Offspring body size and metabolic profile – Effects of lifestyle intervention in obese pregnant women

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Abdominal circumference and weight at birth are both associated with metabolic risk factors in early childhood – results from the Lifestyle in Pregnancy and Offspring (LiPO) study
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INTRODUCTION

Worldwide, the prevalence of obesity has reached epidemic proportions. In Denmark one third of all pregnant women are overweight and 12 % are obese [1]. Even more concerning, a dramatic rise in the number of overweight and obese children has also been evident in recent decades. The World Health Organization (WHO) estimates that 42 million children under the age of five were overweight in 2010, and the number is believed to increase to 60 million in 2020 [2]. In USA more than 35% of school children are overweight or obese [3] and in Denmark, approximately 14% of school children are overweight and 2.5% obese [4]. Already in preschool years are children affected, with 9% being overweight and 2% being obese in Denmark [5].

Obesity and overweight in children is associated with a wide spectrum of adverse outcomes and can negatively affect virtually every organ in the body. Consequences can be hypertension, dyslipidemia, insulin resistance and fatty liver disease [6]. Recent evidence has even linked childhood obesity with liver cancer in adulthood [7]. In addition, overweight and obese children are often stigmatized and might experience social problems with their peers [8]. Obesity in childhood tracks into adulthood [9, 10], and it is estimated that up to two thirds of affected children become obese adults [11, 12], thus potentially creating a life-long condition.

The obesity epidemic is not simply a consequence of poor diet or sedentary lifestyles [13]. Obesity is a multifactorial condition in which environmental, biological and genetic factors all play essential roles. Furthermore, even though a number of genes have been linked with obesity and the metabolic syndrome [14], genes alone cannot explain the dramatic rise in the prevalences of the conditions. In this context, the Developmental Origins of Health and Disease (DOHaD) hypothesis has highlighted the link between prenatal, perinatal and early postnatal exposure to certain environmental factors and subsequent development of obesity and non-communicable diseases. Maternal obesity and gestational weight gain, resulting in over-nutrition of the fetus, are major contributors to obesity and metabolic disturbances in the offspring [15, 16]. Once present, obesity is difficult to treat and early intervention strategies are urgently needed [17]. Pregnancy offers the opportunity to modify the intrauterine environment, and maternal lifestyle changes during gestation may confer health benefits to the child. In this thesis, focus is on the effects of maternal obesity on offspring body size and metabolic profile, with special emphasis on the effects of lifestyle intervention during pregnancy in obese women.

BACKGROUND

ASSESSMENT OF BODY SIZE AND METABOLIC OUTCOMES

Standard Deviation Scores

Standard Deviation Scores (Z-scores) are often used for analyses of anthropometric data, especially in children. A Z-score expresses how far a value is from the population mean, expressed in number of standard deviations of which it differs. It is used to compare a particular value with the mean and standard deviation for the corresponding reference data, stratified by age and sex, by using the following formula:

$$\text{Z-score} = \frac{x - \mu}{\sigma}$$

Where x is the observed value, μ is the mean of the reference value and σ the standard deviation of the corresponding reference data [18]. The advantages of Z-scores are that they are independent of age and sex of the individual, and that they can be studied as a continuous variable. In the present thesis, Z-scores are used for describing a number of outcomes.

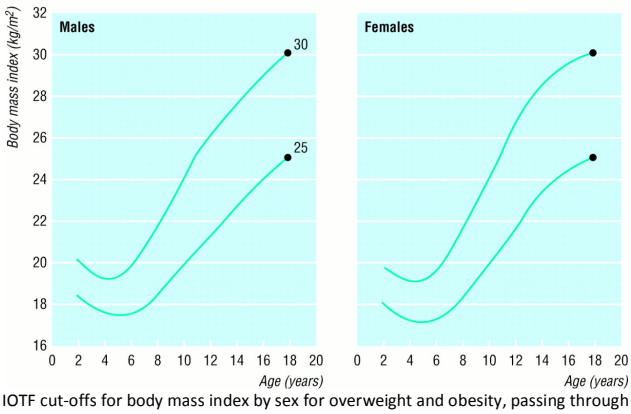
Defining overweight and obesity

Overweight and obesity can be defined as abnormal or excessive fat accumulation that presents a risk to health [17]. Despite being a crude measure, not distinguishing between fat mass and lean mass, the Body Mass Index (BMI) is used as a tool to classify individuals as being overweight or obese on a population basis. Adults are classified as obese if their BMI exceeds 30 kg/m2, or overweight if their BMI exceeds 25 kg/m2, whereas underweight is classified as BMI < 18.5 kg/m2. In children, the use of BMI as a classification tool is challenged by large variation induced by the age and sex of the child, and fixed thresholds such as those used for adults are not applicable. Instead, children´s BMI is classified using thresholds that vary according to the child´s age and sex. These thresholds are usually derived from a reference population, and this means that individual children can be compared to the reference population and the degree of variation from the expected value can be calculated. BMI thresholds are frequently defined in terms of a specific Z-score or centile, and once a child´s BMI centile or Z-score has been calculated, this figure can then be checked to see whether it is above or below the defined threshold. A number of child growth references have been published in recent years [19-21]. Each growth reference tends to have a set of recommended thresholds. These thresholds are usually defined by statistical conventions, for example, a whole number of standard deviations from the mean, or a whole number of centiles (such as the 85th and 95th centiles). One exception is the International Obesity Task Force (IOTF), where the cut-offs correspond to a BMI of 25 kg/m2 or 30 kg/m2 at the age of 18 years, if the child remains at the same centile line during growth [20], Figure 1. There is great debate regarding which references and cut-offs to use. There may be some advantage in using references based on national data, as they give the best description of the background population [19], whereas for international comparisons, using the same cut-offs are essential.

Skinfold thickness, abdominal circumference and the metabolic syndrome in children

As BMI does not distinguish between fat mass and lean mass, other anthropometric measures are needed for the assessment of fat mass. The most commonly used are measures of skinfold thicknesses and abdominal circumference (AC, often referred to as waist circumference). Skinfolds are double, compressed thicknesses of subcutaneous fat and skin and are measured with standardized calipers at selected sites (e.g. triceps, subscapular, and suprailiac sites) [22]. They are considered attractive research tools because measurements are non-invasive and specific to subcutaneous fat, and some have argued that they are the best anthropometric measure of overall adiposity [23]. Furthermore, skinfolds are associated with cardiovascular risk factors such as blood lipid levels, blood pressure, plasma glucose levels and plasma insulin levels [24, 25]. Abdominal circumference is another good indicator of fat mass. It reflects visceral fat better than BMI [26] and increased AC is an essential part of the metabolic syndrome (MS) in adults [27], being an independent predictor of cardiovascular disease, dyslipidemia and insulin resistance [28-30]. Also in children, mounting evidence suggests that central obesity is associated with key components of the metabolic syndrome such as insulin resistance, lipid levels and blood pressure [31-35]. This is reflected in the International Diabetes Federation (IDF) definition for the MS in children, where for children aged 10 years or older, MS is diagnosed by abdominal obesity and the presence of two or more other clinical features (elevated triglycerides, low High Density Lipoprotein (HDL), high blood pressure or increased plasma glucose). For children aged 6 to 10 years, special attention should be brought to those with waist circumference above 90th percentile of a reference population, but MS cannot be diagnosed [36]. Unfortunately, for children younger than 6 years, no recommendations exist due to lack of data [36].

Figure 1



IOTF cut-offs for body mass index by sex for overweight and obesity, passing through body mass index 25 and 30 kg/m2 at age 18.
Adapted from Cole et al. 2000 [20]

BODY COMPOSITION – DUAL ENERGY X-RAY

The body composition describes the percentages of bone mass, fat mass and muscle mass. It is a more sophisticated measure than BMI and the anthropometric measures described above. Numerous assessment methods for body composition exist. The most commonly used methods are bioelectrical impedance, air displacement, Dual Energy X-Ray (DEXA) scans and Magnetic Resonance (MR) [37]. The DEXA scans provide estimates of fat mass, lean mass and bone mass and have several strengths. The scan duration is short, the procedure is non-invasive and the accuracy and reproducibility is high in normal weight individuals [38]. However, the accuracy is reduced in obese individuals [39], which is one of the limitations. Another limitation is radiation, but the effective dose of a total body scan is low (<1.0μSv) and corresponds to less than 5% of a chest X-ray or 5-15% of naturally occurring daily background radiation [40]. Thus DEXA scans are considered safe and reliable for assessing body composition in children.

DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE (DOHAD)

In the 1970s Forsdahl reported that poverty during adolescence, followed by prosperity, was associated with death from cardiovascular disease in adulthood [41]. Also in the 1970s, Ravelli found that maternal exposure to famine in early pregnancy during the Dutch hunger winter in 1944 resulted in increased obesity rates in the adult offspring [42]. A few years later, Barker and colleagues began publishing reports on the associations between an adverse intrauterine environment, using low birth weight as a proxy, and increased risk of type 2 diabetes and cardiovascular disease later in life [43-45]. This led the authors to put forth the "thrifty phenotype" hypothesis, which originally proposed that poor fetal and early post-natal nutrition imposes metabolic adaptations to secure the fetus´ immediate survival, resulting in reduced fetal growth [46]. These adaptations might be detrimental and lead to glucose intolerance, type 2 diabetes, cardiovascular disease (CVD) and hypertension in adulthood if food supply is abundant; a concept termed the mismatch hypothesis [47]. The thrifty phenotype hypothesis has since been supported by further studies of the Dutch hunger winter [48] and has been confirmed by many epidemiological studies in populations worldwide, showing that low birth weight increases the risk of later adverse health (reviewed in [49]). Although the early epidemiological studies focused on the effects of low birth weight, it is now widely recognized that higher incidences of disease occur at both ends of the birth weight spectrum, reflecting a U-shaped curve [50-52].

Inspired by the pivotal work by Barker and colleagues, focus is now also on effects of over-nutrition in uteri. Initially, studies of over-nutrition were investigating the effects of diabetes during pregnancy. Freinkel put forth the term "fuel mediated teratogenesis" and proposed that maternal diabetes could cause obesity and diabetes in the offspring [53]. The fuel-mediated teratogenesis hypothesis has especially been investigated in a population with very high prevalences of obesity and type 2 diabetes (the Pima Indians) [54], but has also been confirmed in many other populations. It is now generally accepted that maternal diabetes has long lasting effects on offspring metabolic health (see also the section on maternal diabetes and offspring outcomes). On a population basis, however, due to the obesity epidemic, the effects of over-nutrition caused by obesity on long-term offspring metabolic health are perhaps of even bigger concern than those caused by diabetes. It is now well documented, that hyperglycemia in pregnancy, excess gestational weight gain and maternal obesity all are sources of intrauterine over-nutrition with programming effects in the offspring ([55], and reviewed in the sections below).

Today, the concept of programming effects of certain events or environmental factors during prenatal, perinatal or early postnatal life is termed the Developmental Origins of Health and Disease (DOHaD). The mechanisms behind these programming effects are poorly understood, but are thought to involve permanent changes in appetite control, metabolism and neuroendocrine function, possibly via epigenetic processes leading to heritable changes in gene expression and function [56]. The epigenetic regulation mechanisms consist of DNA methylation, histone modification and non-cording RNAs [57-59], and these regulatory functions can switch genes on or off, resulting in altered phenotype, whereas the genotype is preserved. Even though the science of epigenetics is in its infancy, emerging data link nutritional environment during embryogenesis, fetal development and early post-natal life with epigenetic alterations [60].

ASSOCIATIONS BETWEEN SIZE AT BIRTH AND LATER OBESITY

A number of epidemiological studies have shown a link between birth weight and BMI in childhood and adulthood [61-63]. In the US growing up today study, a cohort with over 14.000 adolescents, a 1-kg increment in birth weight in full-term infants was associated with an approximately 50% increase in the risk of overweight at 9-14 years [63]. Similarly, a study of Danish military conscripts showed that BMI at ages 18-26 strongly correlated with birth weight [61]. Two recent meta-analyses estimated that high birth weight (>4000g) is associated with an odds ratio (OR) of approximately 2 of becoming overweight or obese later in life [64, 65]. Furthermore, neonatal fat percentage, estimated by total-body electrical conductivity (TOBEC), is a good predictor of increased fat mass in children at the age of 9 years [66]. In fetal life, accelerated growth of the abdominal circumference is a predictor of asymmetric growth and neonatal morbidity [67], and fetal abdominal diameter is associated with BMI at 5 years [68]. In childhood, current abdominal circumference is an independent predictor of insulin resistance [69]. Whether excessive abdominal fat deposition at birth is a better early predictor of later obesity and/or metabolic diseases than high birth weight is, however, not known.

In conclusion, increased size at birth tracks into childhood and adulthood. In this light, knowledge of intrauterine factors influencing fetal growth is imperative.

MATERNAL DIABETES AND IMPLICATIONS FOR THE OFFSPRING

Although the focus of this thesis is on maternal obesity and associated offspring outcomes, a key mechanism proposed for these associations is via maternal hyperglycemia and/or frank diabetes and fetal over-nutrition, and must be recognized. Diabetes mellitus is not a single entity, but covers three main types in pregnancy; pregestational type 1 diabetes, pregestational type 2 diabetes and gestational diabetes (GDM). Mutual for all three is the risk of exposing the fetus to intrauterine hyperglycemia and associated adverse outcomes. Indeed, exposure to a diabetic intrauterine environment has long been recognized as a risk to the fetus and seems to programme long-term effects. Studies have consistently shown that offspring of diabetic mothers have an increased risk of being born with a high birth weight [70], having increased adiposity at birth [71] and during childhood [63, 72], as well as increased BMI and risk of MS in adulthood [73]. These consequences occur independently of genetic dispositions as exemplified in the Pima Indians, with studies of siblings born before and after the mother developed diabetes showing that offspring exposed to maternal diabetes have an increased risk of increased BMI and type 2 diabetes [54]. These results have recently been impressively confirmed in a Swedish study with more than 80,000 sibling pairs [74]. In conclusion, the programming effect of fetal intrauterine over-nutrition caused by maternal diabetes is well described and is perhaps more potent than over-nutrition caused by maternal obesity. However, as maternal obesity is a major problem worldwide, it is essential also to focus on the programming effect of obesity.

MATERNAL OBESITY AND SHORT TERM IMPLICATIONS FOR THE OFFSPRING

The impact of maternal obesity on the fetus has been investigated in several populations, and a range of adverse outcomes such as: Large for Gestational Age (LGA, birth weight > 90th percentile of gestational age- and sex-specific references) and macrosomia (birth weight ≥ 4500g) [55, 75-78], shoulder dystocia [77,

79], birth defects [77, 80], preterm delivery [76, 81, 82], stillbirth [83] and early neonatal death [79, 82] have consistently been reported.
Most relevant to this thesis, maternal overweight or obesity affects the growth of the fetus resulting in increased birth weight [84]. A recent meta-analysis estimated that maternal obesity increases the risk of LGA, high birth weight (> 4000g) and macrosomia, with odds ratios of 2.08, 2.00 and 3.06, respectively [78]. Also results from the large Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study has provided strong evidence of an association between maternal obesity and birth weight [85].
Birth weight is of course a rather crude measure of overweight in the neonate and in recent years, studies have additionally been focusing on neonatal body composition and especially fat mass. Indeed, many studies have shown that maternal obesity is associated with neonatal fat mass, whether it is estimated by measures of skinfold thickness [85-87], TOBEC [88], DEXA [89], magnetic resonance (MR) [90] or by air displacement plethysmography [91]. Recently, Modi et al. have shown that increasing maternal BMI is associated also with increasing abdominal and intrahepatocellular lipid content in the neonatal offspring [90]. Additionally, offspring of obese mothers seem to have increased insulin resistance already at birth [87], indicating very early life effects on offspring metabolic profile.
Interestingly, the increased birth weight in offspring of obese mothers seems to be the result of increased fat mass, rather than lean mass [88, 92], suggesting that the in utero metabolic environment affects primarily growth of fat mass, not lean mass.

MATERNAL OBESITY AND LONG TERM IMPLICATIONS FOR THE OFFSPRING

A large number of studies have consistently linked increased maternal BMI with offspring overweight and obesity, whether it is assessed during childhood or adulthood [93-105]. A recent meta-analysis including four studies with sufficient dichotomous data for prepregnancy BMI and offspring overweight/obesity during childhood estimated that maternal obesity was associated with a three-fold increased risk (OR 3.06) of becoming overweight or obese during childhood [78]. As for studies of implications of maternal obesity on neonatal outcomes, a great interest in the body composition of affected offspring during later life is also present. Again, many studies have consistently shown that increased maternal prepregnancy BMI is associated with increased fat mass in the offspring, whether it is assessed by skinfold thickness [103, 106], DEXA [66, 107-109] or bio-electrical impedance [110]. These associations have been reported from early childhood to adulthood.
Additionally, effects of maternal obesity on offspring metabolic profile have been reported, with studies showing associations between maternal BMI and increased blood pressure [111-113], insulin resistance [112] and dyslipidemia in childhood [112-114], as well as indices of the metabolic syndrome [115] or type 2DM [116] in young adulthood. A recent study of over 37,000 adults with a total of 1.323.275 person years has even suggested associations between maternal obesity and long term increased risk of cardiovascular disease and all course death for the offspring [117].
Interestingly, increased risks of childhood disorders seemingly unrelated to childhood BMI, such as asthma [118-120] and neurodevelopmental cognitive problems and attention-deficit disorders [121-124] have also been linked with maternal obesity.

ANIMAL MODELS OF MATERNAL OBESITY

The epidemiological and clinical studies listed above strongly suggest that maternal obesity affects short and long term outcomes in the offspring. They cannot, however, provide proof of causality. In this regard, animal models are useful and have been used extensively to study the effect of maternal obesity on offspring obesity and metabolic disturbances. Animals are usually fed a high-fat or western style-diet (increased fat and carbohydrate content) to induce obesity. These models have shown that maternal over-nutrition induces adiposity and permanent changes in metabolism in the offspring [125-132], even when the offspring are exposed to normal diets after birth, whether they are cross-fostered onto non-obese animals [129], or weaned to a standard diet [125, 127, 130-132]. A proposed mechanism behind the increased adiposity is a permanent state of hyperphagia in offspring exposed to in utero over-nutrition [130, 133, 134], possibly via programming of central pathways involved in appetite control. Interestingly, in a rodent study Sen and Simmons found that offspring of dams fed a western diet had increased adiposity and impaired glucose tolerance already at 2 weeks. Inflammation and oxidative stress were increased already in pre-implantation embryos, fetuses and newborns. Furthermore, supplementation of antioxidants to the maternal diet decreased adiposity and glucose intolerance in the offspring. This study suggested that obesity is programmed already at the pre-implantation stage of development, and that inflammation and oxidative stress as a result of maternal obesity plays important roles [132]. In favor of the hypothesis of epigenetic modulation as a mediator of obesity programming, as described in the section on DOHaD, is a study using macaque monkeys. The authors found that intrauterine over-nutrition resulted in increased fetal liver lipids and indications of non-alcoholic fatty liver disease as well as global and gene specific methylation and histone modifications leading to alteration in DNA expression, and hypothesized that these modifications were indicators of programming of an obesogenic phenotype [135].

GESTATIONAL WEIGHT GAIN AND IMPLICATIONS FOR THE OFFSPRING

Maternal weight gain during pregnancy is termed gestational weight gain (GWG) and includes the weight of the fetus, uterus, amniotic fluid, placenta, increased maternal blood volume and increased maternal fat and lean mass [136]. Even though GWG is a natural and necessary phenomenon, excessive GWG can be seen as another source of over-nutrition of the fetus. Associations between GWG and birth weight or infant adiposity have been found in many observational studies [92, 137-144]. The associations between GWG and offspring body size continues into early childhood [105, 114, 145-159], adolescence [113, 150, 151, 153, 154, 160-162] and adulthood [101, 150, 151, 154, 163, 164]. A recent meta-analysis including many of these studies estimated that the OR of excessive GWG and childhood overweight/obesity was 1.33 [16]. In the attempt to eliminate confounding factors such as shared genetics, 3 recent large cohorts of 513.501, 42.133 and 136.050 women, respectively [139, 162, 164], followed the women over multiple pregnancies and using a within-subject design, they suggested that GWG was directly associated with offspring birth weight [139] as well as BMI in childhood [162] and adulthood [164].
Overweight and obese mothers tend to gain less weight than lean mothers [165, 166]. This is reflected in the Institute of Medicine (IOM) guidelines [167], Table 1, where obese women are recom-

mended to gain between 5 and 9 kg, whereas normal weight mothers are recommended to gain between 11.5 and 16 kg. Despite this, obese mothers are at higher risk of gaining excessively compared to lean mothers [165, 168]. This is worrisome, as the impact of excessive weight gain is increased in mothers with raised BMI and associated with even higher risks of offspring being large at birth as well as later in life [101, 105, 140, 142, 149].

Table 1
The 2009 Institute of Medicine recommendations for total weight gain during pregnancy

Pre-pregnancy BMI (kg/m^2)	Recommended gestational weight gain (kg)
Underweight (< 18.5)	12.5 - 18
Normal weight (18.5 – 24.9)	11.5 - 16
Overweight (25 – 29.9)	7 – 11.5
Obese (≥ 30)	5 – 9

POTENTIAL MECHANISMS OF IMPACT OF MATERNAL OBESITY AND GESTATIONAL WEIGHT GAIN ON OFFSPRING ADIPOSITY

The mechanisms behind the associations between maternal obesity and/or GWG and offspring adiposity and adverse metabolic profile are not well-determined. According to the original Pedersen hypothesis, maternal hyperglycemia results in fetal hyperglycemia, leading to hyperplasia and hypertrophy of islet tissue in the fetal pancreas. This in turn, leads to fetal hyperinsulinemia and excessive fetal growth of adipose, muscle and liver tissue, often resulting in a macrosomic infant with disproportionate features [169]. Even though the Pedersen hypothesis originally described the influence of diabetes, similar models appear to explain influence of increased maternal glycemia below the threshold. This is exemplified by the large Hyperglycemia and Adverse Pregnancy Outcome Study (HAPO), where birth weight and newborn adiposity increase linearly with maternal glucose concentration [170].

Obesity induces a state of insulin resistance, and it is the strongest predictor of GDM [171]. Pregnancy itself is associated with insulin resistance [172, 173], making the combination of obesity and pregnancy a significant metabolic stress on the female body. For obese women, who have gained excessively during pregnancy, this metabolic stress is even further exaggerated. In addition to insulin resistance, pregnancy also induces significant changes in lipid concentration and function, and especially obese mothers have altered lipid metabolism [55, 174, 175]. In the third trimester, obese women have increased levels of triglycerides, VLDL and lower HDL compared to lean women [176]. Free fatty acids can cross the placenta and become incorporated into fetal lipids [177], and studies have shown correlation between maternal lipids and fetal abdominal circumference [178], birth weight [179, 180] and fat mass at birth [178].

Taken together, these data suggest that in women with obesity and/or associated decreased insulin sensitivity, both increased levels of glucose and lipids may account for a significant proportion of fetal adiposity. These data support the original studies of Freinkel [53], and have further emphasized that fetal overgrowth is the result of multiple nutritional factors, not only glucose. And as fetal overgrowth and consequent neonatal adiposity is highly associated with later obesity and metabolic disturbances, this might be one of the links between in utero over-nutrition (whether it is caused by maternal obesity, GWG, diabetes or a combination) and the long-term adverse offspring outcomes.

GENETIC AND EPIGENETIC IMPACT ON OFFSPRING OBESITY

Based on the many studies on associations between maternal and offspring obesity listed above, it certainly seems that the intrauterine environment contributes to programming of the offspring. Nevertheless, some have argued, that these associations reflect "obesogenic" genes or shared postnatal environment between mother and child rather than the intrauterine environment. If shared genes alone explained the associations, correlations between maternal and paternal BMI and offspring BMI would be the same. Some studies have indeed shown similar effects [106, 181-184]. However, in many of these studies paternal BMI was self-reported, possibly biasing the father-offspring effect. In other studies, maternal BMI seems to be closer associated than paternal BMI to offspring BMI or body composition [98, 185-188]. Also, the studies on associations between GWG and offspring body composition listed above supports an intra-uterine cause rather than genetic or shared lifestyle explanations. Additionally, a British study showed that the BMI of children born to recipients of ovum donation was closer associated with the recipient mother than the ovum donor, suggesting that the genetic component plays a lesser role [189]. Further evidence is provided by studies of siblings born to the same mother before and after bariatric surgery. These demonstrate that bariatric surgery and associated weight loss reduces birth weight and obesity rates, and improves the cardiometabolic profile in the offspring [190, 191]. One of the strengths of these studies is that they have eliminated the confounding factors of genetics and at least to a certain extent also influences of the postnatal environment, as the siblings were brought up in the same family. Interestingly, in a subgroup analysis, the authors found that the siblings born after maternal surgery had different gene methylation and expression compared to the siblings born before surgery, and speculated that this was responsible for the improved cardiometabolic risk profile [192], thus supporting the hypothesis of epigenetic processes as programming factors. As described in earlier sections, the role of epigenetics on the formation of the phenotype is still uncertain. Very few studies have been conducted in humans. However, an interesting study has recently reported links between gene methylation in umbilical cord tissue and later risk of childhood adiposity [193], thus proposing that a substantial component of metabolic disease risk has a prenatal developmental basis. Even though not directly transferable to the human condition, results from animal models suggest associations between in utero over-nutrition and epigenetic changes [60, 135, 194]. As this concept of effect of epigenetic alterations is attracting wide attention, future research will undoubtedly provide further information.

In conclusion, over-nutrition in utero certainly seems to contribute to programming of the fetus. However, whether maternal obesity in humans truly causes long-term programming events in the offspring, whether the associations between maternal and offspring obesity reflects tracking of size at birth, or whether the associations are due to shared genes and lifestyle, is difficult to determine. Solid evidence from intervention studies in obese pregnant women with follow-up in the children is sorely needed.

OTHER EARLY LIFE FACTORS OF CHILDHOOD OBESITY

In addition to the effects of intrauterine over-nutrition caused by diabetes, maternal obesity and/or GWG, many other early life

factors have been linked with childhood overweight and obesity. Rapid growth in the first months of life, for instance, is associated with increased risk of becoming overweight and having increased fat mass in childhood [154, 195-197]. Also maternal smoking during pregnancy has long lasting effects on the offspring. Maternal smoking is associated with a reduction in fetal growth, and often results in children being born small for gestational age (SGA) [68, 198, 199]. Paradoxically, later in life, maternal smoking is associated with increased BMI in the offspring [200-203]. Children born with low birth weight are often subjected to catch up growth during early childhood [204] and are subsequent at risk of increased BMI later in childhood. This might be one mechanism of the effect of maternal smoking. Additionally, cessation of smoking results in weight gain in adult persons, and this might also be the case for the newborn child [202]. But even though attempts have been made to adjust for confounding factors in the listed studies, residual confounding such as living conditions in smoking families might also be an explanation [205].

The effect of breastfeeding on overweight and obesity in childhood has been extensively studied, and several meta-analyses have been conducted in recent years [206-208]. Overall, it seems that breastfeeding has a protective effect against childhood overweight and obesity, albeit the effects on mean BMI might be limited [208]. The large RCT "Promotion of Breastfeeding Intervention Trial (PROBIT)" did not suggest effects of breastfeeding on offspring mean BMI, despite a larger proportion of breastfeeding mothers in the intervention arm compared to the control arm [209]. Furthermore, a study using DEXA scans of 5 year old children did not detect any differences in fat mass between breastfed and never breastfed children [210]. Nevertheless, breastfeeding might have positive effects on the offspring, especially looking at the risks of overweight and obesity rather than BMI as a continuous outcome. Unfortunately, overweight and obese women are less likely to breastfeed [76] and breastfeed for shorter periods [211]. This is particularly a problem, as a significant interaction between maternal BMI and lack of breastfeeding seem to put offspring at an even higher risk of obesity [95].

MATERNAL AND OFFSPRING OBESITY – A VICIOUS INTERGENERATIONAL CYCLE

In conclusion, several influential factors on the development of childhood obesity have been suggested. In this thesis, main focus is on the effect of maternal obesity and taken together, obese mothers are at risk of delivering large babies who become obese during childhood and adulthood, and subsequently obese parents, thus creating a vicious intergenerational cycle of obesity, as initially proposed by Catalano et al. [212], Figure 2. The epidemiological data and animal data listed above suggest that the maternal intrauterine milieu might be favorably altered to confer short and long term benefits to the child. During pregnancy, women have increased motivation to change lifestyle to better their own as well as their unborn child´s health [213]. As a result, a great number of lifestyle intervention studies have been conducted in pregnant women. A recent review and meta-analysis by Thangaratinam et al. included 44 RCTs that examined lifestyle interventions during pregnancy [214]. The authors concluded that interventions based on exercise alone showed a small reduction in birth weight and GWG. Interventions based on diet alone and mixed interventions also resulted in a reduction of GWG, whereas no effect was found on birth weight. Similarly, a number of other systematic reviews or meta-analyses have found that limiting GWG is possible with intervention strategies, whereas effects of interventions on other obstetric outcomes including birth weight are limited [215-221]. However, none of the studies included in the systematic reviews and meta-analyses described above followed the offspring past delivery.

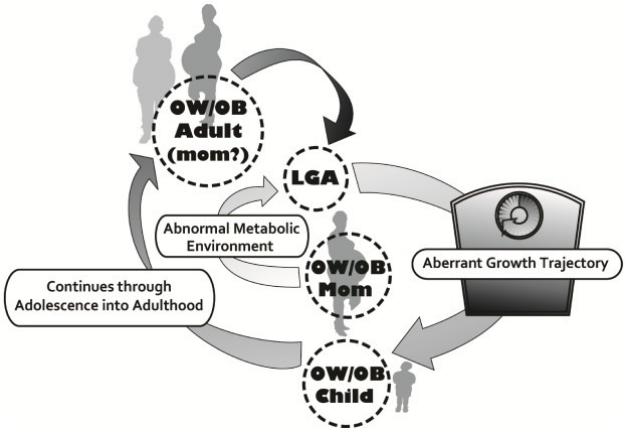


Figure 2
The intergenerational cycle of obesity

LGA; Large for Gestational age, OW; overweight, OB; obese. Adapted from Adamo et al. 2012 [222].

In fact, only two small clinical trials have investigated effects of lifestyle intervention strategies aiming to improve the intrauterine environment on the offspring past birth ([223, 224], Table 2), and none have estimated possible effects into adulthood. Both trials were conducted in Finland by the same research group. In the first trial Mustila et al. used a cluster controlled design to investigate effects of lifestyle intervention during pregnancy on postnatal weight development from 0-4 years in the offspring. This study included 109 pregnant women with all categories of BMI, and women giving birth in intervention centers were given individual counseling on physical activity and diet five times during pregnancy and had the option to attend supervised group exercise sessions. Follow-up rates of the offspring at four years of age was 66%, and no effect of the intervention during pregnancy was seen [223]. In the other trial, the group used a non-randomized design with an intervention and a historical control group to study the effects of lifestyle intervention on offspring weight development from 0-1 years. In this study, 216 women at risk of developing GDM (defined as; body mass index (BMI) ≥ 25 kg/m2, macrosomic newborn (weight ≥ 4500 g) in any previous pregnancy, immediate family history of diabetes and/or age ≥ 40) were included, and follow-up was conducted in 86%. Intervention group participants received two group sessions with diet and physical exercise advice as well as breast feeding advice postpartum. Again, no effect of the intervention was seen in the offspring [224]. Both of these studies were non-randomized and relatively small, and no effect on gestational weight gain was seen. So, whether it is possible to improve weight gain patterns during pregnancy and subsequently confer short- and long-term benefits to the offspring remains to be determined. Fortunately, many large pregnancy lifestyle interventions trials with planned follow-up of the children are being conducted at the moment and results from them will hopefully provide valuable information ([225, 226] and Table 2).

Table 2

Published reports, protocols or registered trials with pregnancy lifestyle intervention programs with follow-up on the offspring

Author and year	Country	Design	Population (n)	Maternal BMI (kg/m2)	Intervention	Offspring age at follow-up	Follow-up rates	Outcome	Results
Published reports:									
Mustila et al. 2012	Finland	Cluster Control-led Trial	109	All BMI categories	5 times individual counseling on physical activity and diet, option to attend supervised group exercise sessions	0-4 years	66 %	Postnatal weight development	No effect
Mustila et al. 2013	Finland	Non-randomized controlled trial. Historical control group	216 (women at risk of develop-ing GDM)	All BMI categories	2 group counseling sessions on diet and exercise, breastfeed-ing advise	0-1 years	86%	Postnatal weight development	No effect
Published protocols:									
Adamo et al. 2013	Canada	RCT, pilot	60	≥18.5	3 group and 2 individual counseling sessions on diet during pregnancy. Group exercise offered twice weekly.	0-2 years		BMI Z-score and skinfolds	
Registered trials:									
Calle-Pascual et al.	Spain	RCT	1000	All	Mediterranean diet, individual counseling sessions and physical activity. Control group also physical activity and dietary advice on less fat intake. Start from 8-12 weeks´ gestation.	0-12 months		Not specified	
Poston et al.	UK	RCT	1564	≥30	Weekly individual counseling sessions on diet and physical activity between 20 and 28 weeks' gestation.	3 years		Not specified	
Joshipura et al.	Puerto Rico	RCT	400	≥25	Counseling on dietary and physical activity.	0-12 months		BMI Z-score	
Gallagher et al.	USA	RCT	210	25-35	Individual and group sessions with dietary and physical activity advice twice monthly and telephone contact every week.	0-12 months		Infant fat percent-age at 14 and 52 weeks	
Chung et al.	USA	RCT	266	25-45	28 home visits both during pregnancy and postpartum. Advice on healthy living in pregnancy and breastfeeding	0-18 months		Not specified	
Van Horn et al.	USA	RCT	300	25-35	Individual counseling sessions on diet and physical activity, daily tracking of diet and activity, and use of pedometer	0-12 months		Postnatal weight development	
Phelan et al	USA	RCT	350	≥25	Not specified in details, but include behavioral strategies to modify diet and physical activity.	0-12 months		Postnatal weight development	
Knowler et al.	USA	RCT	1500	≥25	Not specified, but aims to limit GWG	0-12 months		Postnatal weight development	
Goodman	USA	RCT	150	All	Home visits with advice on limiting GWG and improving breastfeeding	0-12 months		Postnatal weight development	

Own studies

OVERALL AIM OF THE THESIS

The overall aim of this thesis was to investigate the relationship between maternal BMI and offspring body size and metabolic profile. Special focus was put on investigating the effects of lifestyle intervention during pregnancy in obese women on the offspring in early childhood.

SPECIFIC AIMS

Paper I	To examine the impact of maternal pregestational BMI and smoking on neonatal abdominal circumference (AC) and weight at birth. To define reference curves for birth AC and weight in offspring of healthy, non-smoking, normal weight women. To compare the impact of maternal BMI on Z-scores of birth AC and weight and on the ratio between birth AC and weight.
Paper II	To study the effects of lifestyle intervention during pregnancy in obese women on offspring anthropometrics and body composition in early childhood. To compare anthropometrics and body composition in offspring of obese mothers from a lifestyle intervention trial to an external reference group of children born to lean mothers.
Paper III	To study the effects of lifestyle intervention during pregnancy in obese women on offspring metabolic risk factors in early childhood To compare metabolic risk factors in offspring of obese mothers from a lifestyle intervention trial to an external reference group of children born to lean mothers. To study the predictive values of birth weight and birth abdominal circumference on metabolic risk factors in early childhood.

PAPER I – REGISTRY BASED STUDY

The study in paper I is based on data extracted from the Danish Medical Birth Registry. The study was conducted according to the Helsinki Protocol and it was approved by the Danish Data Protection Agency.

MATERIALS AND METHODS

The Danish Medical Birth Registry has information on pregnancies and deliveries since 1973, including 99.8% of all Danish deliveries and has a high reliability and validity [227], especially when it comes to the quantitative data (e.g. birth size and gestational age) [228]. Since 2004 the pregestational height and weight of the mother, as reported by her general practitioner, have been registered.

Nationwide data on pregnant women and their offspring born between January 1, 2004 and December 31, 2010 was extracted. Inclusion criteria included singleton children born in weeks 35+0 to 41+6 (weeks+days) of gestation. Exclusion criteria included stillborn children, children with congenital malformations and children from a multiple pregnancy.

For each mother- and infant-pair, the following variables were recorded: Maternal pregestational weight and height, age, parity, smoking status, any medical condition, gestational age (GA), sex, birth weight and abdominal circumference (AC). Pregestational BMI was calculated and women were grouped into the following five categories: <18,5 kg/m2 (underweight), 18,5-24,9 kg/m2 (normal weight), 25-29,9 kg/m2 (overweight), 30-34,9 kg/m2 (obese) and >35 kg/m2 (severely obese). Maternal and fetal diseases or complications were classified according to the International Classification of Diseases 10th revision.

STATISTICAL ANALYSES

For all analyses STATA 12 software (StataCorp, College Station, TX, USA) was used. Initially, linear regression models were used to estimate the relation of AC and birth weight to different categories of maternal pregestational BMI in non-smoking mothers without medical conditions. Next, the effect of smoking and pregestational BMI on birth AC and weight was quantified using multivariate linear regressions, accounting also for sex, gestational age, maternal age, height, parity and any medical condition.

For construction of normative curves for AC and birth weight only offspring of non-smoking, healthy mothers with normal pregestational BMI were included. Normative curves were produced for 35+0 to 41+6 weeks of gestation. Descriptive statistics (mean and standard deviation) for the normative curves were calculated point-wise for each gestational week and sex. In the corresponding curves, the point-wise estimates were connected by lines.

In addition, we used multivariate linear regressions to analyze whether AC or birth weight had the strongest association with maternal pregestational BMI. Standardized Z-scores of AC and birth weight from our established healthy reference curves were used instead of their "raw" values. The following covariates were included in the model: maternal pregestational BMI (continuous), sex, gestational age (35-41 weeks, continuous), smoking (yes/no), maternal medical condition (yes/no), height (continuous), parity (categorical) and age (continuous). Furthermore, we tested the difference between the two estimated regression coefficients, BMI on AC, and BMI on birth weight, using a method including dummy variables, as described in [229].

Finally, we examined the ratio between AC and birth weight and the impact of maternal pregestational BMI on this parameter, using simple linear regression analyses. For this analysis we only used data on offspring of non-smoking mothers with no medical conditions.

RESULTS

The study included 333,618 healthy singletons born at 35+0 to 41+6 weeks of gestation and their mothers. An overview of sample sizes in the different analyses is given in Table 3.

In a population of non-smoking mothers with no medical conditions, maternal pregestational BMI was directly associated with mean birth AC and weight, and across all BMI categories both outcomes increased significantly ($p<0.0001$). Sex specific curves for mean birth AC and weight, stratified by maternal pregestational BMI according to GA are shown in Figure 3. Corresponding single reference curves and statistics, stratified by sex and maternal BMI category can be seen in the appendix in supplementary material for article I, Figures S1-S4 and Tables S4-S23.

Table 3

Overview of the number of mother-child pairs contributing to the different analyses. All analyses are based on data from 35+0 to 41+6 weeks of gestation

		Abdominal circumference			Birth weight		
Analysis	Inclusion criteria	Boys	Girls	Total	Boys	Girls	Total
Relation of birth AC and weight to pregestational BMI, maternal smoking status, medical conditions, height, age and parity	Non-smokers; no medical condition; all BMI categories	137,825	134,521	272,346	141,654	137,515	279,169
	Both smokers and non-smokers, all BMI, age, height and age categories, medical condition +/-	164,811	160,541	325,352	169,372	164,246	333,618
Normative curves, stratified by sex	Non-smokers; no medical condition; BMI category 18.5-24,9 kg/m2	89,971	87,114	177,085	92,424	89,063	181,487
Comparison of birth AC and weight Z-score with pregestational BMI, adjusted for maternal smoking, height, age, parity and medical conditions	Smoking status, height, age, parity, BMI, birth AC and weight non-missing	164,471	160,202	324,673	164,471	160,202	324,673
Relation of AC/birth weight ratio to pregestational BMI	Non-smokers; no medical condition; all BMI categories, birth AC and weight non-missing	137,825	134,521	272,346	137,825	134,521	272,346

AC; abdominal circumference

In adjusted analysis estimating also the effects of smoking, every increase in pregestational BMI of 1 kg/m2 was associated with an increase in AC of 0.5mm (95% confidence interval (C.I.) 0.5-0.5mm), and an increase in birth weight of 14.2g (95% C.I, 13.9-14.5g), Table 4. An increase in gestational week was associated with an increase in abdominal circumference of 5.0mm (95% C.I. 5.0-5.1mm) and of 162.2g (95% C.I, 161.1-163.3g) in birth weight. In this model, increasing GA had the highest positive impact and smoking had the largest negative impact on both AC and birth weight. Increasing parity and maternal height were also positively associated with both outcomes, whereas sex (girls), advancing maternal age and maternal medical condition (any) were negatively associated, Table 4.

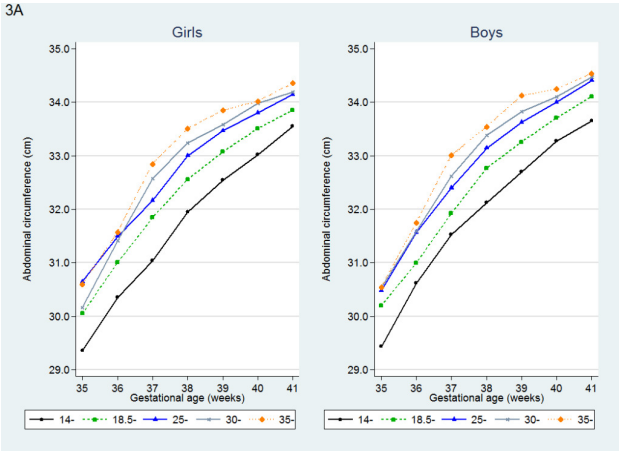


Figure 3A
Figure 3 A) Mean abdominal circumference by maternal pregestational BMI and gestational age, separately for boys and girls (p < 0.0001).

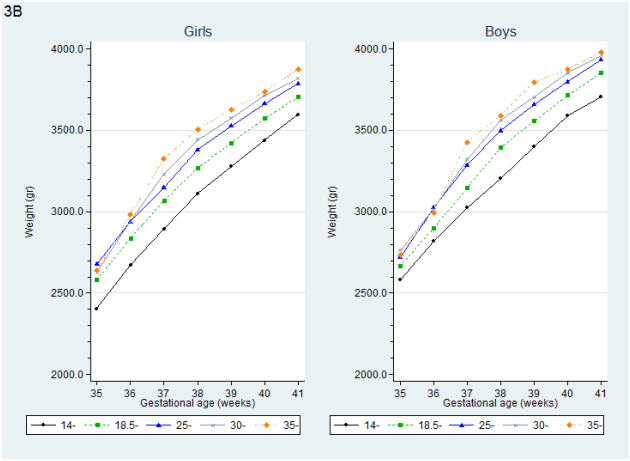


Figure 3B
Mean birth weight by maternal pregestational BMI and gestational age, separately for boys and girls (p < 0.0001)

Table 4

Factors associated with birth AC and weight in multivariate regression analysis (N=333,618 mother-child pairs)

Covariate	Abdominal circumference (cm) Coefficient (95% C.I.)	Birth weight (g) Coefficient (95% C.I.)
Maternal BMI (continuous) †	0.05 (0.05; 0.05)	14.2 (13.9; 14.5)
Gestational Week (35-41, continuous) ‡	0.50 (0.50; 0.51)	162.2 (161.1; 163.3)
Sex (girls vs. boys)	-0.20 (-0.22; -0.19)	-133.2 (-136.0; -130.4)
Smoking (yes vs. no)	-0.45 (-0.47; -0.43)	-172.7 (-176.8; -168.5)
Any medical condition, mother (yes vs. no)	-0.08 (-0.12; -0.01)	-17.4 (-29.7; -5.2)
Parity 1§	0.65 (0.63; 0.66)	153.2 (150.0; 156.4)
Parity 2 or more§	0.73 (0.71; 0.75)	182.8 (178.6; 187.1)
Maternal age (continuous) ||	-0.01 (-0.01; -0.01)	-2.5 (-2.8; -2.1)
Maternal height (continuous) £	0.04 (0.04; 0.05)	13.8 (13.5; 14.0)

†; every increase in pregestational BMI of 1 kg/m2. ‡; every increase in gestational week. §compared to first time pregnancies. ||; every increase in maternal age of 1 year. £; every increase in maternal height of 1 cm.

Sex specific normative curves for birth AC and weight by GA, based on offspring of healthy, non-smoking, normal-weight mothers are presented in Figure 4 and 5. Corresponding statistics are shown in the supplementary material for paper I, Tables S5, S10, S15 and S20.

Finally, we found that birth weight had a stronger association with maternal pregestational BMI than birth AC. For every increase of 1 kg/m2 in pregestational BMI, birth AC Z-score (95% C.I.) increased by 0.02 (0.02-0.03), whereas birth weight Z-score increased by 0.03 (0.03-0.03), after adjusting for smoking, maternal medical conditions, age, parity and height. The difference was statistically significant (p<0.0001). In accordance with these results, the ratio between AC and birth weight decreased with increasing maternal pregestational BMI. For every increase of 1 kg/m2 in pregestational BMI, the birth AC:weight ratio decreased by -0.02 cm/kg (95% C.I. -0.02 to -0.02, p<0.0001).

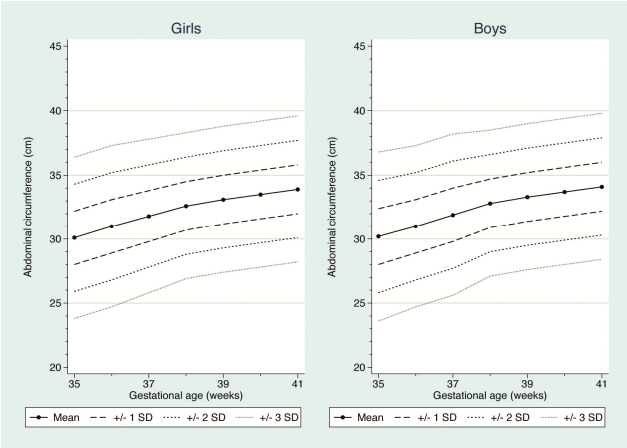


Figure 4
Normative curves for abdominal circumference by gestational age, for healthy singletons of non-smoking mothers with normal pregestational BMI

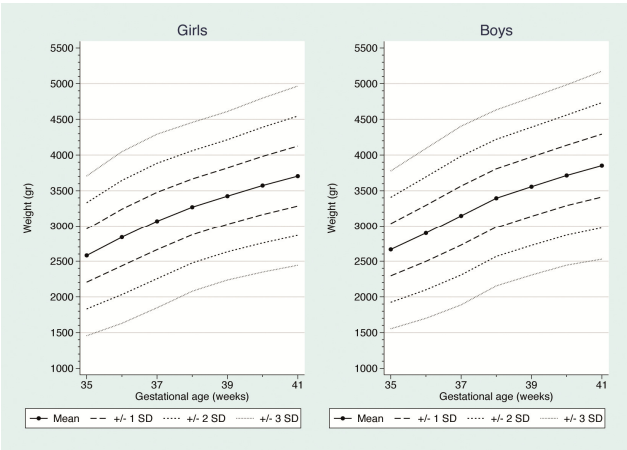


Figure 5
Normative curves for birth weight by gestational age, for healthy singletons of non-smoking mothers with normal pregestational BMI

DISCUSSION

In this registry based study, we have demonstrated that maternal pregestational BMI is associated with both birth weight and birth abdominal circumference. The associations were, however, strongest between maternal pregestational BMI and birth weight. In accordance, the ratio between AC and birth weight, which to some extent is a measure of the degree of abdominal obesity in relation to weight, decreased with increasing maternal BMI. This could imply that intrauterine over-nutrition results in a general weight gain of the fetus rather than just fat accumulation around

the abdomen. However, another explanation could be the difference in accuracy of the measurement methods. AC is often rounded into whole centimeters and lack precision, whereas birth weight is reported in smaller units and thus with more accuracy. Despite the closer correlation between BMI and birth weight, our findings from this study do not tell us whether birth AC is a weaker or better predictor of future metabolic risk factors than birth weight, which is one of the aims for paper III. With our normative birth AC and weight reference curves we have provided a research tool for evaluation of this hypothesis. We additionally found that maternal smoking has a negative effect on fetal growth, as reported by many others [68, 198, 199].
The strengths of this study are the size of the cohort, the high validity of the data and the possibility to stratify for smoking, BMI and diseases. Limitations include inability to estimate the effect of gestational weight gain, ethnicity, paternal BMI and subcategorize the effect of smoking (binary data only), as this information is not available in the Danish Medical Birth Registry.

PAPER II AND III – THE LIFESTYLE IN PREGNANCY AND OFFSPRING (LIPO) STUDY

The studies in paper II and III are based on a follow-up of a randomized controlled trial (RCT) involving lifestyle intervention during pregnancy in obese women. The RCT was the basis for a previous PhD thesis [230].

MATERIALS AND METHODS

Description of the Lifestyle in Pregnancy (LiP) Study

The Lifestyle in Pregnancy (LiP) study was a randomized controlled trial with lifestyle intervention in obese pregnant women running from 2007 to 2010 in two University Hospitals in Denmark; Odense University Hospital and Aarhus University Hospital, Skejby [231]. The LiP study was approved by the local ethics committee of the Region of Southern Denmark (S-20070058) and the Danish Data Protection Agency, and was registered at www.clinicaltrials.gov as NCT00530439. A total of 360 women aged 18–40 years were recruited at 10–14 weeks of gestation. The inclusion criterion was a BMI of 30–45 kg/m2 based on prepregnancy weight, or first measured weight in pregnancy. Inclusion and exclusion criteria can be seen in Table 5. Participants were randomized in a ratio of 1:1 to i) lifestyle intervention including dietary advice, coaching and exercise or to ii) routine obstetric care. A doctor and a research midwife enrolled the patients and they were randomized using computer-generated numbers in closed envelopes, which they themselves picked up from a basket and opened. Subsequently, there was no blinding to patients, care givers or the doctor. The intervention in pregnancy consisted of two major components: i) dietary counseling and ii) physical activity. Dietary counseling was performed individually by trained dieticians four times during pregnancy. The aim of the counseling was to limit gestational weight gain to five kg. Trained dieticians carried out individual dietary counseling four times during pregnancy. The counseling was based on the evaluation of each participant´s dietary history, weight and level of activity and led to a personalized diet. The dietary advises were based on the official Danish recommendations. At the last visit before delivery, intervention group participants were given material on breastfeeding advice. The physical activity component consisted of encouragement to be moderately physically active for 30-60 minutes daily. Participants were equipped with a pedometer (Walking Style II, Omron Healthcare, Japan) and informed about the general advice of walking approximately 10,000 steps daily. Each participant was given free, full-time membership in a fitness center, where they could choose between several different types of aerobic classes or weight training. Additionally, for one hour each week, a closed aerobics class was arranged with a physiotherapist, and participants were requested to attend this session. After physical training the participants were grouped 4-6 times during pregnancy together with the physiotherapist. In these group sessions the physiotherapist used coaching inspired methods to improve participant´s integration of physical activities in pregnancy and daily life.
Women in both groups were monitored three times during pregnancy with fasting blood samples, oral glucose tolerance tests (OGTTs) and weight. As part of the LiP study, women were seen six months postpartum, where breastfeeding information was gathered.

Table 5

Inclusion and exclusion criteria for the LiP study

Inclusion criteria
Age 18-40 years
BMI 30-45 kg/m2
Exclusion criteria
Prior serious obstetric complications (e.g. stillbirth, preterm delivery, second trimester or habitual abortion)
Chronic diseases (e.g. hypertension, diabetes, severe asthma, severe psychiatric disorder, severe disorders in musculoskeletal system)
Positive oral glucose tolerance test in early pregnancy
Alcohol or drug abuse
Non-Danish speaking
Late referral to Department of Gynecology and Obstetrics (>14 weeks of gestation)
Multiple pregnancy

Summary of results from the LiP study

The intervention group had a significantly lower median GWG compared with the control group (7.0 vs. 8.6 kg; p=0.01). Surprisingly, neonates from the intervention group had a higher birth weight compared to the control group (median 3742g vs. 3596) [231]. No significant differences were seen in the five main clinical outcomes between groups (gestational diabetes, preeclampsia/pregnancy induced hypertension, cesarean delivery, infants born large for gestational age or infants admitted to neonatal intensive care unit). Additionally, no differences in breastfeeding patterns were detected postpartum. The compliance with the LiP intervention program was good regarding the dietary counseling sessions; 92% of the women completed all four sessions and 98% completed at least three sessions. When asked if participation in the LiP study had resulted in more healthy eating habits, 85% of women in the intervention group responded affirmatively. However, 21% of women in the control group also reported that they had adopted more healthy eating habits as a result of being in the trial. Compliance with the physical component of the intervention was not as good as that of the dietary sessions. Mean attendance for the 20 aerobic classes was 10.4 hours, and 56% of women in the intervention group attended the aerobic classes for at least half of the lessons. Among women in the intervention group 78% undertook leisure time sporting activities in addition to the aerobic classes. However, also 65% of control group women did some sort of leisure time sporting.

Description of the Lifestyle in Pregnancy and Offspring (LiPO) study

The Lifestyle in Pregnancy and Offspring (LiPO) study was based on a follow-up of the LiP study. Additionally, an external reference group (ER) of lean mothers and their offspring was included. The study thus included three sets of mother and child dyads:

- LiPi (from LiP intervention group)
- LiPc (from LiP control group)
- ER (from external reference group of lean mothers and their offspring)

The LiPO study was planned in 2010, while the LiP RCT was still ongoing. The study was approved by the local ethics committee of the Region of Southern Denmark (S-20100070) and by the Danish Data Protection Agency. It was registered at www.clinicaltrials.gov as NCT01918319 for comparison of offspring of mothers participating in the LiP study and as NCT01918423 for comparison of offspring from the LiP study with the reference group of children born to lean mothers. Written informed consent was obtained for each participant, initially as part of the LiP study and again for participants of the LiPO follow-up.

Inclusion criteria for the LiPi and LiPc groups were mothers who had completed the LiP study until birth and their offspring (Figure 6). The ER group was recruited from lean mothers who had given birth in Odense University Hospital within the same time period as the offspring of LiP participants were born. Maternal inclusion and exclusion criteria for the ER group were similar to those for the LiP study (Table 6), with a few exceptions; pregestational BMI was restricted to 18.5-24.9 kg/m2 and late referral to Department of Gynecology and Obstetrics was not an exclusion criteria. Additional exclusion criteria for the reference group were: children born before 37 or after 41 completed weeks of gestation and children with significant medical conditions (defined by being hospitalized for more than 10 days in the first year of life). These additional exclusion criteria were added as we wished to have as normal a reference group as possible.

Table 6

Inclusion and exclusion criteria for the external reference group (ER) in the LiPO study

Inclusion criteria
Age 18-40 years
BMI 18.5-24.9 kg/m2
Completed questionnaire at the child´s second birthday
Exclusion criteria, maternal
Serious obstetric complications
Chronic diseases (i.e. hypertension, diabetes, severe asthma, severe psychiatric disorder, severe disorders in musculoskeletal system)
Positive oral glucose tolerance test in pregnancy
Alcohol or drug abuse
Non-Danish speaking
Multiple pregnancy
Exclusion criteria, offspring
Born outside gestation 37+0 to 41+6 (weeks + days)
Severe medical conditions

The ER group was identified after pregnancy from electronic patient records. We wished to obtain further information on maternal smoking status during pregnancy, socioeconomic status, breastfeeding, paternal height and weight, child morbidity and diet of the ER group; information which was unavailable in the patient records. In order to limit faulty recall, we chose to mail a questionnaire to potential participants before they were formally invited to the clinical examination. In the accompanying letter, brief information of the follow-up study was given, although a formal invitation for the clinical examination was not included. A number of 2292 normal weight mothers without pregestational diabetes or gestational diabetes gave birth to singleton children born at term from September 2008 to September 2009 in Odense University Hospital. For the first child born on each day from September 2008 to June 2009, the electronic patient record was reviewed. If they fulfilled criteria for participation, a questionnaire was sent to the mother. If the criteria were not fulfilled, the patient record of the second child born on that particular day was reviewed and so forth. From June 2009 to September 2009 the number of questionnaires sent was increased to two each day. We reviewed the electronic patient records of 532 potential participants, and a total of 484 mothers were sent questionnaires, with the original plan of sending the same questionnaires to participants twice; at the child´s first and second birthday. However, the questionnaire survey for the ER group started September 2010, which meant that very few received both questionnaires. We therefore chose to use only data on the ER group from the second questionnaire in our analyses. Out of 484 potential ER group mothers who were sent questionnaires, 325 replied and were eligible for the follow-up.

Of the initial 360 included women in the LiP study, 304 participated in the trial until birth (Figure 6). At delivery, three children were stillborn (two in the intervention group and one in the control group). Accordingly, 301 mother and child dyads were eligible for the LiPO infant follow-up study. Eligible LiP participants were mailed the exact same questionnaires as the potential ER group, and as the questionnaire survey for the LiP groups was started already July 2010, the majority received both questionnaires; at the child´s first and second birthday.

Those fulfilling criteria for the LiPO follow-up received written information about the study when the child was approximately 2.5 years old. They were also given access to a website, which described the study (www.lgos.dk). The mothers were encouraged to contact Mette Tanvig if they wished to participate or wanted to know more about the study and were subsequently verbally informed about the study. Of the 301 eligible LiP study mother and child dyads, 157 (52.2%) were seen for the LiPO follow-up (Figure 6). Of the 325 eligible reference group mother and child dyads, 97 (29.8%) were seen (Figure 6).

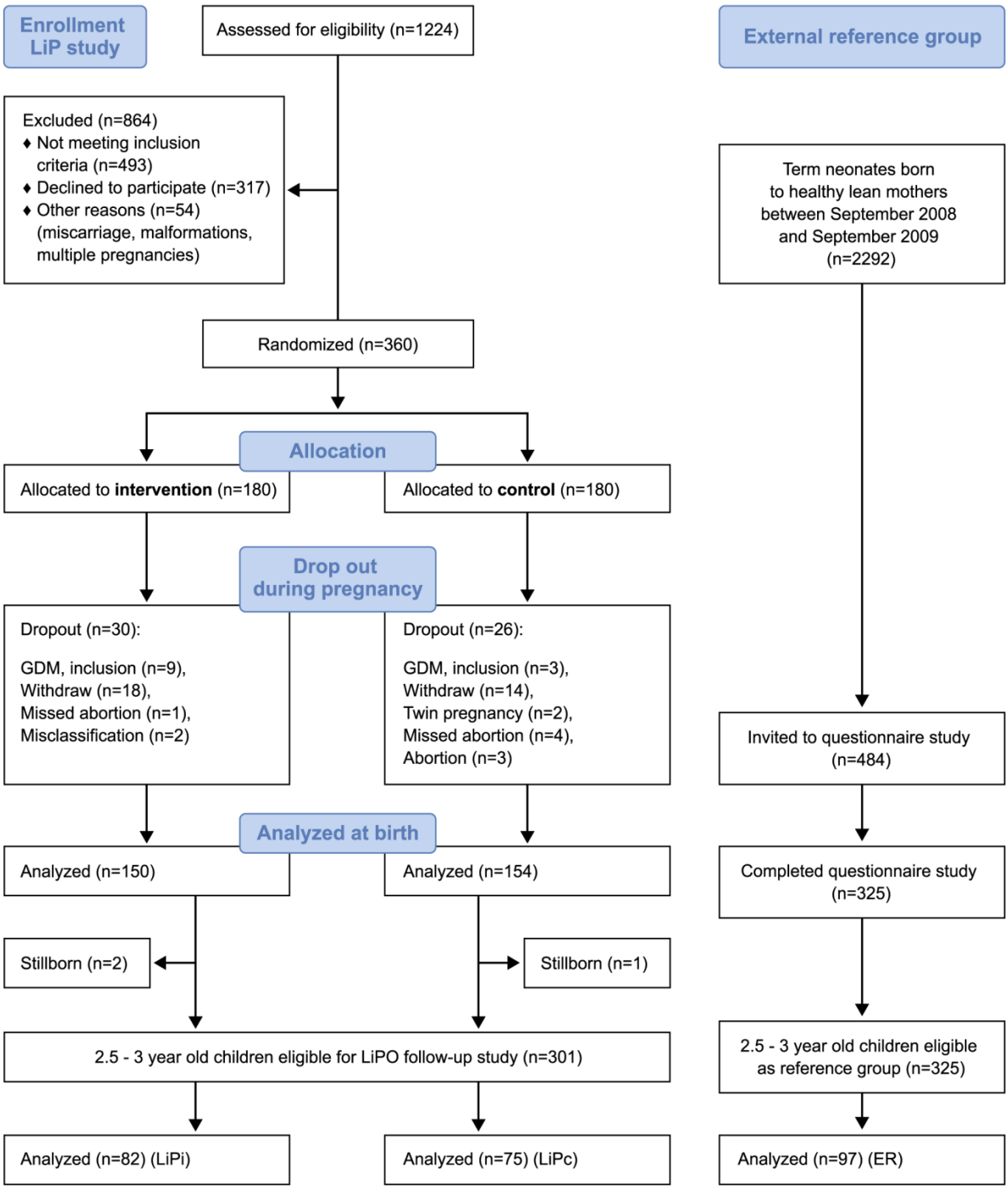


Figure 6
Flowchart for participation in the LiP and LiPO studies

Study visit
Follow-up visits between the age of 2.5 and 3 years were conducted at Odense University Hospital or Aarhus University Hospital between February 2011 and November 2012. All children were examined by the same medical doctor (M.T.), blinded to the RCT intervention. Information on who had received intervention was revealed after data collection was complete. Due to identifiable differences in maternal BMI, it was not possible to blind M.T. to the reference group.

Anthropometry
Weight in light indoor clothing was measured to the nearest 0.1 kg using a digital weight (model 704, Seca, Hamburg, Germany). Height was measured to the nearest 0.1 cm using a portable stadiometer (model 214, Seca, Hamburg, Germany). Triceps and subscapular skinfold thickness was measured to the nearest 0.1 mm using a Harpenden skinfold caliper (Chasmors Ltd, London, UK). Abdominal circumference at the umbilical level and hip circumference at the widest diameter of the buttocks was measured to the nearest mm with a non-stretchable tape measure. Blood pressure was measured using an electronic device (model 420, WelchAllyn, Skaneateles Falls NY, USA) with the child resting in supine position. Measures were performed in triplicate and averaged.

Blood samples
After a 4 hours fast, blood samples were collected from the antecubital vein. Fasting plasma glucose was measured using venous blood and analyzed photometrically in a HemoCue analyzer (HemoCue Glucose 201 RT-system, Ängelholm, Sweden). Serum levels of insulin were analyzed by time-resolved fluoroimmunoassay (AutoDELFIA, Wallac Oy, Turku, Finland). Plasma concentrations of High Density Lipoprotein cholesterol (HDL) and triglycerides (TG) were determined (Modular, Roche Diagnostics, Basel, Switzerland).

DEXA Scans
Dual Energy X-ray absorptiometry (DEXA) scans were performed in Odense University Hospital only. A GE Lunar Prodigy (GE Medical Systems, Madison, WI, USA), equipped with ENCORE software (version 12.3, Prodigy; Lunar Corp, Madison WI, USA), was used to measure estimates of lean mass (LM), fat mass (FM) and body fat percent. Machine calibration and quality assurance tests were performed daily as recommended by the manufacturer. The scanner computer selected the scanning mode (thin, standard or thick) after the data of height and weight of the subject was entered to the machine. The typical scan duration was 4 minutes depending on the child´s height and weight. A trained research bioanalyst and M.T. performed all scans. The children were positioned on the scanner table by M.T. and were instructed to lie still in a supine position wearing underwear and a thin blanket for the duration of the scan. The positioning of the child and the quality of the scan were checked immediately and if these were unsatisfactory, the scan procedure was either ended and restarted or performed again. The GE Lunar Prodigy has good reproducibility with 2.01% Coefficient of Variation (CV) for LM, 1.94% CV for FM and 1.29% CV for body fat percent in children and adolescents aged 5-17 years [38]. The reproducibility of the DEXA scans performed in the present studies was not examined due to ethical consideration. However, repeated daily scans of a phantom were performed to assess the CV during the test period. The CV values were 0.27-0.33% and corresponded well with the above mentioned study. Due to the young age of the children, the quality of the DEXA scans varied and some were inadequate. Consequently, scans were categorized as previously suggested [232]: i) perfect, ii) good with minor irregularities, iii) several irregularities, iv) unusable. Scans graded iii) or iv) were excluded from further analyses.

Outcomes
We assessed a number of anthropometric, body composition and metabolic outcomes. The primary outcome was child BMI Z-score. BMI was calculated as weight (kg) divided by the square of height (m2) and expressed as a continuous Z-score based on age and sex-specific Danish standards [19]. Other outcomes were BMI, triceps skinfold thickness, mid-scapular skinfold thickness, abdominal circumference, hip circumference, abdominal/hip circumference ratio, the DEXA values of total fat mass, total lean mass and fat percentage, blood pressure, fasting plasma glucose, fasting insulin, fasting TG and fasting HDL. Furthermore, overweight or obese children were identified using the criteria defined by the International Obesity Task Force (IOTF) Childhood Obesity Working Group [20].
In order to investigate the associations between birth weight (BW) contra birth abdominal circumference (BAC) and metabolic risk factors (Paper III), we expressed BW and BAC as continuous Z-scores according to our gestational age- and sex-specific normative curves (from paper I). We used the standards based on children born to healthy, non-smoking mothers with a normal pregestational BMI [233].

Statistical analyses
All analyses were performed using STATA 12.0 software (StataCorp, College Station, TX).
With no previous studies available on which to base a power calculation, we originally aimed to include 90 in each randomization groups (in total 180) of 360 mother and child dyads from the LiP study. Given an alpha of 0.05, a beta of 0.80 and a BMI Z-score SD of 1.0, a true difference between the LIP intervention and the control group in offspring BMI Z-score of 0.417 could be detected. However, as only 301 mothers completed the LiP study until birth, we adjusted our power calculations and aimed instead to include 160 of the 301 eligible children (53%). Using the same method for power calculation (an alpha of 0.05, a beta of 0.80 and a BMI Z-score SD of 1.0), we had enough power to detect a difference between the LIPi and LiPc group of 0.447 in BMI Z-score. In order to have a sufficient reference group, we aimed to include a minimum of 90 children born to women with a normal BMI.
A number of different statistical analyses were used. Differences in baseline characteristics and outcomes between groups were analyzed with Chi2 test for categorical variables. For analyses of continuous variables between two groups (e.g. between LiPi and LiPc) Student's t-test was used when data were normally distributed; otherwise Mann-Whitney U test was used. For analyses of continuous variables between more than two groups (e.g. LiPi, LiPc and ER) One-way Anova was used for normally distributed data and Kruskal-Wallis for non-normally distributed data. We did not perform statistical testing for baseline differences between randomized groups and the ER, as the latter was selected from a different population (lean mothers) with the purpose of serving as a normative reference, and was thus by default different.
Linear and multiple regression models were used for analyses of associations between size at birth and metabolic outcomes, as well as for adjusting for potential confounders in the analyses of difference in BMI Z-score between all three groups.

RESULTS
The results of paper II and III are summarized in this section after an overall description of baseline maternal and neonatal characteristics, breastfeeding and infant growth.

Baseline Characteristics
Overall, participants from the LiPi (n=82) and LiPc group (n=75) did not differ with respect to maternal or neonatal baseline characteristics (Table 7). At baseline, there were no differences between those who attended and those who were lost to follow-up except for 2-h OGTT plasma glucose values performed at 28 weeks gestation; median (interquartile range) intervention and control group attendees 6.1 (5.4-7.2) and 6.1 (5.4-6.9) mmol/L vs. lost to follow up 6.6 (5.6-7.5) mmol/L, p = 0.021 (Table 7). Compared to women from the LiP study, those from the reference group had a lower BMI, higher educational level and higher GWG. Children from the reference group had a lower mean birth weight, whereas there were no differences in abdominal circumference or length at birth. Of 250 children with breastfeeding data, 124 (49.6%) were exclusively breastfed (never formula fed) and 66 (26.4%) were exclusively breastfed for at least 5 months, with no differences between the LiPi, LiPc or ER groups. Additionally, there was no difference in postnatal weight development between the LiPi, LiPc or ER groups. Among the LiP children born preterm, two children from the intervention group and one child from the control group had severe medical conditions. None of the children born at term had severe medical conditions.

Anthropometric and body composition outcomes (paper II)
Anthropometric measures and DEXA scan results are presented in Table 8. No significant differences were seen in the primary outcome, BMI Z-score; median (interquartile range) BMI Z-score in children from the LiPi vs. LiPc groups (0.10 (-0.58-0.69) vs. -0.09 (-0.87-0.50)), nor were there any statistically significant differences in BMI Z-score between the LiP offspring and the ER group (-0.32 (-0.75-0.40)). In the linear regression models which analyzed differences in BMI Z-score between the three groups, LiPi group children had an non-significant trend towards a higher BMI Z-score (coefficient 0.27, p=0.069 [crude values]) compared to the reference group, but this was not seen after adjustment for gestational weight gain, parity, smoking during pregnancy, maternal age, educational level (school ≥ 12 years), breastfeeding (exclusive breastfeeding for at least 5 months), birth weight Z-score and post natal growth (change in weight Z-score between 0 and 12 months). No differences between LiPi, LiPc or ER group were seen for the secondary outcomes: BMI (16.4 vs. 16.1 vs. 16.0 kg/m2); the percentage of overweight or obesity (10.9 vs. 6.7 vs. 4.1%), or for weight, length, skinfold thicknesses, abdominal circumference, hip circumference, or abdominal to hip ratio (Table 8). DEXA scanning was successful in 123 (83.7%) out of 147 children (Lipi n=37, LiPc n=30, ER n=56). No differences were detected in total fat mass (2.5 vs. 2.4 vs. 2.3 kg), total lean mass (11.3 vs. 11.2 vs. 10.9 kg) or fat percentage (21.6 vs. 21.6 vs. 21.3%) (Table 8).

Metabolic risk factors and associations with size at birth (paper III)
For the analyses in paper III, we chose to include only children born at term. This meant that 7 preterm children (5 from the LiPi group and 2 from the LiPc group), were excluded. Fasting plasma glucose was measured in 206/247 (83%) of all children (LiPi group: n=59, LiPc group: n=59 and ER group: n=88). Other blood samples were successful in 150 (61%) children (LiPi n=39, LiPc n=51 and ER group n=60), and blood pressure measurement was successful in 200 (81%) children (LiPi n=63, LiPc n=54 and ER group n=83). Metabolic risk factors are presented in Table 9. No significant differences were seen in abdominal circumference (median) LiPi vs. LiPc (48.2 vs. 48.0 cm, p=0.157) or in the fasting plasma measures of glucose, insulin, HDL, TG or in systolic or diastolic blood pressure. Similarly, no differences in metabolic risk factors were detected between LiPi, LiPc and ER group (Table 9).

Associations between size at birth and metabolic risk factors
Results from multiple linear regressions on associations between BW contra BAC and metabolic risk factors at the age of 2.8 years are given in Table 10. The results shown are adjusted for sex, pregestational BMI, GWG, maternal age, educational level, smoking during pregnancy, parity, gestational age at birth, breastfeeding and postnatal growth. BW Z-score was positively associated with (regression coefficients (95% CI), p-value): abdominal circumference (1.56 (1.10; 2.01), p<0.004), fasting blood glucose (0.17 (0.07; 0.26), p<0.004), fasting insulin (5.40 (2.47; 8.33), p<0.004), fasting triglycerides (0.10 (0.02; 0.18), p<0.020) and systolic blood pressure (1.92 (0.71; 3.12), p<0.004). No significant associations between BW Z-score and HDL or diastolic blood pressure were detected. Similarly, BAC Z-score was positively associated with (regressions coefficients (95% CI), p-value): abdominal circumference (1.23 (0.79; 1.68), p<0.004), fasting plasma glucose (0.15 (0.06; 0.24), p<0.004), fasting insulin (5.93 (3.14; 8.72), p<0.004) and fasting triglycerides (0.11 (0.03; 0.18), p<0.004), after adjusting for the above mentioned variables. No significant associations were found between BAC Z-score and HDL, systolic or diastolic blood pressure.

Table 7

Baseline, pregnancy, neonatal and early postnatal outcome data in trial groups from the LiP study and from an external reference group of children born to lean mothers

	Participants in the LiP study			External reference group	
	Intervention (LiPi) n=82	Control (LiPc) n= 75	Lost to follow-up n=144	Children born to lean mothers (ER) n=97	Missing numbers (intervention/ controls/lost to follow-up/reference) percentage
Maternal					
Age at delivery (years)	30.5 (28.2-32.7)	30.2 (26.6-33.4)	29.8 (27.0-32.5)	30.2 (28.0-33.1)	.
Primiparous	42 (51.2 %)	42 (56.0 %)	74 (51.4%)	41 (47.7%)	.
Prepregnancy BMI (kg/m2)	33.2 (31.6-35.8)	33.0 (32.0-36.9)	33.6 (31.7-37.0)	22.1 (20.7-23.4)	.
Smoking in pregnancy	4 (4.9 %)	7 (9.3 %)	17 (11.8%)	10 (10.5%)	.
School ≥ 12 years	62 (75.6%)	48 (64.0%)	96 (66.7%)	97 (100.0%)	.
Further education ≥ 3 years	42 (51.2%)	33 (44.0%)	66 (45.8%)	85 (87.6%)	.
Gainfully employed	54 (65.9%)	55 (76.4%)	97 (67.4%)	78 (80.4)	.
Gestational weight gain	7.1 (5.2-10.8)	8.8 (5.9-11.4)	7.5 (4.4-10.8)	15.0 (12.0-19.0)	6/3/2/2
75-g OGTT at 28 weeks of gestation:					
Fasting plasma glucose (mmol/L)	4.8 (4.5-5.1)	4.9 (4.5-5.1)	4.7 (4.6-5.1)	.	10/1/13/100
2-h plasma glucose (mmol/L)	6.1 (5.4-7.2)	6.1 (5.4-6.9)	6.6 (5.6-7.5)	.	15/7/19/100
Neonatal					
Sex, female/male	41/ 41	33/42	66/78	47/50	.
Gestational age at birth (days)	282 (274-289)	284 (275-288)	283 (273-289)	282 (277-286)	.
GA < 37+0	5 (6.2%)	2 (2.3%)	6 (4.2%)	.	.
Birth weight (g)	3720 (3382-4070)	3520 (3330-3900)	3745 (3460-4040)	3548 (3212-3864)	.
Birth weight Z-score	0.23 (-0.46-0.91)	0.01 (-0.69-0.75)	0.20 (-0.40-0.81)	-0.03 (-0.67-0.68)	.
BAC (cm)	34.0 (32.0-35.0)	34.0 (32.0-35.0)	34.0 (33.0-36.0)	34 (33-35)	4/3/7/0
BAC Z-score	0.25 (-0.53-0.65)	0.06 (-0.54-0.58)	0.27 (-0.55-0.66)	0.11 (-0.37-0.65)	4/3/7/0
Birth length (cm)	53.0 (51.0-54.0)	52.0 (51.0-54.0)	53.0 (51.0-54.0)	53.0 (51.0-54.0)	.
Exclusively breastfed	36 (45.0%)	36 (48.0%)	.	52 (54.7%)	2/0/1002
Exclusively breastfed for at least five months	20 (25.0%)	26 (35.6%)	.	20 (20.6%)	2/0/100/2
Weight development 0-5 months (change in weight Z-score)	0.33 (-0.64-0.96)	0.49 (-0.36-1.42)	.	0.72 (0.03-1.36)	12/13/100/16

Data are given as median (interquartile range) or frequency

Since we had several missing values on DEXA derived and metabolic outcomes, we subsequently compared our primary outcome, BMI Z-score, between participants with information on all outcomes (n = 81) and participants with at least one missing value (n = 173) in order to investigate the possibility of bias. There was no difference in BMI Z-score between these two groups; mean BMI Z-score in participants with information on all outcomes vs. participants with missing values (-0.12 vs. -0.12, p=0.994).

Table 8
Anthropometric outcomes and body composition according to LiP intervention and external reference groups

	LiP Offspring		External reference group
	Intervention (LiPi) n=82	Control (LiPc) n=75	Children born to lean mothers (ER) n=97
BMI Z-score	0.10 (-0.58-0.69)	-0.09 (-0.87-0.50)	-0.32 (-0.75-0.40)
Weight (kg)	14.5 (13.5-15.6)	14.2 (13.4-15.8)	14.5 (13.4-15.4)
Height (cm)	95.0 (92.5-96.5)	94.0 (92.5-97.2)	95 (92.1-97.0)
BMI (kg/m2)	16.3 (15.6-17.2)	16.1 (15.4-17.0)	15.9 (15.4-16.8)
Overweight or obese	9 (10.9%)	5 (6.7%)	4 (4.1%)
AC (cm)	48.2 (46.8-51.3)	48.0 (45.9-50.3)	48.0 (46.0-49.8)
Hip (cm)	51.0 (49.0-53.0)	50.0 (45.9-50.3)	50.3 (48.3-52.3)
AC/hip ratio	0.97 (0.92-0.98)	0.97 (0.93-0.99)	0.96 (0.93-0.98)
Triceps skinfold (mm)	8.2 (7.1-9.8)	8.3 (6.7-9.8)	8.0 (7.1-9.4)
Subscapular skinfold (mm)	6.0 (5.4-6.4)	5.9 (5.1-6.8)	5.7 (5.0-6.7)
DEXA scan	n= 37	n=30	n=56
Total fat (g)	2354 (1719-2770)	2437 (1996-2964)	2110 (1697-2953)
Lean body mass (g)	11 923 (10 415-12 143)	11 250 (10 504-11 947)	10 987 (10 095-11 547)
Total fat (%)	20.9 (14.6-24.8)	21.5 (18.6-24.6)	20.0 (15.7-26.7)

Data are given as median (interquartile range) or frequency. At a significance level of 0.05 (two-sided), there were no statistically significant differences in any variables between the LiPi and LiPc groups or between LiPi, LiPc and ER groups. AC; abdominal circumference, DEXA; Dual Energy X-ray.

Table 9
Metabolic risk factors in children born at term according to LiP intervention and external reference groups

	LiP Offspring		External reference group
	Intervention (LiPi) n=77	Control (LiPc) n=73	Children born to lean mothers (ER) n=97
AC (cm)	48.2 (46.8-52)	48.0 (46.1-50.3)	48.0 (46-49.8)
Systolic blood pressure (mmHg)	98.3 (93.7-105.3)	97.3 (94.3-101.3)	97.0 (91.7-103.7)
Diastolic blood pressure (mmHg)	64.3 (61.0-67.3)	62.0 (60.3-65.3)	63.3 (60.3-68.0)
Fasting blood glucose (mmol/L)	5.2 (4.6-5.6)	5.1 (4.7-5.5)	5.0 (4.7-5.4)
Fasting insulin (mU/mL)	16 (8-33)	12 (8-18)	11 (8-15)
Fasting HDL (mmol/L)	1.2 (1.1-1.4)	1.3 (1.1-1.5)	1.2 (1.0-1.4)
Fasting triglycerides (mmol/L)	0.7 (0.6-1.1)	0.9 (0.6-1.0)	0.7 (0.5-1.0)

Data are given as median (interquartile range) or frequency. At a significance level of 0.05 (two-sided), there were no statistically significant differences in any variables between the LiPi and LiPc or between LiPi, LiPc or ER groups. AC; Abdominal Circumference, HDL; High Density Lipoprotein

Difference in size at birth between offspring of women participating in the LiP study and a matched control group from the background population

As described above, LiP intervention group offspring had a larger median birth weight than control group offspring, despite a lower GWG in the intervention group. This was a surprising result, for which we have no explanation. Other outcomes were comparable between groups, and GWG was quite low in both groups. We therefore speculated that all women in the LiP study had benefited from being in the trial, regardless of the randomization, and that the children were smaller at birth than offspring of obese women in the background population. In order to test this hypothesis, we needed an extra control group of obese women not participating in the trial. Unfortunately, we were not given permission by the ethics committee to investigate the outcomes of eligible patients who had declined to participate, which would have been the ideal control group. We were, however, given permission to use the Danish Medical Birth Registry to match obese mothers who participated in the trial to a comparable group of mothers who did not participate, and subsequently compare the offspring size at birth between these two groups (Danish Data Protection Agency J.nr. 2013-41-2343). We therefore set up a small case-control analysis in which we matched mothers from the LiP study (cases) with offspring of obese mothers not participating in the trial (controls).

Table 10

Associations between weight and abdominal circumference at birth and metabolic risk factors in children born at term.

	Birth Weight Z-score Adjusted*		Birth Abdominal Z-score Adjusted*	
Outcome	Coefficient (95% C.I.)	R2	Coefficient (95% C.I.)	R2
AC	1.56 (1.10; 2.01) †	0.35	1.23 (0.79; 1.68) †	0.29
F-plasma glucose	0.17 (0.07; 0.26) †	0.12	0.15 (0.06; 0.24) †	0.12
F-insulin	5.40 (2.47; 8.33) †	0.16	5.93 (3.14; 8.72) †	0.20
F-HDL	NS	-	NS	-
F-triglycerides	0.10 (0.02; 0.18) ‡	0.17	0.11 (0.03; 0.18) †	0.18
Systolic BP	1.92 (0.71; 3.12) †	0.18	NS	-
Diastolic BP	0.86 (0.12; 1.84) ||	0.16	NS	-

* adjusted for sex, pregestational BMI, gestational weight gain, maternal education (more than 12 years of school), parity, exclusive breastfeeding for at least 5 months, smoking during pregnancy, maternal age, gestational age at birth and postnatal weight gain. †; p<0.004, ‡; p <0.020, §; p =0.045, ||; p =0.084. Z-score; standard deviation score, C.I.; Confidence Interval, R2; Coefficient of Determination, AC; abdominal circumference, HDL; High Density Lipoprotein, BP; blood pressure.

In order not to include those who were invited into the LiP study, but declined to participate, we restricted the controls to women who had given birth in Odense University Hospital or Aarhus University Hospital three years prior to the LiP study.
Mothers were matched using the following criteria: Date of birth minus three years +/- 30 days, same fetal/child sex, same gestational age +/- 7 days, same maternal BMI category and same parity.
Controls were only used once for each case. We stratified the analyses into two sets; one with outcomes for BMI category 30-34.9 kg/m2, and one with outcomes for BMI category 35-45 kg/m2. The analyses were done in two steps; first all who had participated in the LiP trial until birth and their controls, and second all who had participated in the LiPO follow-up and their controls. We subsequently tested if the difference was significant using a linear regression model, which took cluster effects into account.
Out of 301 LiP participants, the registry identified 274 LiP mother and child dyads with valid information for the first set of analyses. Out of 157 LiPO follow-up attendees, the registry identified 143 dyads for the second set of analyses. Results of the analyses can be seen in Table 11 for comparison of LiP offspring with a matched control group and in Table 12 for comparison of LiP offspring attending the LiPO follow-up with a matched control group. Offspring of LiP participants had a smaller mean birth weight compared to their matched control group (3684 vs. 3722 g, p=0.021 for BMI category 30-34.9 kg/m2, and 3649 vs. 3869 g, p=0.004 for BMI category 35-45 kg/m2), Table 11. Mean abdominal circumference was also significantly smaller in LiP offspring compared to controls in BMI category 35-45 kg/m2 (33.8 vs. 34.6 cm, p=0.007), whereas the difference was not significant for BMI category 30-34.9 kg/m2, Table 11.

Table 11

Birth weight and abdominal circumference in LiP offspring (n=274) compared to background population of offspring of obese women

	LiP participants (Cases)	Background population (Controls)	Linear regression, coefficient (95% C.I.)
Birth weight (g)			
BMI 30-34.9 kg/m2	3684 (575), n=180	3722 (512), n=376	-93.6 (-172.9; -14.3), p=0.021
BMI 35-45 kg/m2	3649 (551), n=94	3869 (536), n=123	-219.6 (-365.8; -73.5), p=0.004
Birth abdominal circumference (cm)			
BMI 30-34.9 kg/m2	33.8 (2.3), n=170	33.8 (2.2), n=348	-0.22 (-0.54; 0.11), p=0.191
BMI 35-45 kg/m2	33.8 (2.1), n=91	34.6 (2.0), n=117	-0.8 (-1.37; -0.23), p=0.007

Data are given as means (SD) and linear regression coefficient (95% C.I.) for difference between LiP offspring and background population of offspring born to obese mothers.

For the LiP offspring, who had attended the LiPO follow-up, mean birth weight was also smaller compared to matched controls (3616 vs.3792 g, p=0.001 for BMI category 30-34.9 kg/m2, and 3582 vs. 3860 g, p=0.01 for BMI category 35-45 kg/m2), whereas no significant difference was found in abdominal circumference, Table 12. Additionally, it seems that the follow-up attendees were smaller at birth compared to the total group of LiP offspring (e.g. birth weight 3616 vs. 3684 g for BMI category 30-34.9 kg/m2).

Table 12

Birth weight and abdominal circumference in LiP offspring attending the LiPO follow-up (N=143) compared to background population of offspring of obese women

	LiP participants (Cases)	Background Population (Controls)	Linear regression, coefficient (95% C.I.)
Birth weight (g)			
BMI 30-34.9 kg/m2	3616 (629), n=98	3792 (530), n=182	-206.2 (-322.2; -90.2), p=0.001
BMI 35-45 kg/m2	3582 (597), n=45	3860 (470), n=66	-278.1 (-485.4; -70.8), p=0.01
Birth abdominal circumference (cm)			
BMI 30-34.9 kg/m2	33.4 (2.6), n=92	33.9 (2.0), n=170	-0.4 (-0.90; 0.09), p=0.107
BMI 35-45 kg/m2	33.5 (2.3), n=44	34.6 (1.9), n=63	-0.71 (-1.77; 0.36), p=0.187

Data are given as means (SD) and linear regression coefficient (95% C.I.) for difference between LiP offspring attending the LiPO follow-up and background population of offspring born to obese mothers.

Even though this small analysis suggests a difference in offspring size at birth between LiP offspring and a matched control group, it

must be interpreted with consideration. First, we used a control group, which had given birth three years prior to the LiP study. The ideal control group would have been the women who were eligible for the LiP study, but declined to participate. As we were not allowed to use this group, we had to use a historical control group of women giving birth in the same hospitals as the LiP participants instead. By doing this, we were certain not to include women who had been in contact with the LiP study, and the control group was likely composed of women with the same social background, as they were from the same living area. Second, we did not have thorough information on all exclusion criteria as listed in the LiP study. This means that we did not exclude women with diabetes or other chronic diseases, which might have resulted in larger offspring in the control group. Third, we did not match on smoking status, as this would have resulted in difficulties in retrieving enough matches. However, the number of smokers in the obese background population is believed to be similar to the numbers of smokers in the obese women participating in the LiP study, limiting bias on this account. Fourth, a number of mother and child dyads from the LiP study did not have valid information on all variables in the Danish Medical Birth Registry. This meant that not all participants could be used as cases.

Nevertheless, from this small case-control analysis, we found that LiP offspring in general were smaller in birth weight and abdominal circumference compared to their matched control group. This support our hypothesis that women across groups in the LiP study had benefited from being in the trial, and that the children were smaller at birth than we could have expected based on their maternal BMI alone.

DISCUSSION

In this follow-up of a randomized controlled trial involving lifestyle intervention during pregnancy, we have demonstrated that the lifestyle intervention did not result in improved anthropometric, body composition or metabolic outcomes in the offspring. We also found that offspring of obese mothers participating in the trial were comparable to the external reference group of children born to lean mothers, and that both abdominal circumference and weight at birth are predictors of later metabolic profile. Discussions about details in each of the two components of the LiPO study are found in the respective papers. The following discussion regards the overall issues of the LiPO study.

METHODOLOGICAL CONSIDERATIONS

Study design

The study was designed as a follow-up of a randomized controlled trial, with the addition of an external reference group which was recruited after pregnancy. In the preceding trial, the participants and health care professionals were not blinded. However, in the follow-up, the evaluating doctor was blinded to the intervention groups during assessment of the outcomes. According to the flowchart for participation (Figure 6), the participating women in the LiP study were a selected group of obese pregnant women. Among eligible obese women more than 40% were primarily excluded due to the strict criteria. Among those fulfilling all criteria and invited for participation, a number of 317 women declined. The women´s participation in the study was based on the willingness to change lifestyle during pregnancy. Hence, women in the control group were as motivated as those in the intervention group and many of the women also expressed their disappointment about being randomized to the control group. Women in the control group were seen more often during pregnancy than obese women not included in the study and they received the same information about purpose and content as the intervention group, including access to a website with advice on healthy lifestyle in pregnancy. Study visits during pregnancy included repeated measures of maternal weight, blood pressure and OGTT, and a physical step test. The results of these measures might have been an eye-opener to many women and it is likely that behavior have changed towards a healthier lifestyle. Such cross-over from the control group could explain some of the null results between the randomization groups. Therefore, the control group might have acted more as a "passive intervention group" rather than an "unspoiled" control group. This might also explain the general lower birth weight in Lip offspring compared to the background population, as estimated from our case-control analyses.

The external reference group was recruited from lean women. Unfortunately, we had no information on glucose levels during pregnancy in this group and background information was collected retrospectively, and GWG was based on self-reported values. Furthermore, breastfeeding data was based on the postal questionnaires answered two years postpartum. Especially the self-reported GWG and breastfeeding data might be submitted to faulty recall. Women in the ER group had higher educational levels compared to LiP participants, but this might partly be due to the difference in data collection (two years postpartum for the ER group, compared to during early pregnancy for LiP groups). Nevertheless, the ER group might also represent a selected, well-educated group willing to participate in a clinical exam involving blood samples and DEXA scans of the children. However, the purpose of this group was to serve as a normal reference, not to study the effects of GWG, breastfeeding, glucose values during pregnancy, socioeconomic status or other potential factors on offspring adiposity.

Study power

The study was powered to detect a difference in BMI Z-score between LiPi and LiPc groups if we had achieved an attendance rate of 53% (160 individuals). We almost met our aim with an attendance rate of 52.2%, which is comparable to other follow-up studies [234, 235]. But the power calculation used to calculate this number was based on the expectation of a large difference in BMI Z-score (1SD), which in hindsight probably was unrealistic given the small difference in GWG in the LiP study. However, as the LiPO follow-up was planned while the LiP study was still running, and as we had no previous studies on which to base power calculation, we had no better option. Accordingly, a larger number of follow-up participants might have provided more reliable information.

Internal and external validity

The lost to follow-up group from the LiP study was characterized by having higher 2-h plasma glucose values from OGTT performed at 28 weeks gestation compared to the follow-up attendees. This is a potential weakness, as the higher levels of glucose might increase offspring adiposity in the lost to follow-up group. However, in all other maternal baseline characteristics follow-up attendees were comparable to the lost to follow-up group.

The women who entered the Lip study were all very motivated to change lifestyle and they were highly selected as suggested above. This is making it difficult to extrapolate our results to the general population.

Information bias

The LiP groups were followed throughout pregnancy and the background information for this group is not likely to have been biased, as most outcomes were measured by trained health care

professionals. However, the information on breastfeeding might have been submitted to faulty recall. In order to limit this problem, the information on breastfeeding patterns were predominantly taken from interviews which were part of the six months postpartum visit. However, if the participants had not attended this visit, information from the 12 months postpartum questionnaire was used. The background information for the ER group is much more problematic, as this relies heavily on non-validated questionnaires performed 24 months postpartum or information gathered at the study visit. Accordingly, the self-reported GWG and breastfeeding data might be the subject of faulty recall.
The information on weight and height measures at 5 and 12 months of age was gathered from general practitioners for all participants. And even though some measurement errors could exist, it is unlikely that a systematic bias is present regarding these measures.
To reduce the risk of information bias in study visit outcomes, anthropometric measures were performed after standardized guidelines in triplicates and were subsequently averaged. The DEXA scan is a highly validated tool to measure body composition, and great efforts to maximize the quality and reproducibility of the scans was made. All exams were performed by the same person, eliminating inter-observer variance, whereas some intra-observer variance might have been present. However, as the examiner was blinded to the LiPi and LiPc group we expect potential misclassification to be primarily non-differential. On the other hand, as the examiner was not blinded to the ER group (due to obvious differences in maternal physical status), this might have biased the results.
Unfortunately, we had several missing values both for variables from the DEXA scans and from the blood sampling. Due to logistical challenges we were only able to perform DEXA scans in one of the centers (Odense University Hospital), and not all children were able to lie still during the scan procedure. When performing the blood sampling, we chose to stop the procedure, if the child got upset. This was due to an ethical consideration, as we found it unethical to frighten the child unnecessarily, which might have made it difficult for the child to participate in future more important examinations (for instance if the child was hospitalized later due to illness). Some mothers also refused to let their children participate in the blood sampling, making the numbers even smaller. The result of the several missing values is of course a weakness in our data, and some information might have been hidden due to this. However, our post hoc analyses did not suggest that there was a difference in our primary outcome, BMI Z-score, between the participants with information on all outcomes and those with minimum one missing value. We therefore do not believe that a systematic bias is present on this account.

Confounding factors

The study contains information on many different potential confounders and mediators. When reporting a RCT, mediators and confounders are likely to be distributed equally amongst groups, making adjustment for them unnecessary. However, as we also introduced an extra group (the ER group), which was not part of the original RCT, we had to take measures to make adjustments. We did this on our primary outcome, BMI Z-score, and found that adjustment for known potential confounders did not alter the conclusion; that no difference was present between the three groups (LiPi, LiPc and ER). In the analyses on the associations between size at birth and metabolic risk factors in study III, adjusting for confounders also did not change the conclusions. Nevertheless, residual confounding is always potentially present.

INTERPRETATION OF RESULTS

Overall, there were no differences between the LiPi and LiPc groups, which could imply that the lifestyle intervention during pregnancy had no effect on the offspring. There may be several explanations for this null result. First of all, it may reflect the limited difference in GWG between the randomized groups and the generally low GWG in both groups [231], as well as the lower birth weight compared to the background population. Secondly, it may reflect the timing of intervention with inclusion of women between 10 and 14 weeks of gestation. Previous studies have suggested that only weight gain in the first trimester of pregnancy is associated with increased offspring adiposity [113, 155]. This means that our intervention during pregnancy might have been initiated too late to confer long-term benefits for the offspring. Thirdly, our follow-up study might have been conducted too early in childhood to detect differences between groups.
We have no similar large offspring RCT follow-up studies to compare our results with. A pilot study including questionnaire data on 72 offspring of mothers from an intervention trial, where the intervention group received advice on physical activity and diet, found no effect on weight development from 0-4 years [223]. However, the mothers were mainly lean, making comparisons with our study difficult. In another trial, women with mild GDM between 24 and 34 weeks gestation were allocated to dietary advice, blood glucose monitoring and insulin therapy if necessary, or to routine control. Despite a reduction in the prevalence of macrosomia at birth, no difference was seen in child BMI Z-score at the age of 4-5 years [234]. Finally, a trial aiming to improve lifestyle in women at risk of developing GDM, followed the offspring until the age of one year. Again, no effect of the intervention was seen in the offspring [224]. This is raising general doubts about the effectiveness of pregnancy interventions in preventing childhood obesity. However, it is also possible that the effects of intrauterine over-nutrition are hard to detect early in childhood, and may predominantly appear later in childhood. This hypothesis is supported by studies of offspring exposed to maternal diabetes. Silverman et al. found that children of diabetic mothers were heavier than controls at birth, but not at ages 1,2 or 3 years. However, when the children turned school age, the difference in weight reappeared [236]. Also studies on the Pima Indians found that GDM influenced offspring BMI from the age of nine years, but not earlier [54]. Similarly, in Indian children, the effects of maternal diabetes on offspring metabolic profile and adiposity was stronger at age 9.5 than at age 5 [72]. Furthermore, in the Exploring Perinatal Outcomes among Children (EPOCH) study, the effect of exposure to gestational diabetes became apparent around the child´s age of ten, where exposed children started to have a higher BMI growth velocity compared to controls [237], whereas no difference in growth trajectory was seen in infancy or early childhood. Even though these studies have been conducted in offspring exposed to diabetes during pregnancy, a similar pattern might be seen for children exposed to over-nutrition caused by obesity or high glucose levels below the threshold of diabetes diagnosis.
Thus, it seems to be too soon to conclude that maternal lifestyle intervention during pregnancy cannot improve offspring health. In our study, LiP offspring were comparable in all outcomes assessed in early childhood to the external reference group of children born to lean mothers. This was not expected on the basis of the many observational studies linking maternal obesity with offspring BMI, obesity or metabolic risk factors [78, 94, 112]. In our study, only 9% of the children of obese mothers were classified as overweight or obese at the age of 2.8 years, which is com-

parable to the Danish background population [5]. This is in support of the hypothesis that participating mothers in the LiP study represented a selected health-promotion motivated group of obese women, and that the children had benefited from their mothers voluntarily participation in the LiP study, regardless of the randomization. From our small case-control analysis we found that offspring of LiP participants were smaller at birth compared to a matched control group of obese women from the background population, which further supports our hypothesis.

Associations between size at birth and metabolic risk factors in early childhood

We found that both BW and BAC Z-score were associated with metabolic risk factors in early childhood. In general, BAC reflects both abdominal visceral size and fat stores. However, liver size is a main determinant of BAC [238, 239], and increases in intrahepatic lipid content can result in larger liver size, which in turn leads to increased BAC. In children, abdominal circumference is an indicator of MS [240, 241], whereas less is known about the predictive value of intrahepatic lipid content. We speculated that excessive abdominal fat deposition, both in the liver and other visceral compartments, as well as weight at birth are early predictors of later metabolic risk factors. BW and BAC Z-score showed similar associations with AC, fasting plasma glucose, fasting insulin and fasting triglycerides at the age of 2.8 years, and BW Z-score was additionally associated with systolic blood pressure, after correction for confounding factors. Whereas the predictive value of BW is well established, we believe we are the first to show similar associations between BAC and metabolic outcomes. We could, however, not determine if BAC is an even better predictor than BW, and one reason for this might be the difference in accuracy of the measurement methods, as discussed in the discussion section regarding results from paper I. In order to come closer to an answer on the importance of abdominal adiposity contra weight at birth, more refined methods such as Dual Energy X-ray scans might be helpful. Nevertheless, we have shown that birth abdominal size, in addition to birth weight, is a good predictor for later adverse metabolic profile.

SUMMARY OF STRENGTHS AND LIMITATIONS

The main strength of this study is the detailed examinations of mother and child dyads from the RCT in both pregnancy and early childhood. Limitations include the risk of selection bias towards a group from the LiP study who were highly motivated to improve lifestyle irrespective of the randomization. In addition, there was a low attendance rate and differences in data collection between the LiP study and the reference group, and the many missing values in the DEXA scan and blood sample outcomes.

6. Conclusions and future directions

The development of obesity comprises a complex interplay of genetic susceptibility, intrauterine and postnatal factors. There is vast evidence that maternal obesity and GWG influences the growth of the fetus as well as put offspring at risk of later obesity and associated metabolic conditions. As maternal obesity increases the size of the offspring at birth, which in turn is strongly associated with later weight development, interventions during pregnancy aiming to limit GWG and subsequently fetal excessive growth are intuitively a good tool for limiting downstream obesity.

In our studies, we have clearly demonstrated that maternal BMI is linearly associated with both birth weight and birth abdominal circumference (paper I). We also found that both weight and abdominal circumference at birth are good predictors of later metabolic profile (paper III). We could, however, not demonstrate that lifestyle intervention during pregnancy in obese women resulted in a healthier BMI or metabolic profile in the offspring (paper II and III). Taken together, the results from this thesis suggest that the size at birth is important for early childhood metabolic outcomes.

However, whether it is possible to halt the intergenerational cycle of obesity by lifestyle intervention strategies during pregnancy, cannot be determined by our studies. As discussed above, offspring from the LiP study were comparable to an external reference group of offspring of lean mothers, and furthermore it seems the LiP offspring were smaller at birth than what could have been expected on the basis of the maternal BMI. What is learned from this study is that having an optimal control group is essential to make valid conclusions. The LiP control group acted as more of a “passive intervention group” as discussed about the study design. In hindsight, it would have been optimal to have an additional “unspoiled” control group, which received a minimum of information about the study and was only seen at inclusion and end of trial.

Only two very small studies have investigated the effects of lifestyle interventions on offspring outcomes past birth [223, 224]. It is of paramount importance that larger well-designed lifestyle intervention studies investigate the effects on the offspring. Fortunately, several are on their way as described and results from them will hopefully provide new information on which ways to go in the future.

The ideal management of maternal obesity and associated risk for the offspring is prevention. Perhaps an even better starting point for intervention programs is prior to pregnancy or in between pregnancies to help women conceive at an optimal weight. Such intervention programs should also include follow-up into childhood. Alternatively, prevention strategies could be planned for infants with known high risk of obesity, e.g. children born to obese mothers, mothers who gained excessively during pregnancy or smoked during pregnancy or children with high birth weight and/or large abdominal circumference.

FUTURE RESEARCH

During the LiPO study period a large amount of data was collected. Some of these data were analyzed and presented in this thesis, but much still needs to be analyzed and interpreted. Such additional information include data on regional fat deposition and bone density as estimated from DEXA scans, X-ray scans of the children´s hands to determine bone age, vitamin D levels in early childhood and data from questionnaires (e.g. postnatal dietary information, allergic diseases and general morbidity). Furthermore, a biobank has been established and contains stored maternal blood samples from the three visits during pregnancy and cord blood from the LiP study, as well as blood samples from LiPO participants at the age of 2.8 years.

This large amount of data makes it possible for us to investigate a number of associations and outcomes. We are particularly interested in investigating effects of maternal and child vitamin D status on offspring bone density and bone age, and metabolic and anthropometric outcomes. We also have the ability to investigate epigenetic markers in the cord blood as well as in the offspring in early childhood, possible subtle effects of the lifestyle intervention, and associations with anthropometric and metabolic outcomes. Furthermore, it might be interesting to examine if there is a difference in regional fat deposition between the LiP offspring and the external reference group.

If a lifestyle intervention program can have impact on the most critical time of fetal development, as in the first trimester of pregnancy, intervention must be initiated before conception. An

obvious potential target would be obese women planning to become pregnant, for instance women with one previous pregnancy that could be recruited in the postpartum period. Such an intervention study including follow-up of the offspring would be interesting to conduct with all the experience and knowledge that we have obtained by performing the LiP and LiPO studies.
In our study, we concluded that abdominal circumference at birth (BAC) was a good predictor of later metabolic profile. In order to further investigate this finding, it would be interesting to compare twins discordant for BAC and follow-up them up during childhood to see if the associations were independent of genetic factors. This is possible using the Danish Twin Registry, and such a study might provide valuable information.

SUMMARY

Worldwide, the prevalence of obesity has reached epidemic proportions. In Denmark one third of all pregnant women are overweight and 12 % are obese. Perhaps even more concerning, a dramatic rise in the prevalence of childhood overweight and obesity has also been evident over recent decades. The obesity epidemic is not simply a consequence of poor diet or sedentary lifestyles. Obesity is a multifactorial condition in which environmental, biological and genetic factors all play essential roles. The Developmental Origins of Health and Disease (DoHaD) hypothesis has highlighted the link between prenatal, perinatal and early postnatal exposure to certain environmental factors and subsequent development of obesity and non-communicable diseases. Maternal obesity and excessive gestational weight gain, resulting in over-nutrition of the fetus, are major contributors to obesity and metabolic disturbances in the offspring. Pregnancy offers the opportunity to modify the intrauterine environment, and maternal lifestyle changes during gestation may confer health benefits to the child.
The overall aim with this PhD thesis was to study the effects of maternal obesity on offspring body size and metabolic outcomes, with special emphasis on the effects of lifestyle intervention during pregnancy. The thesis is based on a literature review, description of own studies and three original papers/manuscripts (I, II and III).
In paper I, we used data from the Danish Medical Birth Registry. The aim of this paper was to examine the impact of maternal pregestational Body Mass Index (BMI) and smoking on neonatal abdominal circumference (AC) and weight at birth and to define reference curves for birth AC and weight in offspring of healthy, non-smoking, normal weight women. Data on 366,886 singletons were extracted and analyzed using multivariate linear regressions. We found that birth AC and weight increased with increasing pregestational BMI and decreased with smoking. Reference curves were created for offspring of healthy, non-smoking mothers with normal pregestational BMI.
Paper II and III are based on an offspring follow-up of a randomized controlled trial (RCT) with 360 obese pregnant women. The intervention during pregnancy consisted of two major components: dietary advice and physical activity. The intervention resulted in a small, but significant difference in gestational weight gain compared to the control group. A number of 301 completed the trial and were eligible for the follow-up. We managed to include 157 mother and child dyads in the follow-up, which was conducted in Odense University Hospital and Aarhus University Hospital, Skejby between February 2010 and November 2012. At that time the children were in the ages 2.5-3 years. In addition to the children from the RCT, a group of 97 children born to lean mothers were included as an external reference group. The follow-up consisted of a clinical examination with anthropometric measures, DEXA scans and fasting blood samples for evaluation of metabolic outcomes.
In paper II the effect of the maternal intervention on offspring body composition and anthropometric outcomes was studied. The primary outcome was BMI Z-score and secondary outcomes were: body composition values by DEXA (fat mass, lean mass and fat percentage), BMI, percentage of overweight or obese children and skin fold thicknesses. We found no significant differences in offspring outcomes between randomized groups of the preceding RCT. Neither was any differences detected between offspring of the RCT or the external reference group born to lean mothers.
Paper III focused on the metabolic outcomes in the offspring. We additionally studied the predictive values of birth weight (BW) and birth abdominal circumference (BAC) on metabolic risk factors. We found that both BAC and BW were significantly associated with several risk factors in early childhood. All metabolic measurements in RCT offspring were similar, and no differences were detected between the RCT offspring and the external reference group of offspring of lean mothers.
Lifestyle intervention in obese pregnant women has the potential to modify the intrauterine environment and confer long-term benefits to the child. In this follow-up study, lifestyle intervention in pregnancy did not result in changes in offspring body composition or metabolic risk factors at 2.8 years. This might be due to a limited difference in gestational weight gain between follow-up attendees. When comparing offspring of obese women with offspring of normal weight mothers all outcomes were similar. We speculate that obese mothers entering a lifestyle intervention RCT regardless of the intervention have a high motivation to focus on healthy lifestyle during pregnancy, which makes it difficult to determine the effects of the randomized lifestyle intervention compared to an unselected control group of obese women. Our studies (paper I and III) on birth abdominal circumference show that abdominal size at birth is a good predictor of later adverse metabolic profile. Abdominal circumference at birth may reflect visceral adiposity and this measurement together with birth weight are strongly associated to later adverse metabolic outcome. Future studies should be performed in other populations to confirm this.

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