Long-term effects of dietary lipid structure in early life
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Chapter 7

General discussion and conclusion
Suboptimal early life nutrition can have a life-long effect on the risk to develop obesity and its associated detrimental health afflictions such as type-2 diabetes, atherosclerosis, and cardiovascular disease [24-31]. Mother’s own milk with vitamin K supplementation is considered the most optimal source of early life nutrition for nearly all term infants [50, 56, 57]. The alternative, namely infant milk formula, is (among other long-term health effects [28-31]) epidemiologically associated with a higher incidence of childhood, adolescent, and adulthood obesity [24, 25], and higher blood pressure and plasma cholesterol levels in adulthood [26, 27]. It appears that differences between breast-feeding and formula-feeding, whatever they may be, are associated with persisting effects on later life health [24-31]. Increasing the initiation rate and duration of breast-feeding are expected to decrease the aforementioned non-communicable disease risk factors [54]. In the Netherlands, the rate and duration of breast-feeding are mainly determined by socioeconomic and legislative factors [54-56]. Alternatively, the gap between breast-fed and formula-fed infants, with regards to the incidence of later life obesity, could be narrowed by further ‘humanization’ or otherwise improvement of infant formulae [18, 72, 92, 237]. Preclinical research indicates that mimicking the physicochemical structure of human milk lipid globules in infant formula, versus control infant formula, lowers the body weight and fat mass gain in later life upon a Western-style diet challenge [8, 9, 80, 96]. In this thesis I aimed to determine possible mechanism(s) of metabolic programming of adult life body weight and fat mass gain after feeding mice an early life diet containing large phospholipid-coated lipid globules.

The robustness and limits of early life IMF-induced programming

In a series of preclinical experiments, it was shown that mimicking the physicochemical structure of human milk fat globules in an IMF fed in early life lowered fat mass gain upon a Western-style diet challenge in later life [8, 9, 80, 96]. On a logical basis, it was not possible to conclude whether the observed lower fat mass (gain) was a consequence of a lower ‘response’ to the Western-style diet, or due to an incapability to gain fat mass per se [9, 80, 96]. Data in Chapter 2 showed that the beneficial effects of eIMF versus cIMF with regards to later life body weight and fat mass gain were transient when the exposure to a high-fat diet was continued [8]. This
observation provided evidence that the effects on body weight and fat mass gain were not due to an incapability to gain body weight and/or fat mass *per se* [238-240]. If this was the case, the effect could logically not have been transient [8]. This distinction is important as the inadequacy to respond to a high-fat diet (*i.e.* lower body weight and fat mass gain) may in fact indicate a pathological condition instead of a beneficial (programming) effect [171, 207, 210]. Data in Chapter 2 did not indicate signs of IMF-induced (long-term) pathology with regards to the assessed parameters [8]. Also peak body weight was similar between eIMF and cIMF-fed mice [8]. The observation that the programming effect (*i.e.* the ability to partially resist HFD-induced weight gain) can be overruled by a prolonged exposure to a HFD challenge is therefore considered a sign that the mice are able to exhibit their natural (body weight accruing) phenotype to their full potential given sufficient time [8].

Mice are born more altricial (immature, less developed) compared to humans, *e.g.* without developed ears and eyes [102]. In terms of brain development, the third trimester of human pregnancy approximately corresponds to murine postnatal day (PN) 0-10 [100, 101]. Artificial methods of rearing rat and mouse pups are colloquially named “pup in a cup” models [101, 241, 242]. These models are possible as early as postnatal day 1 through invasive methods [243]. Artificial rearing models *versus* standard dam rearing, however, may result in undesirable long-term consequences such as anxiety-like behavior [244] and thus by itself are a likely programming factor. I chose to avoid the inherent stress associated with artificial rearing models. A time period for the dietary intervention was chosen in Chapter 2-4 wherein pups eat food on their own. The programming diets were provided from PN16 onwards; the approximate age from which mouse pups are able to (and do) eat solid food by themselves [245]. In mice, the quantity of secreted milk reduces from PN17, and breast-feeding ceases entirely after PN22 [245]. Milk production and thereby milk feeding reduces earlier (and natural weaning then also occurs earlier) in smaller nests compared to larger nests [245]. The IMF diets were provided during the periweaning (PN16-21) and postweaning (PN21-42) period [8, 245, 246]. Plasma lipid data in Chapter 3, in particular the phospholipid levels, suggested that the weanlings had eaten from the IMF diets (shortly) prior to sampling at PN21. However, dietary
phospholipids may alter the phospholipid content of breast milk \[247\]. Thus, it cannot
be stated conclusively whether the pups (mostly) ate the IMF diets during the
periweaning period or not. It was not further tested which days within the PN16-42
period were (non-) essential for the effects on later life body weight and fat mass
gain. Others have shown that central mechanisms important in dietary preferences
can be programmed in mice during the period PN21-28 \[248\]. It is assumed that IMF
feeding does not impede or otherwise disturb breast-feeding and that eIMF’s long-
term beneficial effects on body weight and fat mass gain occurred in (fully) breast-
fed mice. If that assumption is valid, it indicates that eIMF acted independently from,
or in combination with, breast-feeding in mice \[8, 9, 80, 96\]. If eIMF exerts a similar
long-term beneficial effect on body weight and fat mass gain in humans, it may do
so either with or without concomitant breast-feeding. Proving the long-term (multi-
decade) efficacy of eIMF in humans is not practically feasible. As per European law,
it is required to show the (short-term) safety and tolerance of a new infant formula
prior to allowing it on the commercial market. The eIMF was recently evaluated for
safety and tolerance in a trial (Dutch Trial Register NTR3683) \[249\]. The eIMF was
found to be safe, and well tolerated (i.e. number, severity, or relatedness of adverse
events) \[249\]. In healthy infants, early life growth (daily weight gain) was equivalent
between eIMF and commercially available control IMF \[249\]. Follow up (5 years) of
infants fed eIMF in early life (Dutch Trial Register NTR5538) is expected to provide
insights into body mass index, and the prevalence of overweight and obesity at 3, 4,
and 5 years of age.

**Energy balance**

After establishing that the long-term effects on body weight and fat mass gain
were not a result of an incapability to store fat mass \[8, 207, 238-240\], possible underlying
mechanisms with regard to body weight and fat mass gain were explored in **Chapter
3** and **4**. Fundamentally, weight gain by fat mass gain is the result of a positive energy
balance of the organism \[250\], i.e. energy intake exceeds total energy expenditure.
Energy intake is determined by food intake and the efficiency of absorption. Total
energy expenditure is the sum of basal metabolic rate, adaptive thermogenesis and
physical activity \[250\]. The basal (resting) metabolic rate is the amount of energy
consumed at rest by the myriad of biochemical processes necessary to sustain life at thermoneutrality \[150, 250\]. When adequately corrected for tissue weights, only subtle changes in basal metabolism occur due to age \[150\]. Adipose tissue consumes very low amounts of energy per day for its basal metabolism \[150\]. Whole body basal metabolic rate (when expressed per kg) may therefore decrease upon fat mass gain. The remainder of energy expenditure depends on heat loss to the environment and bodily activity and is highly variable. The concept of ‘slow metabolism’, \textit{i.e.} a low energy expenditure per kg of body weight, is clearly erroneous \[251\]. This concept led to years of futile clinical studies on obese humans seeking to find obese individuals with a slow metabolism; none were found \[251\]. A plethora of studies indicate that total energy intake and/or energy intake per unit of body weight was lower in free-living obese \textit{versus} normal weight human subjects \[252-259\]. The premise that obesity is not linked to food intake is, however, absurd and misleading. The contribution of dietary (energy) intake to weight gain and obesity is heavily confounded by the difficulty of attaining accurate measurements of caloric intake and by human psychology \[250, 260, 261\]. Caloric intake is underreported by as much as 47\%, and physical activity is overreported by as much as 51\% by obese subjects \[260\]. The discrepancy between self-reported energy intake and actual intake is not unique to the obese, as it is also seen in athletes \[261\]. It appears that self-reported data on energy intake and energy expenditure is not sufficiently accurate to assess energy balance \[260, 261\]. Using doubly-labeled (\textit{heavy}) water, it is possible to calculate total energy expenditure in free-living humans \[262\]. Using that method, total energy expenditure in men and women increases steadily with increasing BMI \[263\]. Under controlled metabolic chamber conditions, both the basal metabolic rate, and the metabolic rate during sedentary activities is higher in obese \textit{versus} normal-weight subjects \[264\]. In men and women, a clear relationship exists between investigator-measured caloric intake and weight gain or weight loss \[165\]. Between 1980 and 2005, physical activity expenditure (measured by doubly-labeled water) has not declined in Europe and North America \[265\]. The additional costs of moving a higher body weight and the additional (lean) body mass in the obese may offset less overall movement \[265\]. Energy expenditure in Western societies is not lower compared to that of inhabitants of third world countries, and not relatively lower compared to wild terrestrial
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It may still be that total energy expenditure per person was higher prior to 1980, but no accurate data exists to (dis)prove this. Estimations of total energy fluxes from 1980 onwards indicate that substantial increases in total energy intake are more likely to have driven the increases in body weight rather than a decrease in energy expenditure [6, 265]. Preventing (too) high energy intake is key and early life programming provides a unique opportunity to set later life susceptibility to weight gain and subsequent obesity [24-27].

Energy balance by expenditure

The basal metabolic rate has been presented as a static number, highly dependent on tissue mass and (to a minor extent) age in humans [150]. The recent increase in the prevalence of overweight among Western European citizens is more likely to be caused by a higher energy intake than a lower energy expenditure [6, 265]. For reasons of completeness, I will nonetheless discuss the biological plausibility that later life energy expenditure can be affected by early life factors. The basal metabolic rate of animals is responsible for a significant part of total energy expenditure. One would expect, given that basal metabolic rate is a large contributor to total energy expenditure, that it strongly affects reproductive success and thereby has a high heritability [266]. However, in wild leaf-eared mice (Phyllotis darwini) heritability of basal metabolic rate is low, and is associated instead more strongly with maternal and environmental factors [267, 268]. In zebra finches (Taeniopygia guttata), later life basal metabolic rate can be programmed in early life by altering the growth rates via the diet [266]. It appears that later life basal metabolic rate is higher when finches are primed in early life with a poor quality diet, and fed a high-quality diet in later life, compared to when diets match [266]. In rats, protein restriction during pregnancy and/or lactation results in permanently growth-retarded offspring with permanent and selective changes in organ weights, versus ad libitum fed controls [269]. Essential organs like the brain and lungs were unaffected, whereas the liver, pancreas, skeletal muscles and the spleen were smaller and lighter upon protein restriction [269]. In growth-retarded offspring, liver metabolism appears permanently biased towards a starved-like condition in that the activities of key hepatic enzymes of glycolysis were lower and of gluconeogenesis were higher [269]. Individuals with smaller organs will
have a lower basal metabolic rate at the expense of a lower maximal metabolic rate \cite{268}. Energy spent on movement, or the amount of spontaneous movement (i.e. pacing or fidgeting), may be a modifiable trait \cite{270}. Rat pups, born from undernourished versus nourished mothers and weaned on an ad lib control diet, were less active in adolescence (PN35) and later life (PN145 and PN420) \cite{270}. This later life effect was exacerbated by high-fat diet feeding \cite{270}. A similar phenomenon was observed in mice \cite{271}. Mouse pups, born from protein-malnourished versus nourished dams, and breast-fed (cross-fostered) by protein-nourished dams, engaged in less voluntary wheel running exercise in later life \cite{271}. Mouse pups, breast-fed in litters of 4 pups versus 9 pups (a model of postnatal overnutrition; i.e. more milk per pup), had lower bodily activity and lower energy expenditure in later life (PN180) \cite{272}. Data in Chapter 2 indicated that early life eIMF feeding, under well-nourished conditions, did not significantly change later life (PN154) energy expenditure (per kg body weight) in HFD-fed sedentary males \cite{8}. The reader is advised to be critical of measurements involving (indirect) calorimetry \cite{251}. Subtle differences in energy expenditure (and intake, for that matter) can account for marked differences in accumulated expenditure over time \cite{251}. Such subtle differences in energy expenditure can fall below the limits of the discriminatory capacity of the technique used to assess it \cite{251}. Interestingly, the maximum mitochondrial oxidative capacity in eIMF versus cIMF-fed mice, fed high-fat diet in later life, was higher in skeletal (\textit{M. tibialis}) muscle and retroperitoneal white adipose tissue (at PN98) \cite{166}. These findings indicate that early life environmental factors such as maternal undernutrition \cite{270, 271}, postnatal overnutrition \cite{272}, and even the postnatal diet’s physicochemical structure \cite{166}, can change parameters of energy expenditure. It is unclear whether the observed differences in mitochondrial oxidative capacity \cite{166} underlie the long-term beneficial effects on body weight and fat mass gain. Even if this is the case, it still remains unclear how a relatively subtle change in the physicochemical structure of an early life diet changes the later life mitochondrial capacity. Chapter 3 showed, at least in liver tissue, that surrogate markers of mitochondrial capacity were already higher during eIMF-feeding. It is, however, unreasonable to assume that a higher capacity to utilize substrates always results in a higher long-term rate of substrate utilization (i.e. a higher energy expenditure). A
dramatic example of this is seen in endurance athletes, who have a much higher total energy expenditure during competitions compared to during the (pre-competition) training season \cite{261}. Endurance training in elderly subjects (aged 56-78 years) notably increases maximum oxygen consumption without a concomitant increase in total energy expenditure \cite{273}. Similarly, aerobic training in young non-obese sedentary women increases maximum oxygen consumption without increasing the total energy expenditure \cite{274}. In both studies, it is thought that the energy costs of training are compensated for by a reduction in activity outside of the training sessions \cite{273, 274}. These studies do not argue against the usefulness of endurance training for young and old subjects. Endurance exercise promotes mitochondrial biogenesis in skeletal muscle and enhances muscle oxidative capacity \cite{275}. By inference, it may thus well be that a higher mitochondrial oxidative capacity in (for instance, skeletal muscle) does not automatically result in a higher (whole body) total energy expenditure. It appears that relatively short bouts of higher substrate utilization are sufficient to increase mitochondrial oxidative capacity. It is unclear why eIMF-fed mice have higher hepatic levels of proteins involved in fatty acid oxidation (Chapter 3). It is also not clear which mechanism could underly the higher muscle and adipose tissue mitochondrial capacity in eIMF versus cIMF-primed mice in later life during WSD-feeding \cite{166}. Vors \textit{et al}, administered a breakfast comprising of (biochemically identical) emulsified \textit{versus} spread fats to normal weight and obese men \cite{97}. Stable isotopically labelled triglycerides were added to these breakfasts. They reported that emulsified \textit{versus} spread fat was more rapidly absorbed, led to larger chylomicrons, and a sharper $^{13}$CO$_2$ appearance in obese subjects \cite{97}. These observations suggested that emulsified \textit{versus} spread fats, despite being biochemically identical, are more rapidly oxidized (and converted to stable isotopically labeled CO$_2$) \cite{97}. This is in agreement with the notion that rapid fat absorption results in larger chylomicrons, from which the triglycerides are more rapidly taken up by extra-hepatic tissues \cite{167, 172, 175, 176}. Whether this would then coincide with a higher mitochondrial capacity is unclear. Vors \textit{et al} reported that total fat oxidation was similar after ingestion of emulsified or spread fat \cite{97}. It may be that the peak rate of fatty acid oxidation is different after ingesting emulsified or spread fat. I hypothesize that rapid fat absorption (in early life) results in short bouts of high peripheral lipid oxidation.
These short bouts may then (temporarily) increase mitochondrial oxidative capacity in extra-hepatic tissues. I cannot, with reasonable certainty, state whether this would then explain the differences in liver mitochondrial enzyme levels between cIMF and cIMF-fed mice in Chapter 3. I hypothesize that the higher oxidative capacity in muscle tissue [166] and the higher levels of proteins involved in fatty acid oxidation (Chapter 3) are a consequence of a difference in the handling of absorbed fats. It is unclear whether the observed differences in mitochondrial capacity are mechanistically related to the long-term effects on body weight and fat mass gain. Alternatively, it is possible that whatever factor is responsible for the effects on mitochondria, simultaneously and independently underlies the long-term effects on body weight and fat mass gain per se. The rate of fat absorption is linked to the onset of the sensation of hunger in obese but not in lean subjects [97]. This now makes it tempting to speculate that an aspect of the absorption and/or postprandial handling of fats underlies the effect on mitochondria and perhaps also on food intake.

**Energy balance by intake**

Under conditions of food availability, food intake occurs at the intersect between free will and determinism [276, 277]. Decisions on total energy intake may appear to be free to the individual, but are nonetheless unquestionably subject to (occasionally strong) biological regulation and environmental influences [276-283]. A seemingly perpetual war is waged over the most ideal human diet; low-carb, low-fat, keto, paleo, low-protein, high-protein, plant-based, vegetarian, vegan and endless other diets with disputed short-term and long-term effects on health [165]. These diets arguably cause public confusion and they may lower the (public) trust in (nutritional) science. The seemingly common denominator of these widely diverse diets is the avoidance of so-called ‘ultra-processed foods’ [165, 284]. These are described as “formulations mostly of cheap industrial sources of dietary energy and nutrients plus additives, using a series of processes”, and contain low amounts of whole foods [165]. Men and women offered (ad libitum) an ultra-processed diet versus a mostly unprocessed diet consumed more calories and gained body weight [165]. These observations suggest that attributes of a meal determine (to some extent) satiety. This notion is underlined by the observation that obese (but not lean) subjects, when
offered a breakfast containing spread fat \textit{versus} (biochemically identical, but physicochemically dissimilar) emulsified fat, felt more hungry prior to lunch \cite{97}. Clear differences exist with regards to the rate of fat absorption, when comparing spread \textit{versus} emulsified fat \cite{97}. This concept has been coined ‘slow \textit{versus} fast fat’ \cite{68}, as a homage to slow and fast carbohydrates. In \textbf{Chapter 2}, data indicated that eIMF-programmed mice ate slightly (albeit non-significantly) less of the high-fat diet \cite{8}. Food intake did correlate with long-term (PN42-147) weight gain \cite{8}. The hypothesis that eIMF’s long-term effects on body weight and fat mass gain are caused by a lower food intake is plausible. Given the lack of statistical significance, I cannot conclude with certainty that this is indeed the case. Even if this \textit{is} the case, then it remains unclear how a relatively subtle change in the physicochemical structure of an early life diet changes later life food intake.
Understanding the underlying mechanisms of eIMF-induced programming

Upon absorption, dietary fats are packaged into lipoprotein particles (chylomicrons) by the gut \textsuperscript{136}. These particles are subsequently secreted into the lymph \textsuperscript{136}. Lipoprotein particles consist of apolipoproteins and a phospholipid monolayer containing amphipathic molecules (such as cholesterol) \textsuperscript{138, 175}. The type of apolipoprotein B (ApoB) is unique to the type of particle; ApoB48 for chylomicrons and ApoB100 for VLDL particles in humans \textsuperscript{285}. Chylomicron particles typically contain one ApoB48 protein. The lipoprotein particle membrane encapsulates a core consisting of neutral lipids \textsuperscript{138}. The diameter of chylomicron particles during fasting and during active lipid absorption depends (among others) on the quantity of biliary phospholipids \textsuperscript{136}. In adulthood, active lipid absorption increases the diameter of chylomicrons \textsuperscript{95, 97, 136}. By increasing the diameter, the volume-to-surface area ratio is increased, increasing the lipid carrying capacity \textsuperscript{286}. It is not clear whether dietary phospholipids (such as those present in human milk and eIMF) similarly impact the diameter of lymphatic chylomicrons in infants. Breast-fed infants, \textit{versus} formula-fed infants, have a higher plasma TG-to-ApoB48 ratio \textsuperscript{95}. Triglycerides make up the neutral core (the volume) and ApoB48 is primarily stretched across the phospholipid layer (the surface area) \textsuperscript{285}. This suggests that chylomicrons in breast-fed \textit{versus} formula-fed infants are larger and carry a larger fat load per particle \textsuperscript{95}. Triglycerides (TG), present in the neutral core of chylomicrons, are enzymatically hydrolyzed to free fatty acids by lipoprotein lipase (LPL) \textsuperscript{172, 173}. Free fatty acids are occasionally referred to as non-esterified fatty acids (NEFA). LPL is present in the vascular bed of extrahepatic tissues including the heart, skeletal muscle and adipose tissue \textsuperscript{172, 173}. The rate of TG hydrolysis (by LPL) is higher when chylomicrons have a larger diameter and thus a higher volume-to-surface area ratio \textsuperscript{97}. This peculiar characteristic aids in the rapid clearance of plasma TGs following a fat laden meal \textsuperscript{95, 97}. In adult men, rapid absorption of fat, \textit{versus} slower absorption of fat, results in larger postabsorptive chylomicrons, which in turn lead to (transiently) higher plasma NEFA levels \textsuperscript{97}. It is not known whether differences in the rate of absorption such as those seen in breast-fed infants \textit{versus} formula-fed infants \textsuperscript{95}, have a lasting effect on the rate of
absorption in later life. Given the data collected in adult men \cite{97} and infants \cite{95}, it was originally hypothesized that eIMF would program the rate of fat absorption. Our present data, however, do not support this notion. Instead, data in Chapter 4 do demonstrate that postabsorptive lipid handling is a trait that is modifiable by early life feeding with eIMF. Similar to what was seen in adult men \cite{97}, the effects on postabsorptive lipid handling seen in Chapter 4 are unique to high-fat diet feeding. No explanation currently exists for this HFD-exclusivity. If the long-term effects of eIMF on body weight and fat mass gain rely on postabsorptive lipid handling, it may be that weight gain from fat is affected, whereas weight gain from non-fat is not or to a lesser extent. Alternatively, it may be that the regulation of postabsorptive metabolism differs between low-fat and high-fat diet fed mice (and men). In humans, adipose tissue and heparin-releasable LPL activity is much higher in obese versus lean subjects \cite{287}. Weight loss in obese subjects further increases adipose tissue LPL activity, returning to its pre-weight loss levels upon rebound weight gain \cite{177}. It has been suggested that LPL plays an important role in the ‘adipostat’, a hypothetical regulatory system controlling how much fat is stored in the body \cite{287}. To some extent, LPL protein levels are regulated via epigenetic means by DNA methylation of the LPL promotor region \cite{173}. The degree of LPL promotor methylation positively associates with abnormalities of the metabolic profile and basal and postprandial triglycerides \cite{173}. In healthy and obese men and women, major determinants of adipose tissue LPL activity are obesity itself and its associated hormones (leptin and insulin) \cite{288, 289}. In these human studies, it is not clear what is cause and what is effect \cite{173, 288, 289}. In addition to the long-term regulatory mechanisms of LPL \cite{173, 288, 289}, short-term regulatory mechanisms exist that are affected by fasting and the postabsorptive state \cite{290-293}. In obese but not in lean mice, LPL activity is increased in skeletal muscles \cite{290} and adipose tissue (only per gram of adipose tissue protein, not per cell) by leptin infusion \cite{290, 291}. In rats, adipose tissue LPL activity is down-regulated at the post-translational level by fasting within hours \cite{292}. The nutritional state is thought to modify LPL activity via ANGPTL4, GPIHBP1 and LMF1 \cite{293}. ANGPTL4, also known as Fiaf is an endogenous inhibitor of LPL that is secreted into the blood stream by the liver, intestines, white adipose tissue and brown adipose tissue \cite{294}. It is thought that ANGPTL4 inhibits LPL by promoting the conversion of
active LPL dimers into inactive monomers\textsuperscript{295}. High expression levels of ANGPTL4 (and subsequent inhibition of LPL) is suggested to be responsible for the lean phenotype of germ-free mice \textit{versus} conventional mice\textsuperscript{294}. Intestinal ANGPTL4 expression is selectively suppressed in conventional mice\textsuperscript{294}. Germ-free mice lacking ANGPTL4 have similar amounts of body fat compared to conventional mice\textsuperscript{294}. Despite much higher adipose tissue and heart LPL activity levels (in conventional \textit{versus} germ-free mice)\textsuperscript{294}, and thereby shorter chylomicron plasma residence time, plasma triglycerides are similar between these mice\textsuperscript{296}. This discrepancy is explained, at least in part, by the higher VLDL-TG secretion rates seen in conventional \textit{versus} germ-free mice\textsuperscript{296}. This possibly illustrates an elegant regulatory system maintaining plasma TG (and NEFA) levels by increasing VLDL secretion upon shorter chylomicron residence time. These data do not rule out the myriad of other mechanisms through which the microbiota (in conventional \textit{versus} germ-free mice) may or may not affect adiposity. Gross (3~6 fold) transgenic overexpression of ANGPTL4 leads to a reduction in adipose tissue weight and body weight which is at least in part explained by a higher fatty acid oxidation and uncoupling in adipose tissue\textsuperscript{297}. These data suggest that postabsorptive lipid handling, mediated via the endogenous inhibition of LPL, can impact whole-body adiposity and body weight\textsuperscript{294, 296, 297}. LPL is transported across and anchored to the capillary endothelial surface by GPIHBP1\textsuperscript{298}. The active (homodimer) form of LPL is stabilized by GPIHBP1\textsuperscript{295}. Absence of or specific mutations of GPIHBP1 lead to LPL dysfunction and severe hypertriglyceridemia in humans\textsuperscript{299}. Interestingly, the amount of GPIHBP1 protein in the visceral adipose tissue of men and women correlates negatively with plasma insulin and glucose levels\textsuperscript{298}. Fasting increases GPIHBP1 expression levels in the heart, white adipose tissue and brown adipose tissue\textsuperscript{300}. GPIHBP1 expression is specifically induced by PPAR\textsubscript{\gamma} agonists\textsuperscript{300}. It is not clear to what extent GPIHBP1 is relevant for LPL activity under physiological conditions\textsuperscript{295}. The antidiabetic thiazolidinedione BRL 49653, a high affinity PPAR\textsubscript{\gamma} agonist, induced LPL expression in rat adipose tissue\textsuperscript{301}. A sequence element (PPAR response element; PPRE) is present in the human LPL promoter. This PPRE is responsible for the functional responsiveness of the adipose tissue to fibrates and thiazolidinediones\textsuperscript{301}. In humans, LPL synthesis in adipose tissue is
relatively inefficient as approx. 25-50% of the synthesized polypeptide forms inactive, high-molecular weight aggregates destined for degradation \cite{295}. LMF1 is an endoplasmic reticulum chaperone involved in the maturation of homodimeric lipases (such as LPL) \cite{295}. In mice, Lmf1 overexpression increases LPL activity without changing the total amount of LPL protein \cite{302}. It is not clear to what extent, if at all, LMF1 is involved in the regulation of LPL activity under physiological conditions.

The data presented in Chapter 2-4 \cite{8}, and in the manuscript of Kodde et al \cite{166} make it tempting to speculate that eIMF exerts its long-term effects via the LPL regulatory system \cite{294, 296, 297}. Early life eIMF feeding results in higher gene and protein expression of mitochondrial oxidative capacity markers in skeletal muscle and adipose tissue in later life during HFD feeding \cite{166}. Concomitantly, early life eIMF feeding changes postabsorptive lipid handling in later life during HFD feeding. Early life eIMF feeding lowers later life PPARγ expression in white adipose tissue depots during HFD feeding \cite{96}, which may lower GPIHBP1 expression levels \cite{300}. Currently, there is no data indicating that the microbiota is involved in the observed long-term effects of eIMF on body weight and fat mass gain. This would have to be tested in an independent study. Given the body of data accrued, it appears more plausible that the proposed mechanism of action of eIMF lies in the LPL regulatory system. The latter may be responsible for the observed long-term effects on mitochondrial capacity, and body weight and fat mass gain. This hypothesis will need to be tested in an independent study.

Several questions remain open at this point. Is the proposed mechanism of action unique to eIMF, or does it also underlie the long-term effects of breast-feeding *per se*? Are the differences in postabsorptive metabolism caused by differences in LPL activity \cite{294, 297}? If this is the case; are these differences in LPL activity caused by DNA methylation of LPL \cite{173} and/or ANGPTL4 \cite{303}?
**Vitamin K interactions**

Vitamin K (VK) is a fat-soluble vitamin which facilitates blood coagulation by activating clotting factors II (prothrombin), VII (proconvertin), IX (Christmas) and X (Stuart–Prower), and plasma Protein C, S and Z in the liver \[71, 111\]. VK has functions beyond blood coagulation, such as in the carboxylation of osteocalcin and matrix Gla-protein \[304\]. In humans, VK can be obtained via the diet \[182\]. Colorectal (microbially synthesized) VK absorption is poor \[182\]. Vitamin K (VK) deficiency (VKD) can cause bleedings (VKDB) \[111\]. At birth, neonates have low amounts of VK stores due to limited placental transfer of VK and due to the limited capacity to store VK \[53\]. Human milk typically contains low concentrations of VK. The result of the combination of these unfortunate factors is that unsupplemented breast-fed neonates are vulnerable to develop VKDB and its consequences \[111\]. In ~50% of neonatal VKDB cases, bleedings occur intracranially and associate with high morbidity and mortality \[53\]. To prevent VKD, breast-fed newborns are prophylactically supplemented with VK \[52\]. Formula-fed infants are typically not supplemented with VK, as the formula provides sufficient VK. In spite of supplementation, VKDB empirically occurs more often in breast-fed infants with (yet undiagnosed) impairments of bile flow, such as biliary atresia \[52, 53, 109, 112, 113\]. It appears that formula-feeding protects infants, including those with yet unidentified cholestasis, from VKDB \[52\]. This observation is puzzling, as the VK dosages are similar or even higher for supplemented breast-fed infants versus unsupplemented formula-fed infants. In Chapter 5, the hypothesis that (human milk) cholesterol disrupts vitamin K absorption was tested. Data in Chapter 5 suggest that dietary cholesterol may be responsible for the aforementioned observation. These data were obtained by feeding rats for 4 days a chow diet with or without cholesterol (chronic study). This is in agreement with experimental data obtained by others \[114, 179, 187\]. Upon testing the VK absorption using stable isotopically-labeled VK (acute study), plasma VK levels following human milk ingestion were paradoxically higher compared with plasma VK levels following infant formula ingestion. This is seemingly not compatible with the notion that formula feeding is the most effective prophylaxis to prevent VKDB \[52, 111\]. Ezetimibe, the NPC1L1 inhibitor known to disrupt cholesterol and VK absorption \[71, 179, 186, 191\], was used as a control. In the
‘chronic study’, the addition of ezetimibe to the high-cholesterol diet did not further lower plasma VK levels. It is undisputed that ezetimibe is able to disrupt the absorption of cholesterol and VK \cite{179, 191}. However, it has been noted that under certain conditions, ezetimibe cannot further inhibit absorption if the absorption is already inhibited via other means \cite{192}. It may be that high-cholesterol feeding inhibits VK absorption, and that ezetimibe cannot further inhibit this process. In the ‘acute study’, the group fed a diet containing cholesterol and ezetimibe had a similar plasma VK response as the group fed the same diet without ezetimibe. This study lacked a group fed a low-cholesterol diet, thus it is not possible to speculate whether the absence of ezetimibe’s effect is due to saturation of the inhibition \cite{192}. Given the observations in Chapter 5, it appears likely that cholesterol can disrupt the absorption of VK in rats. Whether this is the reason why VKDB occurs more often in breast-fed infants with (yet undiagnosed) impairments of bile flow remains up for discussion and requires a new study.
**Spontaneous murine liver disease**

Throughout **Chapter 2-4**, a standard inbred mouse strain (C57BL/6JOlaHsd; a substrain of C57BL/6J [197]) was employed [8, 198]. These mice were fed so-called ‘semisynthetic’ diets made from purified ingredients [118]. The composition of these diets was based on guidelines for rodent diets from the American Institute of Nutrition (AIN) [118]. Occasionally a high within-group variability in liver weight was noted. This was attributed to natural variability, though it was speculated that it may be caused by social hierarchy ranks [198]. 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 could be detected in other experiments.

Semisynthetic diets are manufactured from purified ingredients such as soybean oil, sucrose, casein and maltodextrin [118]. Semisynthetic control diets fulfil all known nutritional requirements of a mouse [118]. This manufacturing method allows researchers to easily manipulate the macro- and micronutrient composition of the diet, such as in a high-fat diet or a methionine- and choline-deficient diet. The usage of purified ingredients minimizes variability between food batches [118] and allows for fair comparisons between studies. Non-purified rodent diets (chow) are typically made from natural products such as wheatfeed, dehulled soybean meal, barley, and fish meal. These natural products inherently show natural and seasonal variability in their exact composition. Chow diets have (subtle) differences between batches [118], making comparisons between studies more complicated. Chow diets are primarily used due to their low costs (compared to semisynthetic diets) while still fulfilling all known nutritional requirements of the mouse. It is likely that chow diets contain a more diverse array of (nutritional) components. It has been noted that mice fed a semisynthetic diet (AIN-93G) compared to a non-purified (chow) control diet have a lower liver mass [235]. The rationale behind using inbred mouse strains and semisynthetic diets is to minimize genetic and environmental heterogeneity in experimental models of human diseases. In spite of these efforts, (occasionally high) phenotypic heterogeneity has been observed in inbred C57BL/6J mice in response to (semisynthetic) high-fat diets [171, 210, 305].
ApoE*3Leiden.CETP mice (C57BL/6J background strain) show an extremely heterogenic response to a (semisynthetic) high-fat high-cholesterol diet with regards to body weight, and lipid and glucose metabolism \cite{213}. It is now recognized that this phenotypic heterogeneity is, at least in part, caused by the presence of distinct subpopulations within cohorts of mice \cite{207, 213}. These sub-cohorts are named ‘responders’ (R) and ‘non-responders’ (NR) (occasionally ‘low-responder’) \cite{207}. ApoE*3Leiden.CETP mice are typically categorized into the aforementioned groups at PN42, when they are still fed chow, based on an arbitrary plasma TG or TC cut-off value \cite{207}. In later life, NR versus R mice under identical experimental conditions develop a more severe inflammatory liver condition, lower liver weight, higher liver TG, higher plasma liver enzymes and higher plasma bile acids \cite{207}. The NR phenotype occurs in approx. 25% of apoE*3Leiden.CETP mice \cite{207} (TNO DEC 3068, 3095, 3112, 3126). Studies using this mouse strain do not always disclose whether the ‘non-responders’ have been excluded from analyses. It was hypothesized that the NR phenotype was due to a low ApoE*3Leiden \cite{TNO DEC 3068} or a low CETP expression \cite{207}. Recently, it was demonstrated that CETP activity was similar in NR and R mice \cite{207}. Up till now, it has remained unclear what causes the NR phenotype in ApoE*3Leiden.CETP mice \cite{207, 213}. Without a clear understanding of why the ‘NR’ phenotype occurs, it seems ethically challenging and scientifically questionable to exclude 25% of a cohort. From the point of animal welfare (replacement, reduction, refinement; the 3Rs), the exclusion of 25% of mice is undesirable \cite{306}. Nonetheless, retaining ‘NR’ in cohorts may lower discriminatory capacity of studies (i.e. statistical power) \cite{129}, which also goes against animal welfare \cite{306}.

Substantial neurochemical variability has been noted in C57BL/6J, but not in mouse strains NMRI, BALB/c, ICR CD1, NUDE, NOD-SCID, and SV 129 \cite{211, 212}. Approximately 25% of C57BL/6J mice have threefold elevated cerebral glutamine levels, with concomitantly lower myo inositol, taurine and total choline independent of diet or superimposed genetic manipulation to model human disease states \cite{211, 212}. In the Q140 knock-in mouse model of Huntington’s disease (C57BL/6J background strain) and its WT control group, (unspecified) high brain glutamine levels were also seen in some mice but not in others \cite{307}. These mice were
excluded from subsequent analyses [307-309]. Such large differences in glutamine were well in excess of any treatment effect observed in studies performed at the research center in question [212]. Glutamine can be synthesized in the central nervous system from ammonia via the astrocyte-specific enzyme glutamine synthethase [212]. Elevated glutamine/glutamate peaks, coupled with lower myo-inositol and choline peaks (measured noninvasively using 1H-MRS) may represent disturbances in cell-volume homeostasis secondary to brain hyperammonemia [310]. Clinically, this is referred to as ‘hepatic encephalopathy’ (HE), typically associated with cirrhosis and portal hypertension or portal-systemic shunts (PSS) [310]. C57BL/6J mice with high cerebral glutamine levels appeared to concomitantly have a PSS that resembled incomplete ductus venosus closure [212]. Cerebral glutamine levels were similar between mice that would later be identified as having a PSS or not at PN10, only mildly elevated at PN20, and grossly elevated from PN30 onwards [212]. PSS mice can be distinguished from non-PSS mice by performing portal angiography (a terminal procedure) or by measuring glutamine and myo-inositol levels in the brain [211,212,308]. Plasma ammonia, total bilirubin, and aspartate/alanine amino transferase were within normal limits in these mice [211,308]. Brain glutamine concentration can be measured biochemically (post-mortem) or non-invasively via magnetic resonance spectroscopy (1H MRS) [211]. The spontaneous PSS could not be attributed to any environmental cause [211,212]. Offspring of PSS mice did not show neurochemical anomalies, suggesting a non-Mendelian inheritance pattern [212]. It has been noted that two third of C57BL/6J Nrf2-/- mice have a congenital portacaval shunt. [311]. This is in contrast to WT C57BL/6J mice, where PSS is observed in “only” 25% of mice [211,212]. In outbred ICR Nrf2-/- mice, PSS is not found, suggesting a complex multiple gene inheritance pattern of congenital PSS [311]. The metabolic consequences of portal blood directly entering the systemic circulation (i.e. PSS) are profound [211]. By inference, one would expect that the metabolic consequences of PSS are more severe in mice fed a high-carbohydrate diet versus a low-carbohydrate diet (i.e. a high-fat diet). Absorbed (long-chain) fats are carried via the lymphatic system into the vena cava. To an extent, the glycemic index of the diet determines the carbohydrate uptake rate. Semisynthetic diets typically contain carbohydrates with a high glycemic index (sucrose and maltodextrin) [118], whereas chow diets typically
only contain carbohydrates with a low glycemic index (starch). In rats with versus without PSS, the liver to body weight ratio is lower \[^{312}\]. In dogs with a congenital PSS, liver volume rapidly normalizes after surgical correction (narrowing or full ligation of the shunt) \[^{313}\]. Congenital PSS in WT C57BL/6J versus non-PSS is associated with delayed and less efficient postprandial glucose clearance and mild steatosis \[^{211, 212}\]. Beyond these observations, congenital PSS of WT C57BL/6J has not been thoroughly characterized \[^{211, 212}\]. PSS in C57BL/6J may be related to the aryl hydrocarbon receptor \((Ahr)\) \[^{212}\]. C57BL/6J mice express an allelic variant \((Ahr^b)\) which translates into a high affinity ligand binding form of Ahr \[^{314}\]. Other mouse strains express other allelic variants of \(Ahr\), with a lower affinity \[^{314}\]. \(Ahr\) knockout mice all have PSS \((via\ patent\ ductus\ venosus)\), suggesting that Ahr plays a role in the resolution of fetal vascular structures during development \[^{315}\]. In these mice, PSS correlates with a small liver size \[^{315}\]. However, the inheritance pattern of \(Ahr\) and of WT PSS is different, and \(Ahr\)-KO mice have additional vascular anomalies not present in WT PSS mice \[^{212, 315}\]. At this time it is not known what causes congenital PSS in WT C57BL/6J mice \[^{211, 212}\]. The exclusion of mice with high cerebral glutamine, which can represent 25% of a given cohort, seems ethically challenging and scientifically questionable \[^{306}\]. Cudalbu et al have raised questions regarding the suitability of the C57BL/6J mouse strain for neurobiology in absence of tools to screen the animals for high cerebral glutamine levels \[^{212}\]. Soares et al indicated that, given the affected hepatic lipid content and glucose homeostasis, that the incidence of PSS in C57BL/6J affects should be taken into account \[^{211}\].

Data in Chapter 6 show that heterogeneity in C57BL/6JOlaHsd can, to an extent, be explained by the presence of distinct subpopulations within cohorts \[^{200}\]. These sub-cohorts are named ‘small liver’ (SL) and ‘normal liver’ (NL) for their most prominent feature upon initial identification \[^{200}\]. Data in Chapter 6 suggest that the observed heterogeneity may be an inherent attribute of the C57BL/6JOlaHsd strain and possibly also the C57BL/6J mouse strain \[^{200}\]. J and JOlaHsd are recognized as separate substrains which differ genetically and phenotypically \[^{197}\]. Attributing the presence of the ‘SL’ phenotype solely to OlaHsd may be short-sighted as there are no clear data on its (non-) occurrence in C57BL/6J. As noted in studies using
apoE*3Leiden.CETP mice, it is possible that ‘SL’-like C57BL/6J mice are excluded from studies on suspicion of being statistical outliers. An outlier is an observation that appears to be inconsistent with other observations in the data set [316]. It would certainly be reasonable to assume that grossly inconsistent measurement values are more likely to originate from analytical errors than from seemingly random model-specific idiosyncrasies. This is especially reasonable when group sizes are small, and the ‘SL’ phenomenon only occurs in 1 or 2 mice per group. The ‘SL’ phenotype is not discussed in Chapters 2-4. It is, nonetheless, present in all assessed cohorts, occurring approximately equally in eIMF and cIMF-fed mice. Curiously, the ‘SL’ phenotype appears to occur more often in LFD-fed versus HFD-fed cohorts. These mice had not been omitted from our analyses, and their presence did not alter the direction or the validity of any conclusion. The very nature of metabolic programming studies, i.e. (relatively) large group sizes, consistent usage of standardized (semisynthetic) diets, and long follow-up, likely created the perfect milieu to recognize the SL phenotype. Similarly, the ‘NR’ phenomenon was likely noticed in apoE*3Leiden.CETP mice as their phenotype (high plasma TG and TC) is grossly affected by differences in liver function [207].

The pathological liver condition described in Chapter 6 occurred in one third of C57BL/6J mice upon semisynthetic diet feeding. The condition correlates with low liver weight, low VLDL secretion (and low fasting plasma TG and TC), high plasma bile acids, liver steatosis, fibrosis and inflammation, and mitochondrial dysfunction [200]. The SL phenotype appears partially similar to what is observed when mice are infected with Helicobacter pylori [317], Helicobacter hepaticus or Helicobacter bilis [318]. Hepatic manifestations of Helicobacter infections typically show ballooning of hepatocytes, which is not seen in mice with the SL phenotype. Helicobacter agents are highly contagious. Mice with the SL condition occasionally share a cage with mice that do not have this liver condition. This does not fully rule out that the SL phenotype is not caused by an infectious etiologic agent. It is, nonetheless, unlikely that an infected mouse can share a cage with an uninfected mouse without transmitting its infectious agent. In the apoE*3Leiden.CETP casus, it was confirmed that mice with the ‘NR’ phenotype were not infected with Helicobacter [207]. The
importance of the gut microbiota for the phenotype of an individual is nowadays recognized by the scientific community \cite{207,319}. It may be that the microbiota play a role in the SL phenotype (or vice versa). Contrary to what one would expect, housing mice from different suppliers (with different microbiota compositions) in the same cage does not fully normalize their microbiota \cite{319}. It is suggested that some aspects of the microbiota are established in early life and are difficult to displace post-weaning due to limited transfer between animals \cite{319}. Arguing for a microbiological origin of the ‘SL’ phenotype may, however, merely shift the issue of why ‘SL’ occurs towards why only certain mice exhibit phenotypes whereas others do not.

At weaning (PN21), we have not been able to identify characteristic aspects of the SL phenotype, in contrast to the situation at PN42, when several parameters were discriminatory in one third of mice when fed a low-fat semisynthetic diet. It is not clear whether a similar phenomenon would occur upon standard chow feeding. Until such data is available, it is too speculative to conclude that the pathological condition occurs (in some mice) as a consequence of semisynthetic diet feeding. Regardless if this is the case, it would remain unclear why only certain mice develop this liver condition whereas others do not. It may be that specific experimental conditions amplify an otherwise unremarkable (epi)genetic heterogeneity. For maintaining a high standard of preclinical experimentation with the C57BL/6J mouse strain, identification of the cause of the SL phenotype would be very helpful. If the SL phenotype would only be manifested in (some) mice fed semisynthetic diet but not when fed chow, it may be possible to prevent the onset of the SL phenotype via the diet. If the SL phenotype is (epi)genetic, it may be possible to selectively breed the cause out of the C57BL/6J (epi)genome. It is, however, a possibility that congenital WT PSS, the ApoE*3Leiden.CETP ‘NR’ phenotype, and the WT C57BL/6J\textit{OlaHsd} ‘SL’ phenotype are all one and the same thing. If this is the case, it may explain that ‘NR’ and ‘SL’ occur as a consequence of PSS, but not why (seemingly spontaneous) congenital PSS occurs. As the inheritance pattern of C57BL/6J congenital PSS is currently not understood, this may be the most significant problem associated with C57BL/6J inbreeding \cite{212}. The acceptance of the existence of sub-cohorts would be a big step forward to understanding and preventing this source of significant heterogeneity and potential bias in preclinical research using C57BL/6J.
Conclusions

An early life diet containing large phospholipid-coated lipid globules programs mice for a transiently lower body weight and fat mass gain later in life on high-fat diet [8]. The possible underlying mechanism is now hypothesized to be due to a long-term modulation of postabsorptive lipid metabolism. The concept that postabsorptive lipid metabolism can affect adiposity and body weight is at this moment poorly understood, yet recognized as an important factor for energy expenditure and energy intake [97, 294, 296, 297]. These data indicate that infant milk formulae, despite currently achieving a high level of quality and safety [73], can be improved further (by modifying the physicochemical structure) for the benefit of infants who, for whatever reason, are not breast-fed [54].

Taken together, this thesis highlighted the prominent role of early life nutrition in determining adult life handling of fats. The way that dietary fat is ‘packaged’ in infant milk formula could be a powerful tool that can be used to improve long term (adult) health and potentially contribute to the prevention of various diseases of civilization.