed. En, o wonder, ik vond het nog interessant ook! Sindsdien heb je me keer op keer weten te enthousiasmeren voor (afstudeer)vakken met betrekking tot (neuro) endocrinologie bij de vakgroep Fysiologie van mens en dier van de, toen nog Landbouw Universiteit in Wageningen. En daarna voor alle experimenten die we voor Nutricia/Numico/Danone in elkaar hebben geknutseld. Ik bewonder je niet aflatende drive en ambities, je wetenschappelijke visie en je vermogen om me na een overleg altijd met hernieuwt enthousiasme en nieuwe ideeën de deur uit te laten gaan. Het is mijn ambitie om dat effect ooit nog te krijgen op de mensen in mijn (werk)omgeving.

Beste Martine, de steun, het vertrouwen en de vrijheid die je me hebt gegeven waren onontbeerlijk om eindelijk te kunnen promoveren. De tijd die ik heb mogen besteden aan de laatste fase van mijn proefschrift was zeker niet direct ten gunste van het platform of mijn taak als teamleider. Dat maakt het extra bijzonder dat je me de ruimte gaf om hier toch in te investeren. Dank daarvoor! Daarnaast heb je me vooral veel inzichten gegeven over mijzelf en mijn manier van werken en communiceren (soms confronterend, maar altijd waardevol) om te kunnen groeien als lijnmanager en wetenschapper. Ik heb veel bewondering voor de tijd en energie die je investeert om jouw mensen te laten groeien en je bereidheid om zonder voorbehoud informatie, kennis en vaardigheden te delen voor "the greater good". Ik hoop dat we nog vaak kunnen sparren!

Lieve Diane, natuurlijk heb ik dit eigenlijk allemaal gedaan zodat jij eindelijk paranimf kan zijn in oogverblindende jurk. Sorry dat het zo lang heeft geduurd en echt heel erg dat ik uit mijn "LaDress" ben gegroeid. Voor alle studies in dit proefschrift hebben we samen vele uren in de kelder van Numico en het CKP gestaan. Altijd ambitieuze experimentele planning (NATUURLIJK kunnen we nog een experiment parallel opstarten en/of naar voren schuiven. Nee hoor, als we vier man nodig hebben, dan moet het met twee ook wel lukken). En toch heb ik de beste herinneringen aan het uitwisselen van nieuwtjes, ideeën en slechte (=goede) grappen tijdens het inzetten van de muizenfok, DEXA, kooien mesten, canuleren, sectie, bloed tappen en (het absolute hoogtepunt van onze carrière) muizen melken met aangepaste kolfpomp. Het kosten een paar jaren, maar dan

heb je ook wat (publicaties). We werken niet meer zo intensief samen nu jij een rol hebt veroverd als DE project- and portfolio manager pre-clinical studies van Danoontje (Powerrrrrrrrrrrr). Je daadkracht, betrokkenheid en doeltreffendheid zijn niet te evenaren. Als ik weer eens heel veel tijd en woorden nodig heb om mijn punt te maken, denk ik wel eens: Misschien kan Diane even mijn punt maken, want zij kan tot de kern van de zaak komen in gemiddeld twee zinnen. Dus binnenkort maar weer eens strategisch overleg en sushi?

Lieve Karen, vele jaren mijn roomie in het Wageningse. Samen met Tiemen gek doen op de vrijmibo momenten (die ook rustig op maandagochtend voor konden komen, want gezelligheid kent geen tijd). Je hart kunnen luchten op de kamer van "niemand" in mindere tijden, zowel privé als werkgerelateerd. Ik herinner mij een maandagochtend waarin ik ongeveer 2 kg chocola, een fles port en een poster van Brad Pitt op mijn bureau vond (met dank aan jou en Diane?). Ik heb me zelden zo goed gevoeld op zo'n slecht moment. Nog steeds hebben we onze momenten, nu in de cognac-kamer, maar dat is toch niet helemaal hetzelfde, vind je niet? Al dat glas. Ook al hebben we nooit echt samengewerkt op projecten, je praktische no-nonsense insteek, je luisterend oor en je relativeringsvermogen waren altijd waardevol. Ik heb me jarenlang vergaapt (en doe dat nog steeds) aan je gestructureerde manier van werken (leer ik nooit), je efficiëntie (misschien ooit) en de kennis die je weet te extraheren uit ogenschijnlijk weinigzeggende data door je grondige manier van werken en je beroemde vrijdagmiddag experimentjes "on the side" (ik ga z.s.m. bij je in de leer).

En dan mijn superteam! Vier verschillende persoonlijkheden, vier verschillende manieren van (samen)werken, maar wat jullie gemeen hebben is je gedrevenheid en ambitie. Van jullie leer ik het meest. Eefje, je geeft me inzicht in het belang van het groepsproces en wijst me op punten die daarin aandacht verdienen. Lidewij, als warrige geeltje kijk ik met bewondering naar jouw focus, doelgerichtheid, directheid en scherpe waarnemingen. En nu ook nog de openbaring dat dat softe communicatie gedoe werkt!!? Andrea, tijd nemen, geduld hebben en reflectie leveren verrassend veel op. Annemarie, je enthousiasme, energie en onbevangenheid werken aanstekelijk. Nooit mee ophouden!
Zonder al teveel ophef verzetten jullie bergen werk en genereren binnen no time met beperkt budget waardevolle kennis voor onze projecten. Het werd

misschien niet door iedereen positief geïnterpreteerd, maar ik kan met trots zeggen: "We are fast, flexible and cheap" ;-). Bedankt!

Ink! Always looking forward to our next talk about our day to day life and all that is bothering or pleasing us, preferably over drinks and good food. I love your enthusiasm for your peculiar home projects and you deep loyalty towards friends and family and admire your perseverance and profound approach when it comes to work. Respect, Master Yoda!

Tiemie! Wanneer doen we nou weer eens een Katie? En heb je al nieuwe data?

Voormalige Numicoters en huidige collega's: Inge, Annelies, Jan, Harm, Karin, Dianne, Chantal, Kate, Arjan en Sander. Het was en is altijd bijzonder gezellig.

Zonder alle wetenschappelijke terminologie en poespas laat de kern van dit proefschrift zich heel simpel omschrijven en ook nog toepassen op alles in het leven: Als de basis goed is, ben je opgewassen tegen alle tegenslagen die op je pad komen. Voor mij is de basis familie en goede vrienden waar je op terug kan vallen in geval van nood. Wees gewaarschuwd, ik ben nogal familieziek, dus daar komen ze…

Lieve Hes, wat heerlijk dat je al weer een flinke tijd naast mijn vriendin ook mijn collega bent. Als ik even mijn hart moet luchten stiefel ik gewoon naar Orange village 4c. Fijn om een vriendin te hebben die zo goed kan luisteren. Misschien gaan we, als onze kleine parasietjes wat groter zijn, nog eens met z'n tweeën vulcanelli zoeken op Sicilië? Maar alleen als jij rijdt!

Lieve Maai, we gaan al terug sinds 1996! (17 dagen wandelen met groep pubers in Tsjechië) Over 2 jaar een jubileum om te vieren! Sindsdien studiegenoot en vriendin. Bovendien nuchtere en pragmatische ervaringsdeskundige en vraagbaak met betrekking tot promoveren. Ik mis je. Wanneer kom je nou eens terug naar Nederland? (moest ik van mijn moeder vragen).

Lieve mama, als er iemand de sterke basis vormt, dan ben jij het. De wetenschap dat je onvoorwaardelijk van ons houd, dat je letterlijk altijd voor ons klaar staat ("Wacht even, mama komt zo!!!") en dat we in geval van nood altijd op je

kunnen rekenen, geeft zekerheid en zelfvertrouwen. Je pakt problemen altijd met wortel en al aan. Ik ken niemand anders die zo kwetsbaar over kan komen, maar stiekem zo taai is als jij.

Hannie, zus en beste vriendin, niemand begrijpt zo goed wat ik bedoel zonder dat ik het zeg. Dat al die jaren kibbelen zo'n sterke band heeft opgeleverd. Je bent best aardig opgedroogd, hoor. Arbeidsjuriste, moeder van Sam Sam, trouwe vriendin en vrouw van Heike: "Nee joh, ik ben mijn eigen persoon!". Sistahs 4 ever!

Lieve Kleine Smurf, je bent zo lekker bezig! Stoere, slimme, zelfstandige chica guapa ben je geworden. Niet zo streng voor jezelf zijn. "Keep your head up, keep your hard strong, keep your mind set in your ways". Ik moet altijd aan jou denken als ik dat hoor.

Lieve Bro, zuster Remmelt, jij ook al zo goed bezig in big city Amsterdam. Je saaie oude zus is trots op je. Fijn om te weten dat jij voor pa en ma gaat zorgen op hun oude dag...

Mijn lieve Daantje banaantje, het mooiste moment van de dag is 's avonds thuiskomen van werk en begroet te worden door een klein mannetje dat op de arm van papa met alle vier zijn pootjes aan het zwaaien is. Als je altijd zo ondeugend blijft lachen, dan is het leven goed.

Lieve lieve Menno, ik ben menigmaal bijzonder onredelijk tegen je geweest in de afronding van dit proefschrift en heb ongeveer alle andere lopende zaken in ons leven op jouw schouders geladen. Bedankt voor je eindeloze geduld, liefde en steun. Ja, die wereldvreemde wetenschappers leven in een parallel universum en zijn niet (altijd) van deze wereld. Overigens zullen we dat te allen tijde ontkennen. Gelukkig kan ik altijd terugvallen op jouw analytisch vermogen, relativeringsvermogen, positieve instelling, realisme, ambitie en creatieve oplossingen. Je zorgt voor de benodigde rust en creëert overzicht als ik weer eens ongecoördineerd, ongecontroleerd en zonder plan door het leven stuiter. Regeren is vooruitzien? Vooruitzien is regeren!

Biografie

Annemarie Oosting werd geboren op 13 september 1979 te Oss. Het grootste deel van haar lagere en middelbare schooltijd bracht zij door in Woubrugge. In 1997 behaalde zij haar Atheneumdiploma aan het Ashram college in Alphen aan den Rijn. Hetzelfde jaar startte zij de studie Voeding en Gezondheid aan Wageningen Universiteit. Haar afstudeervak werd in 2001 gevolgd bij de vakgroep Fysiologie van mens en dier onder supervisie van dr. Annelieke N. Franke en had betrekking op verminderde fertiliteit tijdens veroudering in vrouwelijke ratten als gevolg van deregulatie van de endocriene hypothalamus-hypofyse-gonaden-as. Onder supervisie van dr. Pierre Demacker volgde zij in 2002 een stage met betrekking tot postprandiale lipide responsen na een parenterale vetemulsie bij de Afdeling Interne geneeskunde aan de Radboud universiteit te Nijmegen. Na haar afstuderen begon zij in 2003 als onderzoeker bij de afdeling Condition and Disease Specific Research van Numico Research. Haar onderzoeksprojecten hadden betrekking op gezondheidsbevorderende effecten van voedingscomponenten in medische voeding voor verschillende patiënten populaties (onder andere oncologie, type II diabetes en cardiovasculaire aandoeningen). In 2008, met de overname van Numico door Groupe Danone, werd zij aangesteld als teamleider binnen de New Health Benefits groep (onder leiding van dr. Eline M. van der Beek) van de Baby Nutrition Divisie van Danone. Sinds 2011 is zij met haar team onderdeel van het platform Nutrition & Developmental Physiology onder leiding van dr. Martine Alles. Het onderzoek van dit team (Nutrition & Metabolic Programming) richt zich op de effecten van voeding in het vroege leven op obesitas and metabole ziekten later in het leven. Het deel van dit onderzoek aangaande de kwaliteit van vet in de vroege postnatale voeding, is beschreven in dit proefschrift. Deze is tot stand gekomen middels een promotietraject dat in 2011 officieel startte onder supervisie van prof. dr. Henkjan J. Verkade van de afdeling Kindergeneeskunde van het Universitair Medisch Centrum Groningen.

List of Publications

Effect of dietary lipid structure in early postnatal life on mouse adipose tissue development and function in adulthood.
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