Long-term effects of dietary lipid structure in early life

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DOI:
10.33612/diss.136676657

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 1

General introduction
Outline of the thesis
In the 20th century, life expectancy gains exceeded those in all previous periods in human history [1]. These gains are attributed to a combination of improved nutrition, sanitation, hygiene, and health care [1]. With improved living conditions and higher life expectancy disease patterns changed from infectious diseases towards (chronic) degenerative and man-made "diseases of civilization" [2, 3]. The latter encompass non-communicable diseases (NCDs) such as ischemic heart disease, stroke, chronic respiratory disease, type-2 diabetes and certain types of cancer [3, 4]. A variety of environmental risk factors contribute to the incidence of NCDs. In developed countries, these are mainly: tobacco use, high blood pressure, alcohol consumption, high cholesterol levels, high Body Mass Index (kg/m²; BMI), low fruit and vegetable intake and physical inactivity [3-5]. Some NCD risk factors may contribute to another. High BMI, blood pressure and cholesterol levels are, at least in part, related to the overt increase in caloric intake and supposed decrease in physical activity which occurred in Western European and North American countries in the 20th and 21st century [3, 5, 6]. Reductions in NCD morbidity and mortality occur when exposure to the aforementioned risk factors is reduced [3, 5, 7].

Many strategies can be devised for the reduction in the exposure to NCD risk factors. In this thesis, I will discuss possible mechanisms by which an early life diet can lower the incidence of later life obesity [8, 9]. Here I briefly summarize the current understanding of the relationship between early life nutrition and later life body weight and fat mass gain. The second part of this introduction will discuss the composition and structure of human milk, infant formulae, and how to more closely mimic the composition and structure of human milk in infant formulae. Finally, the aims and scope of this thesis will be presented.

Developmental Origins of Health and Disease (DOHaD)

Originally, the phenotype of an organism was viewed as the expression of its genotype in response to environmental cues [10]. This view was first challenged by the studies on the 1944-1945 Dutch Famine Birth Cohort (the ‘Hongerwinter’, literally ‘hunger winter’), which convincingly demonstrated that poor maternal nutrition (as a consequence of a severe famine at the end of the second world war in
the Western part of the Netherlands) increases the susceptibility of the offspring to diabetes, obesity, cardiovascular disease, and microalbuminuria in adult life [11-15]. It was reasoned that suboptimal intrauterine conditions adversely affect the short- and long-term health of the offspring long after the original stressor was removed [13, 16]. The Dutch famine birth cohort helped build the framework for what would later be coined the Developmental Origin of Health and Disease (DOHaD) and the research field of metabolic programming [10, 12, 15]. From an evolutionary point of view, a mechanism which enables developmental plasticity, i.e. the ability of the genotype to produce different phenotypes in response to different environments [10], provides a clear advantage with regards to survival and reproductive success when the environment changes at a fast rate. This early life adaptation is referred to as a predictive adaptive response [10, 17]. Disease as a consequence of this early life predictive adaptive response can occur upon a mismatch between adaptations in early life and different environmental exposure in later life. The paradigm of the DOHaD is schematically visualized in Fig. 1 [10].

![Fig. 1. The match/mismatch paradigm of DOHaD. Exposure to an environmental parameter (represented by a vertical gradient) in early life ‘primes’ (illustrated with a lightning bolt), i.e. adapts, the organism for its later life environment. If early and later life environments match, the organism can survive and reproduce optimally. Upon mismatch of early and later life environments, survival and reproduction success may be less optimal. Figure adapted from [10].](image-url)
Chapter 1

Metabolic programming

The process hypothesized to underlie the DOHaD concept is called ‘metabolic programming’ [10, 12]. Metabolic programming is defined as a stimulus / insult during a critical or sensitive window of development, with long-term (even lifelong) effects on an organism [18-20]. Mechanistically, metabolic programming is thought to be effectuated by changes in the epigenome such as DNA methylation and/or histone tail modifications [21]. The molecular mechanisms responsible for specific changes in the epigenome in response to environmental cues is poorly understood [21]. Nonetheless, descriptive studies provide insight into methylation patterns of genes relevant for NCDs [21-23]. Adults who were in utero exposed to the conditions of the Dutch Hongerwinter had, six decades later, a lower degree of DNA methylation of the insulin-like growth factor 2 (IGF2) gene compared to unexposed same-sex siblings [21]. In offspring which were in utero exposed to preeclampsia (high blood pressure of pregnancy), methylation levels of IGF2 were also lower [23]. In contrast, overnutrition during infancy in rats is associated with hypermethylation of key genes involved in insulin signaling (Irs1 and Glut4) in skeletal muscle in later life [22]. Importantly, early life overnutrition in rats leads to higher ad libitum food intake and consequently higher body weight in adulthood.

Of interest, breast-feeding is epidemiologically associated with a lower incidence in childhood, adolescent and adulthood obesity, compared to infant milk formula (IMF)-feeding [24, 25]. Breast-feeding is also associated with lower blood pressure and lower plasma cholesterol levels in adulthood [26, 27]. Human milk versus formulae has beneficial long-term effects on neurodevelopment and cognition [28], which will not be extensively described in this thesis. The reader is referred to [29-31] for an in-depth analysis of the link between human milk and neurodevelopment. Overall, the incidence rate and severity of certain non-communicable diseases (NCDs), such as ischemic heart disease, stroke, chronic respiratory disease, type-2 diabetes and certain types of cancer are modifiable by intrauterine [11-13, 32] and postnatal [24-27, 33, 34] conditions. Early postnatal life is a sensitive developmental period wherein metabolic set points, relevant for long-term disease risk, can be modified. It appears that early life nutrition can (be used to) modify these parameters [18].
**Obesity**

Overweight (BMI: 25-29.99) and obesity (BMI > 30) are defined as an abnormal or excessive body fat accumulation that may impair health [35, 36]. The gold standard of assessing body fat mass is hydrostatic underwater weighing [37], whereby a subject is weighed while fully submerged in water to calculate body density. Alternative techniques to assess (a surrogate measure of) body fat mass include body weight and height (BMI), skinfold thickness, dual energy X-ray absorptiometry, air displacement, nuclear magnetic resonance (imaging), and electrical skin conductivity. Each method has advantages and disadvantages with regards to ease of use, costs, and proximity to true body fat mass [37]. Epidemiological studies often rely on BMI as a surrogate marker for body fat mass [38]. The correlation between BMI and body fat mass is affected by age, sexual maturation, race, sex, lean mass, and the distribution of fat [39]. Notwithstanding, BMI is an at-home measurable and trackable surrogate parameter that strongly correlates with insulin resistance, hypertension, and dyslipidemia [40]. Losing excess weight (*i.e.* decreasing one’s BMI and fat mass) by reducing food intake strongly correlates with improved plasma lipids, lower blood pressure, lower glucose and lower insulin levels [41]. Limiting caloric intake has a powerful protective effect against obesity, high blood pressure, insulin resistance and atherosclerosis [41].

In the north of the Netherlands, approx. 40% of the general adult (18 years and older) population is overweight and another 16% is obese [36]. During the period 1981-2018 the incidence of overweight in the Netherlands has increased drastically for both males and females (Fig. 2 A & B). The incidence of overweight is not equal for all age groups and appears to occur much more often in males and females aged 40-75 years compared with younger ages (Fig. 2 C & D). Children and adolescents who are overweight or obese *versus* those who are normal weight are more likely to stay obese into adulthood [42]. Childhood obesity profoundly affects short-term physical health, social, and emotional well-being, and self-esteem [42]. Affecting the (eating) habits of children is expected to have a much greater impact on adulthood obesity than tackling adulthood obesity itself. Trends in overweight and obesity are of concern given the strong relationship between obesity, NCDs and health care.
utilization [40, 43]. It is expected that a reduction in obesity rates leads to a reduction in NCD rates. The high obesity incidence is attributed to an obesogenic environment, *i.e.* one that promotes (too) high caloric intake and (too) low energy consumption such as body activity or exercise [44]. Obesity has been considered as “normal physiology within a pathological environment” [45]. Solving the complex issue of obesity requires a collaboration between policy makers, industry and health care to eliminate as much as possible the obesogenic environment [44]. Proof that a holistic approach can be successful in combatting a population-scale NCD risk factor can be observed from the decline in tobacco usage following fierce population-based tobacco control campaigns and policies [46, 47]. In my view, the prevention of NCDs by early life metabolic programming fits a holistic approach to decrease the exposure of a population to NCD risk factors.

**Fig. 2. Overweight epidemiology in the Netherlands from 1981 to 2018 for males and females aged 4 years and older.** For individuals over the age of 18, a BMI of 18.5 to 24.99 was considered normal weight. For individuals under the age of 18, cut-off values were chosen based on established international standards [48, 49]. The incidence of overweight in 1981-2018 for individuals aged 4 years and older are shown for males (A) and females (B). The incidence of overweight in 1981, 1991, 2001 and 2011 are shown per age group (indicated on the x-axis) for males (C) and females (D). Data: CBS, 81565NED (CC BY 4.0).
Early life nutrition

Mother’s own milk is the best source of nutrition for nearly all term infants \(^{[50]}\). It is nonetheless advised to supplement breast-fed infants with vitamin K due to low levels in human milk and poor absorption \(^{[51-53]}\). When mother’s own milk is unavailable, donor human milk is considered as the second best choice \(^{[50]}\). Infant formulae provide a substitute to human milk and their compositions aim to mimic its nutritional composition \(^{[50]}\). The World Health Organization (WHO) recommends exclusive breast-feeding for the first 6 months of life \(^{[54]}\), and continued breast-feeding thereafter as long as mother and child wish \(^{[55]}\). The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) similarly underlines that exclusive breast-feeding for around 6 months is a desirable goal. Shorter periods of breast-feeding and a combination of breast-feeding and formula feeding are considered by ESPGHAN as valuable over not breast-feeding at all \(^{[56]}\). Breast-feeding initiation rates vary significantly by country; as low as 65% in the United States, approx. 80% in the Netherlands, and 99% in Norway \(^{[54]}\). Factors associated with initiation of breast-feeding include maternal and paternal education level (higher for higher education level), maternal job status (higher for more hours worked), smoking habits (higher for non-smokers), number of children (lower for more children), gestational age (lower for <38 weeks) and home delivery (higher for home versus hospital delivery) \(^{[54]}\). In the Netherlands, breast-feeding rates in term infants are 51% at 1 month postpartum, decreasing to 37%, 30%, 25%, 19% and 15% at 2-6 months postpartum, respectively. During these 6 months, 11%, 14%, 13%, 14%, 14% and 18% are mixed fed (breast-fed in combination with feeding formula), the remainder is formula-fed \(^{[54]}\). In the Netherlands, it appears that only a minority of infants are mainly breast-fed for the recommended first 6 months. Reasons for introducing formula milk are mainly insufficient breast-feeding technique, not enough milk (perceived or, less common, real), problems relating to breasts and/or nipples, unfavourable previous experience, and returning to work \(^{[54-56]}\). It is suggested that a major contributor to differences in breast-feeding durations between European countries is the duration of paid pregnancy leave \(^{[54]}\). In the Netherlands, paid postpartum pregnancy leave is on average 10 weeks. In Nordic
countries, with paid pregnancy leave up to 1 year, very high (80%) breast-feeding rates are seen at 6 months postpartum age \[54\].

**Human milk**

Human milk is a complex biofluid, intended as the sole nourishment and immunological protection of the infant \[57\]. Milk is classified into colostrum (the first milk produced), transitional milk (from approx. 4 days postpartum) and mature milk (from approx. 1 week postpartum), describing the gradual change in milk composition \[57\]. The work described in this thesis focusses on mature milk. A negative relationship exists between the duration of breast-feeding and the incidence of overweight in later life \[58\]. This association is poorly understood \[24, 25\], but implies that it may not be specific for colostrum and/or transitional milk. Human milk consists of carbohydrates (mainly lactose and Human Milk Oligosaccharides), proteins (grouped into caseins, whey and mucins), and lipids (mainly triglycerides, with minor amounts of di/mono-acylglycerols, free fatty acids, phospholipids and cholesterol) \[57, 59\]. It is beyond the scope of this introduction to fully describe all (bioactive) components of human milk and the intricate differences between human milk and (for example) cow milk. For a review of the current knowledge on human milk, I refer to \[50, 57, 59-62\]. I will briefly describe the lipid compartment of human milk, relevant for this thesis. Lipids in human milk contribute the largest fraction, approx. 50%, of the total energy, and are present as an emulsion of oil in water. The lipids originate from *de novo* synthesis by the mammary gland, from maternal lipid stores, and from the maternal diet \[57, 63\]. Regardless of the lipid source, cytoplasmic lipid droplets enveloped by a phospholipid monolayer, are secreted by the mammary gland cells into the mammary gland alveolar lumen \[59, 64\]. In this process, the lipid droplet attains a phospholipid bilayer from the cell membrane \[65\], net giving a biologically unique phospholipid trilayer \[59\]. The latter is called the milk fat globule membrane (MFGM) \[59, 66-68\]. The diameter of the milk fat globule (a physicochemical property) in mature human milk varies from 0.1 μm to 15 μm \[59\]. From the distribution, the mode diameter (the particle diameter most abundant by volume) can be calculated. Milk fat globules in mature milk have a mode diameter of approx. 3 to 5 μm \[59, 65, 69\], though a second smaller population of Milk Fat
Globules (MFGs), absent in colostrum, have a mode diameter of approx. 0.2 µm\textsuperscript{[69]}. The complex composition and structure of MFGs may be responsible for rapid rise and clearance of plasma triglycerides. The suggested structure-function relationship of the MFG is reviewed in \textsuperscript{[68]}.

Maternal diet does not affect the proportion of milk protein, fat and carbohydrate\textsuperscript{[56]}, with the exception of highly deviant dietary patterns. To some extent, milk is produced at the expense of the mother’s health. Several nutrients reflect the maternal nutrient status and may subsequently lead to low levels in milk\textsuperscript{[56]}. These include the fat-soluble vitamins A, D, E and K, water-soluble vitamins and minerals such as B\textsubscript{1}, B\textsubscript{12}, C, and calcium, copper, iodine, and essential fatty acid species such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)\textsuperscript{[56, 60, 61, 70]}. Maternal diets lacking essential nutrients can result in severe pathologies in the exclusively breast-fed infant; such as growth faltering, megaloblastic anaemia and neurological abnormalities\textsuperscript{[56]}. Human milk usually contains low levels of vitamin K\textsuperscript{[56]}. Vitamin K is necessary for the formation of blood clotting factors\textsuperscript{[71]}. It is recommended to give vitamin K supplementation to the neonate shortly after birth and continually during the first months of life or until the diet consists of at least 50% infant milk formula. It is emphasized that the short-term and long-term positive health effects of breast-feeding are more important than the possibility of (micro) nutrient deficiencies in some infants\textsuperscript{[18, 24, 56]}. Unless there are specific medical contraindications, human milk with vitamin K supplementation is the best source of nutrition for nearly all term infants\textsuperscript{[50, 56]}.

**Infant formulae**

Infant milk formulae are intended as an effective substitute to human milk for infants without unusual medical or dietary problems\textsuperscript{[50, 72]}. Infant formulae are nutritionally (and legally) unique as they must meet and provide all nutritional requirements of the rapidly-growing infant\textsuperscript{[73]}. In the past, criteria for the adequacy of infant formulae were mainly infant mortality\textsuperscript{[74]}. In more recent history, human error and adulteration during the formulation or production of formulae, with serious long-term adverse effects or even death of infants, have led to stringent quality
control measures and legal minima and maxima for most ingredients [73]. In the European Union, the composition of infant formulae is bound by law (directive 2006/141 and regulation 2008/1243, from 22 February 2020 onwards: regulation 2016/127). Current commercially available infant formulae are considered to be of high quality and safety [50, 72, 73]. Today these formulae, nonetheless, are epidemiologically associated with a higher incidence of childhood, adolescent, and adulthood obesity [24, 25], among other long-term ailments [26-31, 56]. Modern infant formulae may have reached close similarity to human milk with regards to their gross biochemical composition [72-74]. Yet, these formulae lack certain components and/or characteristics inherent to human milk which can be responsible for the remaining differences in the (long-term) health of the infant [24-27, 56]. The biochemical and physicochemical composition of infant formulae has continued to be an area of research and innovation, to further ‘functionally humanize’ infant formulae [72], discussed below.

Mimicking the composition of human milk requires careful formulation of ingredients and correcting for the excesses and shortcomings of (for example) cow’s milk [73, 75]. Infant formulae for healthy term infants is made from cow’s, goat’s, ewe’s, mare’s, donkey’s or camel’s milk [50]. In the European Union, only cow’s and goat’s milk-based formula is legal. Alternatively, it can (legally) be manufactured from soy [50]. Here, I will focus on cow’s milk-based formulae, which is the basis of most common formulae intended for healthy term infants. It is beyond the scope of this introduction to fully compare the biochemical composition of human milk and cow’s milk. The reader is referred to [62, 69, 75] for an in-depth comparison of human milk and cow’s milk. In brief, cow’s milk contains higher levels of fat, minerals and protein compared to human milk [50]. The quality, i.e. biochemical composition, of cow’s milk fat [50, 76] and protein (casein-to-whey ratio) [77, 78] is different from that of human milk. During the production of cow’s milk-based infant formulae, these differences must be minimized; in essence ‘functionally humanized’. With regards to lipids, cow’s milk contains a higher percentage saturated fatty moieties and a lower percentage of mono-unsaturated and poly-unsaturated moieties [50, 76]. Essential fatty moieties such as arachidonic acid (ARA), EPA and DHA, important
for growth, immune function, vision and development of the nervous system in humans are especially low in cow’s milk versus human milk \cite{76}. It is currently not possible to (viably) add individual fatty moieties to formulae on a commercial scale and reach the desired composition. For the purpose (among others) of attaining a human-like fatty acid profile, cow’s milk is skimmed (its MFGM and fat removed), and fat from non-bovine sources (predominantly vegetable oils) is added. The choice of the fat blend is dependent on legislation (e.g. required amounts of ARA, EPA, DHA), commercial availability of fat, economics, and ethical perception (e.g. unsustainable farming practices) \cite{76}. Commonly used fats include coconut, palm, corn, soybean, sunflower, safflower, and canola oil \cite{50, 76}. Occasionally, (MFGM-free) milk fat is used \cite{50, 76}. From the current legal requirements of poly-unsaturated fatty acid (PUFA) levels, one can infer that infant formulae can contain a maximum of two-third cow’s milk fat \cite{76}. The common denominator for typical infant formulae is the removal of the MFGM (and cholesterol) during the initial milk processing \cite{59}. The steps involved in milk processing are reviewed in \cite{79}. The fats added back to infant formulae are emulsified and thereby stabilized by (for example) small amounts of soy lecithin and protein aggregates \cite{59, 80}. Typical infant formulae contain fat droplets with a mode diameter of 0.4 µm \cite{59}.

**Is there a reason for the composition of milk?**

Milk is the sole nourishment of the infant \cite{57}. Milk from different mammalian species has, however, widely different compositions, especially concerning concentrations of lactose and fat \cite{81, 82}. The (relative) weaning age is highly different between mammalian species \cite{83, 64}. Milk secretion (and thereby milk itself) likely evolved in order to optimize the later life reproductive success of the suckling (co-evolving) offspring \cite{85, 86}. Herein lies a fine balance between impacting the reproductive success of the mother and the (later life) reproductive success of the offspring \cite{85}. Milk composition is partially constrained by osmolarity, which is mainly determined by lactose content \cite{81}. Lactose content is, due to its water-attracting properties inherently negatively correlated with milk fat content and with total energy density (kcal/g milk) \cite{81}. In contrast, no clear relationship exists between the energy density of milk and protein content \cite{81}. The purposes of fats in milk are
most likely for the delivery of energy and essential fat-soluble compounds, and for antimicrobial effects \cite{86-88}. It is possible that species-specific parameters of milk composition (such as the gross content of carbohydrates, fats and proteins) have been (and are being) selected based on the life history of the species. Certain marine mammals (such as the hooded seal, *Cystophora cristata*) have highly concentrated milk and nurse for a short duration of time (<1 week). Hooded seals must nurse outside of the water, where they are vulnerable to predators, and the young must rapidly accrue several kilograms of body fat to survive the harsh arctic conditions \cite{81}. It is speculated that milk is more dilute in species that require a high amount of evaporative cooling \cite{82}. In primates, milk is dilute likely for a different reason; reflecting the slow rate of postnatal growth \cite{82, 89}. It is poorly understood how slow rates of postnatal growth are advantageous for a species. In the great apes (*Hominidae*), the taxonomical family which humans are a member of, nursing is typically 6 to 8 years in duration, resulting in an interbirth interval of approximately the same duration \cite{84}. The long *Hominidae* nursing period (and interbirth interval) may compete with the capacity at which its populations can grow \cite{83}. This is in sharp contrast to the WHO-advised 6 months of breast-feeding in humans. It is not known for how long pre-historic *Homo sapiens* nursed their young. Natural human breast-feeding duration has been estimated to be 3 years \cite{84, 90, 91} and in food-abundant environments can be shorter still. Mankind’s comparably short nursing period, and thereby short interbirth interval, may have been a (strong) contributing factor to how humans (as a species) overcame the low capacity at which typical *Hominidae* populations can grow \cite{83, 84}. Minor differences in the composition of infant formulae (such as its protein content) can greatly alter infant growth velocity \cite{92}. It appears that our early life development and later life reproductive success is, to some extent, optimized for a specific milk composition and/or early life growth velocity.

*Closer to human milk*

Infant formulae currently achieve a high level of quality and safety \cite{73}. Breast-feeding versus IMF-feeding is epidemiologically and duration-dependently associated with a lower incidence of childhood, adolescent, and adulthood obesity \cite{24, 25, 58}, and lower blood pressure and lower plasma cholesterol in adulthood \cite{26, 27,
It is suggestive that the remaining differences between human milk and formulae are responsible for these long-term effects. The mechanisms of these long-term effects are thought to be (partially) mediated via metabolic programming \[^{56}\]. For an in-depth investigation into the long-term effects of the early life diet on later life cholesterol metabolism, I refer to \[^{93, 94}\]. In this thesis, I will focus on the long-term effects on body weight and fat mass gain. The underlying programming stimulus, responsible for the long-term effects on body weight and fat mass gain, present with breast-feeding and absent with formula-feeding (or vice versa) is unknown. The stimulus has been hypothesized to be linked to the difference in emulsification and/or fat globule diameter \(i.e.\) physicochemical differences \[^{8, 9, 68, 69, 80, 95-97}\]. When the physicochemical structure of human milk is mimicked in a rodent diet mixed with an experimental infant milk formula (eIMF) and fed to mice in early life, these mice transiently gain less body weight and fat mass when challenged with a Western style diet later in life, when compared to a rodent diet mixed with control IMF (cIMF) \[^{8, 9, 80, 96}\]. The eIMF is a concept infant milk formula with large, phospholipid coated lipid droplets (mode diameter 3-5 μm); Nuturis® \[^{59}\]. A schematic representation of the structure is shown in Fig. 3 \[^{59}\]. The beneficial effects of eIMF on body weight and fat mass gain are only seen when adding MFGM as a coating to IMFs containing large lipid globules and not upon adding MFGM as a coating to IMFs containing small lipid globules \[^{9}\]. The beneficial effects on body weight and fat mass gain are also not seen upon adding phospholipids in free form \[^{98}\]. The absence or presence of cholesterol does not appear to play a role in the long-term beneficial effects on body weight and fat mass gain \[^{8, 80, 96}\]. Furthermore, the beneficial effects on body weight and fat mass gain were seen with an eIMF with a fat moiety containing 8% milk fat (remainder being vegetable fats) \[^{96}\], ~25% milk fat \[^{80}\] and even ~50% milk fat \[^{8}\]. It appears that, when adequate amounts of essential nutrients are present, lipid blend \(i.e.,\) vegetable oil with or without milk fat is not a strong determining factor for the observed long-term effects on later life body weight and fat mass gain as long as large MFGM-coated lipid globules are present \[^{9, 59}\]. In this thesis, I use a preclinical mouse model. In an attempt to answer the question ‘is the mouse a suitable model for studying human metabolic programming’, I refer to \[^{32-34, 99}\]. In brief, the early murine postnatal period may represent the late human prenatal period
Studies in mice should be carefully designed and interpreted to maximize their translatability \cite{33}. Mouse models of early life programming are considered an indispensable tool to study the mechanisms metabolic programming in humans \cite{33}. Mice fed a low-fat chow (approx. 6% energy from fat) rarely develop overweight even after extended feeding periods \cite{103}. Mice fed high-fat (such as a diet containing 45% energy from fat) or ‘Western-style’ diets develop overweight within months of feeding. For investigating the effects of an early life diet on later life body weight and fat mass gain, it is necessary to ‘challenge’ the model by feeding a high-fat or Western-type diet.

To better understand the link between the physicochemical structure of early life diet and the later life body weight and fat mass gains on a Western style diet, the work described in this thesis aims to explore the involvement of a set of relevant metabolic parameters, outlined below.

**Fig. 3. Simplified schematic representation of the lipid globule structure in human milk (a), typical infant formulae (b) and eIMF (c).** Neutral lipids (shown in yellow) do not dissolve in water and require emulsification to prevent phase separation. In human milk (a), neutral lipids are emulsified by a triple phospholipid membrane (shown as red circles each with two attached fatty acyl moieties). In typical infant formulae (b), neutral lipids are emulsified by interfacial aggregated protein (shown in green). In eIMF (c), neutral lipids are emulsified by a thin monolayer membrane (5–10 nm), including few protein aggregates. Size of globules is not to scale. Figure adapted from \cite{59}.

**Vitamin K in breast-fed and formula-fed infants**

Vitamin K (VK) is a fat-soluble vitamin required for the synthesis of blood clotting factors II (prothrombin), VII (proconvertin), IX (Christmas) and X (Stuart–Prower), and plasma Protein C, S and Z in the liver \cite{71}. VK cannot be synthesized by human (and murine) cells. VK can be obtained from the diet and from synthesis by the intestinal microbiota \cite{104,105}. The vitamin K status of women of childbearing
age is often inadequate [56]. During pregnancy, limited amounts of vitamin K are transferred via the placenta to the fetus [106, 107]. At birth, term birth infants have low plasma vitamin K levels and only limited VK stores [106]. Human milk contains approximately 5 µg/L vitamin K [51], versus ~15 µg/L in raw cow’s milk [108]. By law, infant formulae contain at least 25 µg/L VK. Breast-fed neonates are vulnerable to develop VK deficiency unless they are supplemented [53]. When the neonatal diet consists of at least 50% formulae (the remainder human milk), supplementation is no longer required [109]. Vitamin K deficiency (VKD) can cause bleedings (VKDB), formerly called "hemorrhagic disease of the newborn" [110]. VKDB in unsupplemented breast-fed infants has an estimated incidence of 0.25-1.7% [111]. Prophylactic VK regimens in the different countries vary in dosages (25 µg up to 2 mg), administration schemes (daily or weekly) and routes of administration (enteral or parenteral) [52, 53]. Absorption of VK relies heavily on its micellization by luminal bile acids. VKDB empirically occurs more often in breast-fed infants with (yet undiagnosed) impairments of bile flow, such as in biliary atresia [52, 53, 109, 112, 113].

In the Netherlands, newborns had been supplemented with 25 µg VK daily until February 2011. Based on demonstrated prophylactic failure in infants with yet unrecognized cholestasis (such as yet unrecognized biliary atresia) this daily dose was revised to 150 µg daily. Surveillance data obtained after introduction of this latter regimen, however, showed a similar failure rate as the daily 25 µg regimen [53]. Intriguingly, formula-fed infants, including patients with yet unidentified cholestasis, who receive approximately 25 to 50 µg VK per day via the formula without additional supplementation, are protected from VKDB [52]. It appears that daily VK supplementation of breast-fed infants with 3-6 times the dose present in infant formula fails to prevent VKDB in biliary atresia patients.

Experimentally, VK absorption can be disrupted by concomitant cholesterol intake [114]. Human milk contains high levels of cholesterol [93], whereas typical infant formulae (with an all plant-derived fat moiety) does not contain cholesterol. To better understand the differences between VKD incidence rates in breast-fed and formula-fed infants with yet unidentified cholestasis, I aim to test whether cholesterol interferes with vitamin K absorption.
Outline of the thesis

Human studies support the notion that breast-feeding versus formula-feeding is epidemiologically and duration-dependently associated with a lower incidence of childhood, adolescent, and adulthood obesity [24, 25]. One distinct difference between human milk and typical formulae is the physicochemical structure of milk fat globules [59, 68, 69]. Preclinical research supports the notion that the rate of later life body weight and fat mass gain can be modified by modest changes in the physicochemical structure of the early life diet [8, 9, 80, 96]. It is not known whether the physicochemical differences between human milk and infant formulae are solely responsible for the lower incidence of later life obesity. The observed (preclinical) beneficial effects on body weight and fat mass gain provide an interesting avenue to explore the potential benefit for infants that, for whatever reason, cannot be breast-fed. The mechanism of early life programming in this paradigm is currently poorly understood. Therefore, the aim of this thesis is to identify the possible mechanism(s) of metabolic programming of later life body weight and fat mass gain after feeding mice an early life diet containing large phospholipid-coated lipid globules.

In Chapter 2, I tested the robustness and limits of early life eIMF programming on later life body weight and fat mass gain using a mouse model of early life programming. Wild-type C57BL/6JOlaHsd mice were fed a rodent diet based on eIMF or cIMF from postnatal day (PN) 16 to 42, after which they were challenged by a Western style diet that was continued until PN 168. The dietary challenge extended further than previous studies and the mice were metabolically characterized by calorimetry to build on previous knowledge.

The work described in Chapter 3 explored a set of parameters that may underlie the long-term effect of eIMF on body weight and fat mass gain. Similar to the mouse model employed in Chapter 2, mice were fed a rodent diet containing eIMF or cIMF from PN 16-42. During this period, early life growth rate and body composition were assessed. At the end of this intervention period (at PN 42), plasma adipokines and
cytokines, parameters of bile acid metabolism, and markers of mitochondrial substrate utilization were quantified.

In **Chapter 4** I tested the hypothesis that early life eIMF feeding alters the absorption or the post-absorptive handling of dietary lipids in later life. Similar to the mouse model employed in Chapter 2, mice were fed a rodent diet containing eIMF or cIMF from PN 16-42. The mice were fed a low-fat or high-fat diet from PN 42-63. At the end of this period (at PN 63), lipid absorption rate and the post-absorptive handling of dietary lipids were quantified using stable isotope-based methods.

Breast-fed neonates are dependent on vitamin K supplementation to prevent vitamin K deficiency bleedings, which occur more often in breast-fed versus formula-fed infants with (yet undiagnosed) cholestasis. This observation may interfere with efforts to increase breast-feeding rates and durations. Evidence suggests that a component in human milk, cholesterol, interferes with vitamin K absorption. Within the frame of lipids in early life nutrition, the intriguing differences in apparent vitamin K absorption with human milk versus formula was investigated in **Chapter 5**.

In Chapters 2-4 a standard mouse model of early life nutrition was employed. I used an inbred mouse strain in combination with standardized (semisynthetic) diets. The rationale behind these choices was to minimize genetic and environmental heterogeneity. During the course of the experiments, occasionally a high within-group variability in liver weight was noted. Initially, this was attributed to natural variability and therefore all samples were retained. In **Chapter 6** this notion was challenged in a series of experiments designed to test whether the observed variability in liver weight was pathological and, if so, how it may interfere and perhaps could be prevented in other experiments.

The implications of these findings and our interpretation of the underlying mechanism are discussed in **Chapter 7**.