Examining the link between maternal nutrition, gestational weight gain, and later offspring health

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Academic Dissertation

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"The first wealth is health"

Ralph Waldo Emerson

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Abstract

Aim: The aim of this Ph.D. thesis is to enhance understanding of the relation between maternal diet, gestational weight gain (GWG), and offspring's cardio-metabolic factors. Moreover, the objective is to identify dietary predictors for excessive GWG.

Methods: This thesis comprises four research papers from two different prospective observational cohorts. Papers I-III were based on data from the observational Danish Fetal Origins Cohort (DaFO88), which was established in 1988-1989 in Aarhus (n=965), with a 20-year follow up. On the other hand, paper IV was based on recently collected data from an Icelandic pregnancy cohort, PREgnant Women of ICEland (PREWICE) (n=2113). Offspring of mothers in the DaFO88 cohort participated in a clinical examination at 20 years of age (n=434). The follow-up data included: anthropometry, blood pressure measurements, and cardiovascular biomarkers. Information regarding the maternal diet was collected in gestational week 30 with an FFQ combined with a dietary interview. Maternal markers of inflammation were also quantified in serum from week 30. Taking advantage of epidemiological findings can be difficult in clinical practice, partially because the dietary assessments used can be very detailed and time-consuming. However, the data from the Icelandic PREWICE study was collected using a short dietary screening questionnaire (40 items) to get a snapshot of the participant's general diet (weeks 11-14 of gestation). Information on maternal weight measurements and birth outcomes was retrieved from maternal records. Data on covariates were obtained from questionnaires and maternal records. Associations were assessed using multivariable linear, Poisson log-linear and logistic regression models.

Results: In the first phase of the project, the DaFO88 cohort was used to examine the relationship between maternal GWG and diet with offspring long-term outcomes. **Paper I:** In adjusted models, GWG was relatively strongly associated with offspring leptin and insulin levels 20 years later. Hence, a 1-kg increase in GWG was associated with 3.7% (95% CI: 1.4, 6.2) higher insulin and 10.7% (95% CI: 5.7, 15.9) higher leptin levels in male offspring. However, GWG was inversely associated with levels of total cholesterol and low-density

lipoprotein (LDL) levels among female offspring. Differences in lifestyle habits may account for these sex differences. **Paper II**: Higher intake of protein during pregnancy, at the expense of carbohydrates, was associated with slightly higher offspring diastolic blood pressure (highest compared to the lowest quintile of protein intake: Δ=2.4 mm Hg; 95% CI: 0.4, 4.4; p=0.03 for trend). Similar differences, although not significant, were found for systolic blood pressure. **Paper III:** In cross-sectional analysis, both excessive GWG and high maternal protein intake were associated with higher concentrations of inflammatory factors during pregnancy. Each 1-kg increase in GWG was associated with 3% (95% CI: 1, 5) higher C-reactive protein (CRP) and 3% (95% CI: 1, 4) higher serum amyloid A (SAA) concentrations, which corresponded to an increase of ~18% to 25% in these inflammatory factors among women with excessive weight gain in pregnancy. With respect to diet, women in the highest compared to the lowest quintile of protein intake had 26% (95% CI: 3, 54) higher CRP concentrations. Intake of animal protein appeared to drive this increase. **Paper IV:** The results from the PREWICE study showed that a dietary risk score (range 0-5), characterized by a non-varied diet, inadequate fruits/vegetables, milk and whole grain intake, as well as excessive intake of sugar/artificially sweetened beverages and dairy, was associated with excessive GWG and macrosomia. Women with poor dietary habits, i.e., with a high dietary risk score (≥4 scores), had higher risk of excessive GWG (RR=1.24; 95% CI: 1.01, 1.52) and higher odds of delivering macrosomic offspring (OR=2.28; 95% CI: 1.18, 4.38), compared to women with the lowest scores (≤2 scores).

Conclusion: The results indicate that maternal diet and GWG may affect levels of inflammatory factors during pregnancy and offspring cardio-metabolic factors at young adult age. Although the observed associations with offspring's long-term outcomes were modest, we cannot exclude the possibility that these modest shifts may become more apparent later in life. The results from the PREWICE cohort also indicate that by asking simple questions about women's dietary habits early in pregnancy, we may be able to identify women in more need of support and counseling to meet the GWG recommendations and find women at higher risk of giving birth to a macrosomic infant. Together, the studies highlight the importance of balanced diet and weight gain during pregnancy in terms of short- and long-term health.

Keywords: Gestational weight gain, maternal diet, inflammation, cardiometabolic factors, dietary screening.

Ritgerð fyrir doktorsvörn í næringarfræði við Háskóla Íslands 2018

Ágrip

Markmið: Markmið þessa doktorsverkefnis er að bæta vísindalega þekkingu hvað varðar tengsl næringar verðandi móður, þyngdaraukningar á meðgöngu og efnaskiptaþátta barna. Enn fremur er markmiðið að greina fæðuþætti sem hafa forspárgildi þegar kemur að óhóflegri þyngdaraukningu á meðgöngu.

Aðferðir: Þessi ritgerð er byggð á fjórum vísindagreinum þar sem notast var við tvær ólíkar framvirkar ferilrannsóknir. Greinar I-III voru byggðar á gögnum frá áhorfsrannsókninni Danish Fetal Origins Cohort (DaFO88), sem hófst 1988-1989 í Árósum (n=965) og var með 20 ára eftirfylgni. Grein IV var aftur á móti byggð á nýlegum gögnum frá íslensku þýði, þ.e. PREgnant Women of ICEland (PREWICE) (n=2113). Börn mæðra í DaFO88 þýðinu tóku þátt í klínískri skoðun við 20 ára aldur. Upplýsingum var þá safnað um líkams-samsetningu, blóðþrýsting og lífvísa sem tengjast hjarta- og æðakerfinu. Fæðuval mæðranna var metið á 30. viku meðgöngu, bæði með fæðutíðnispurningalista og viðtali um fæðuvenjur. Lífvísar fyrir bólgumyndun móður voru einnig magngreindir úr sermi í 30. viku. Það getur verið erfitt að nýta niðurstöður áhorfsrannsókna í klínískum aðstæðum. Að hluta til vegna þess að aðferðir sem eru notaðar til að meta fæðuval í þessum rannsóknum eru oft mjög ítarlegar og tímafrekar. Upplýsingum um mataræði í íslensku PREWICE rannsókninni var aftur á móti safnað með því að nota stuttan skimunarlista fyrir fæðuval sem gaf mynd af almennu fæðuvali þátttakenda (á 11.-14. viku meðgöngu). Þyngdarmælingar á meðgöngu og fæðingaútkomur voru fengnar úr mæðraskrám. Upplýsingar um mögulegar skýribreytur voru fengnar úr spurningalistum og mæðraskrám. Tengsl voru metin með fjölþátta línulegri, Poisson og lógístískri aðhvarfsgreiningu.

Niðurstöður: Í fyrri hluta verkefnisins var DaFO88 þýðið nýtt til að kanna tengsl þyngdaraukningar á meðgöngu, mataræðis og langtímaútkoma hjá afkvæmum. **Grein I**: Í leiðréttu módeli var þyngdaraukning á meðgöngu tengd við leptín- og insúlíngildi afkvæma 20 árum síðar. Það er, hvert kg af þyngdaraukningu var tengt við 3,7% (95% CI: 1,4, 6,2) hærri insúlíngildi og 10,7% (95% CI: 5,7, 15,9) hærri leptíngildi hjá karlkyns afkvæmum. Neikvæð tengsl voru aftur á móti milli þyngdaraukningar á meðgöngu og kólesteróls og lágþéttni fitupróteins (low-density lipoprotein, LDL) gilda meðal kvenkyns afkvæma. Þennan kynjamun má mögulega rekja til mismunandi lífsvenja.

Grein II: Hærri próteinneysla kvenna á meðgöngu, á kostnað kolvetna, var tengd við lítillega hækkaðan hlébilsþrýsting hjá afkvæmum (hæsti fimmtungur borinn saman við þann lægsta: Δ=2,4 mmHg; 95% CI: 0,4, 4,4; p-gildi =0,03 (p for trend)). Svipaðar niðurstöður, þó ekki marktækar, fengust fyrir slagbilsþrýsting. **Grein III:** Þversniðsgreiningar bentu til þess að óhófleg þyngdaraukning og mikil próteinneysla á meðgöngu gæti mögulega ýtt undir bólgumyndun. Hvert kg af þyngdaraukningu var þannig tengt 3% (95% CI: 1, 5) hærri C-reactive protein (CRP) og 3% (95% CI: 1, 4) hærri serum amyloid A (SAA) gildum, sem samsvaraði ~18% til 25% aukningar á þessum bólguþáttum hjá þeim konum sem þyngdust óhóflega mikið á meðgöngu. Konur sem voru í hæsta fimmtungi próteinneyslu voru með 26% (95% CI: 3, 54) hærri CRP gildi miðað við konur í lægsta fimmtungi. Þessi aukning virtist stafa af neyslu dýrapróteina. **Grein IV:** Niðurstöður úr PREWICE rannsókninni sýndu að áhættufæðuskor (sem var á bilinu 0-5), sem einkenndist af lítilli fjölbreytni, ónægri ávaxta-/grænmetis, mjólkur- og heilkornaneyslu, ásamt mikilli neyslu á annars vegar drykkjum sem innihalda sykur/sætuefni og hins vegar mjólkurneyslu, var tengt við aukna áhættu á þyngdaraukningu umfram ráðleggingar og auknum líkum á þungburafæðingum. Konur með óheilsusamlegasta mataræðið, það er með hátt áhættufæðuskor (≥4 stig), voru þannig í aukinni áhættu á þyngdaraukningu umfram ráðleggingar (RR=1,24; 95% CI: 1,01, 1,52) og auknar líkur voru á þungburafæðingum (OR=2,28; 95% CI: 1,18, 4,38) meðal þeirra miðað við konur með lægstu áhættufæðuskorin (≤2 stig).

Ályktun: Niðurstöðurnar benda til þess að næring kvenna á meðgöngu og þyngdaraukning umfram ráðleggingar geti mögulega haft áhrif á gildi bólguþátta á meðgöngu og efnaskiptaþætti barna snemma á fullorðinsaldri. Þrátt fyrir að tengsl við langtímaútkomur barna væru hófleg, er ekki hægt að útiloka að þessar lítilvægu breytingar geti orðið greinilegri síðar á ævinni. Niðurstöðurnar frá PREWICE þýðinu benda einnig til þess að með því að spyrja einfaldra spurninga um fæðuval í upphafi meðgöngu, sé mögulega unnt að finna konur sem þurfa aukinn stuðning og ráðgjöf til að þyngjast í samræmi við ráðleggingar og finna konur í aukinni áhættu á því að fæða þungbura. Saman undirstrika rannsóknirnar mikilvægi jafnvægis þegar kemur að mataræði og þyngdaraukningu á meðgöngu í tengslum við heilsufar, bæði til skemmri og lengri tíma.

Lykilorð: Þyngdaraukning á meðgöngu, næring á meðgöngu, bólga, efnaskiptaþættir, skimun á mataræði.

Contents

List of abbreviations

11β-HSD2	11-beta-hydroxysteroid dehydrogenase 2
AGEs	Advanced glycation end products
ALSPAC	Avon Longitudinal Study of Parents and Children
BMI	Body mass index
CI	Confidence intervals
CRP	C-reactive protein
DaFO88	Danish Fetal Origins Cohort
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DNBC	Danish National Birth Cohort
DOHaD	Developmental Origins of Health and Disease
FFQ	Food Frequency Questionnaire
FIGO	The International Federation of Gynecology and Obstetrics
GDM	Gestational diabetes
GWG	Gestational weight gain
GWG30	Gestational weight gain in week 30
HDL	High-density lipoprotein
HPA	Hypothalamic–pituitary–adrenal
hsCRP	High-sensitivity C-reactive protein
IL	Interleukin
IOM	Institute of Medicine
IGF	Insulin-like growth factor
IGFBP	The insulin-like growth factor-binding protein
IQR	Interquartile range
LDL	Low-density lipoprotein
LGA	Large for gestational age
MoBa	The Norwegian Mother and Child Cohort Study

NCD	Noncommunicable diseases
OR	Odds ratios
PREWICE	PREgnant Women of ICEland
PPWR	Postpartum weight retention
RR	Relative risk
SBP	Systolic blood pressure
SD	Standard deviation
SGA	Small for gestational age
TNF	Tumor necrosis factor
WC	Waist circumference
WHO	World Health Organization

List of figures

List of tables

List of original papers

The thesis is based on the following research papers, which will be referred to in the text by their respective Roman numerals:

I   **Title**: Gestational weight gain in normal weight women and offspring cardio-metabolic risk factors at 20 years of age.
Authors: Hrolfsdottir L, Rytter D, Olsen SF, Bech BH, Maslova E, Henriksen TB, Halldorsson TI.
Status: Int J Obes (Lond) 2015; 39 (4):671-676. doi: 10.1038/ijo.2014. 179

II   **Title**: Maternal Macronutrient Intake and Offspring Blood Pressure 20 Years Later.
Authors: Hrolfsdottir, L., T.I. Halldorsson, D. Rytter, B.H. Bech, B.E. Birgisdottir, I. Gunnarsdottir, C. Granstrom, T.B. Henriksen, S.F. Olsen, and E. Maslova.
Status: J Am Heart Assoc, 2017; 6(4). Doi: 10.1161/JAHA.117. 005808

III   **Title**: Maternal diet, gestational weight gain, and inflammatory markers during pregnancy.
Authors: Hrolfsdottir L, Schalkwijk CG, Birgisdottir BE, Gunnarsdottir I, Maslova E, Granstrom C, Strom M, Olsen SF, Halldorsson TI.
Status: Obesity (Silver Spring) 2016; 24(10): 2133-9. doi: 10.1002/ oby.21617

IV   **Title**: Development of a dietary screening questionnaire to predict excessive weight gain in pregnancy.
Authors: Hrolfsdottir L, Halldorsson TI, Birgisdottir BE, Hreidarsdottir IT, Hardardottir H, Gunnarsdottir I.
Status: Manuscript submitted to Maternal & Child Nutrition.

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Declaration of contribution

The author of this thesis, Laufey Hrólfsdóttir (LH), planned the research work for papers I-IV, with guidance and feedback from her supervisors and doctoral committee.

The observational Danish Fetal Origins Cohort (DaFO88) was established in 1988-89 and the follow-up data were collected in 2008-2009. From this dataset, LH, Sjurdur F. Olsen and Þórhallur Ingi Halldórsson developed the research questions for the first three studies (papers I-III), and LH was responsible for the data interpretation.

LH was one of the principal investigators in the Icelandic study, the PREgnant Women of ICEland (PREWICE) cohort (paper IV). She worked with Ingibjörg Gunnarsdóttir on the study design and development of the research questions. She was also responsible for the data interpretation. LH was responsible for the collection of the web-based data (the dietary questionnaire and background questions). LH also participated in and guided coworkers working on the data collected from the maternal records (maternal and birth outcomes).

LH performed the statistical analyses in all the studies and prepared and drafted the data for presentation and publications. She wrote the first draft of all the manuscripts and had primary responsibility for the final content. During her studies, LH submitted abstracts and prepared and presented the results of the research projects at several international and domestic conferences.

1 Introduction

This Ph.D. thesis aims to enhance understanding of the relation between weight gain during pregnancy, maternal diet, and offspring's cardio-metabolic health. Since the theory of fetal origins of disease [1] was published by David Barker at the University of Southampton, UK and coworkers, numerous studies have shown that adverse exposures during the fetal period may influence the risk of adverse health outcomes later in life [2-4]. The term fetal "programming" has also been used to describe this link. That is, during development of the embryo and fetus, environmental conditions can reset important physiological parameters that may influence later susceptibility to chronic diseases [5]. Pre-pregnancy obesity is a strong risk factor for maternal complications during pregnancy, and an accumulating body of evidence also implicates high maternal pre-pregnancy body mass index (BMI) as a major determinant of offspring's later health [6]. However, during pregnancy, preventive health care must focus on modifiable risk factors at that time point, e.g., maternal diet and gestational weight gain (GWG).

Weight gain is an essential aspect of pregnancy, but excessive GWG, independent of pre-pregnancy BMI, is associated with maternal complications, macrosomia and postpartum weight retention (PPWR) [7-9]. Public health authorities have issued guidelines on optimal GWG. These guidelines are primarily aimed at minimizing the risk of adverse pregnancy and birth outcomes [10], but more information is needed on the role of excessive GWG in offspring long-term health. Recent epidemiological studies suggest that maternal GWG is associated with offspring's BMI later in life, with increased risk of obesity in offspring with excessive GWG [11-13]. However, the association between GWG and comorbidities of adiposity, such as weight-regulating hormones, glucose metabolism, blood lipid profile, and blood pressure, has been minimally explored [14]. In addition to weight gain, maternal diet may have an independent effect on the development of offspring metabolic health, with macronutrient composition identified as a potentially important factor [15].

Previous studies have suggested that inflammation during pregnancy may play a role in offspring programming [16]. Abnormal and untimely inflammation is also linked with pregnancy complications like preeclampsia, gestational diabetes (GDM) and preterm birth [17]. Obesity, increased adipose

tissue as well as suboptimal diets, e.g., the Western diet (characterized by high intakes of refined grains, sweets, French fries and red and processed meats), has been associated with chronic, low-grade inflammation in nongravid individuals [18, 19]. Less is known whether similar associations exist for GWG or suboptimal diets during pregnancy. Further examination of which factors may modulate inflammatory responses during pregnancy may possibly shed light on the mechanisms behind the long-term programming of offspring disease.

A substantial proportion of pregnant women do not comply with current weight gain recommendations [8, 20, 21], and recent results from Iceland and several other countries indicate that the diet of women during pregnancy is, in general, not optimal, i.e., not in line with official dietary recommendations [22-25]. Recommendations on GWG focus on the weight without offering dietary recommendations on how to lower the risk of excessive GWG. Examining this issue is of great importance to preventative measures. Moreover, it is urgent to try to identify modifiable dietary risk factors during pregnancy as this knowledge may help identify women early in pregnancy who may be at higher risk of excessive GWG and adverse pregnancy outcomes.

To address our aims, we used two cohorts. In the first phase of the project, we analyzed data from a birth cohort in Denmark, i.e., the observational Danish Fetal Origins Cohort (DaFO88), to examine the relation between maternal GWG and macronutrient intake with offspring's long-term outcomes (n=915). However, taking advantage of epidemiological findings is difficult in clinical practice. Therefore, in later phases, we collected new data in Iceland, i.e., the PREgnant Women of ICEland (PREWICE cohort) (n=2117), where we used a short dietary screening questionnaire to assess the participants' general diet as well as collect information on GWG and pregnancy/birth outcomes. The long-term purpose is to use the Icelandic data to design a screening tool usable within the healthcare system to find women at higher risk of suboptimal diet and excessive GWG.

2 Background

2.1 Fetal programming

The prevalence of noncommunicable diseases (NCDs) and obesity has steadily increased over the past 40 years. Worldwide, obesity has more than doubled since 1980, but recent World Health Organization (WHO) global estimates indicate that in 2014, 39% of adults were overweight, and an additional 13% were obese. Overweight and obesity are one of the major risk factors for a number of NCDs like cardiovascular diseases, diabetes, and some types of cancer [26]. This increase in weight is, therefore, a significant concern in terms of global public health and public health spending. Evidence suggests that the susceptibility to gain weight and develop NCDs may be markedly influenced during fetal development and infancy. This period may, therefore, offer important opportunities in terms of preventative measures [3].

During early life, rapid growth, development, and maturation of organs and organ systems occurs. Insults during this critical period of development may trigger long-term adaptations in organ structure, physiology, and metabolism. These effects have been termed "programming" [5]. This concept originates partly from the work of Professor David Barker and his research group. Their early findings showed that a population's neonatal measurements, including birth weight and length, were strongly related to the later incidence of cardiovascular diseases and diabetes [1, 27, 28]. Data from the 1944 Dutch famine cohort further supported these results [29]. This work was the primary evidence leading to the evolution of the Developmental Origins of Health and Disease (DOHaD) hypothesis. It is based on the concept that inadequate nutritional status during pregnancy is one of the main programming signals associated with offspring disease susceptibility. Although the initial research mainly focused on birth measurements and programming effects seen in response to fetal undernutrition [2, 29], increasing evidence indicates that exposure to maternal overnutrition also influences offspring's long-term health [30].

2.2 Recommendations regarding maternal diet

The importance of optimal nutrition early in life has been recognized for many decades, but more widespread consensus on the immense consequences of non-optimal nutrition has increased greatly the past 20 years. Good nutritional status and balanced diet during pregnancy is important for both short- and

long-term outcomes, i.e., the health of the mother during pregnancy, as well as the development, growth, and health of the infant [3, 31].

Dietary patterns comprise the whole diet and provide a broader view of food and nutrient consumption, making it possible to account for synergetic effects of combinations of foods and nutrients instead of identifying individual nutrients or foods [32]. Recent results from the Norwegian MoBa (The Norwegian Mother and Child) study, the Danish DNBC (Danish National Birth Cohort) study and other observational cohorts have suggested that those adhering to a dietary pattern during pregnancy based on consuming fruits, vegetables, fish, and unsaturated fat, while at the same time consuming less food with little nutritional value, have a decreased risk of preeclampsia, preterm birth, and various other pregnancy and birth outcomes, compared to those not adhering to such a pattern [33-37].

Current guidelines from public health authorities recommend that all women, regardless of BMI, should be given information and subsequent advice on diet early in pregnancy [38-42]. The emphasis should be on eating a varied nutrient-dense diet, including fruits, vegetables, whole grain, regular fish intake, low-fat dairy products, and using oil instead of solid fats, while limiting processed foods, salt intake, and added sugar. In Iceland, nutritional guidelines call for all women of childbearing age to take folic acid supplements (400µg /day) before pregnancy and during the first 12 gestational weeks, and to add D-vitamin (RDA:15 µg/day) from cod liver oil or supplements [41, 43].

In addition to general recommendations, the International Federation of Gynecology and Obstetrics (FIGO) has recently issued guidelines [44] highlighting the need to recognize and counsel women with maternal micronutrient deficiencies during pregnancy, especially regarding nutrients important for fetal development. These include calcium, iron, iodine, folate, vitamin B12 and vitamin D. FIGO's guidelines called for public health measures to improve nutrition education as women's dietary pattern and nutritional status can influence clinically important pregnancy outcomes [44]. A recent Icelandic study [22], using four-day weighed food diaries to estimate food and nutrient intake, found that only about 20% of the participants had the recommended minimum intake of fiber, and sugar intake amounted to about 12% of total energy. Moreover, about a quarter of the women were found to be at risk of not meeting the need for important nutrients for fetal development, such as iodine, iron, vitamin D and docosahexaenoic acid (DHA) [22]. These results are in accordance with results from other countries [23-25], and they highlight the need for strategies to optimize nutritional status during pregnancy.

2.3 Recommendations regarding gestational weight gain

2.3.1 Why do women gain weight during pregnancy?

Weight gain is a normal part of biologic processes promoting fetal growth. The composition of GWG varies between women and is related to the number of fetuses. For a singleton pregnancy, this weight gain consists of ~8 kg of water, 1 kg of protein and ~1-6 kg of adipose tissue [10]. Overall, conception produces about 35% of GWG, including the fetus, placenta, and amniotic fluid, but the remaining GWG is maternal (e.g., increased blood, extracellular fluid and maternal tissues). To a considerable extent, weight gain in the first two trimesters reflects maternal components of gain: the fat depots accumulate relatively constantly from the start of pregnancy to the late second trimester when the rate of accumulation flattens. On the other hand, GWG in the third trimester largely reflects other factors, such as the growth of the fetus, placenta, and uterus, as well as fluid retention [10, 45, 46].

2.3.2 What is "optimal" weight gain during pregnancy?

Although gaining weight is an essential aspect of pregnancy, ideal weight gain or weight gain range has long been debated. It is important to balance this weight gain, so that it is sufficient for optimal growth of the offspring without jeopardizing the health of the mother, both for the short- and long-term outcomes. The US Institute of Medicine (IOM) published its recommendations for healthy GWG in 1990 [47]. They were updated in 2009, based on the increased incidence of obesity in women of reproductive age [10]. The IOM recommendations for GWG are based on weight before pregnancy. They are now 12.5-18 kg for underweight women (BMI<18.5 kg/m^2), 11.5-16 kg for normal-weight women (BMI 18.5-24.9 kg/m^2), 7-11.5 kg for overweight women (BMI 25.0-29.9 kg/m^2) and 5-9 kg for obese women (BMI≥30.0 kg/m^2) [10]. The main change in the update was a wider interval than before for obese women. Because obese women enter pregnancy with excess adipose tissue, the recommendations primarily cover the natural accrual of water and protein. In Iceland, a weight gain of 12-18 kg is recommended for normal-weight women (BMI <25) and 7-12 kg for women who are overweight or obese (BMI≥25) before pregnancy [41, 48, 49]. The reason for the broader range in the Icelandic recommendations for normal weight women than the IOM recommendations is that former studies of Icelandic women found the upper GWG limit to be higher when looking at the lowest risk of pregnancy-delivery complications [49]. Furthermore, the results of studies showed that most

women regained normal weight 18-24 months postpartum despite weight gains of up to 24 kg [48]. There has been no similar research on overweight or obese women in Iceland, and the recommended GWG is similar to the un-updated version of the IOM [47]. Table 1 shows the IOM and the Icelandic recommendations.

Table 1. The US Institute of Medicine and the Icelandic recommendations for gestational weight gain, depending on the pre-pregnancy BMI

Pre-pregnancy BMI (kg/m2) (WHO criteria)		IOM Range of total weight gain (kg)	Icelandic Range of total weight gain (kg)
Underweight	<18.5	12.5-18.0	12.1-18.0
Normal weight	18.5-24.9	11.5-16.0	12.1-18.0
Overweight	25.0-29.9	7.0-11.5	7.1-12.0
Obese	≥30.0	5.0-9.0	7.1-12.0

Abbreviations: BMI, body mass index; IOM, Institute of Medicine; WHO, World Health Organization.

The recommendations regarding maternal GWG are based on specific maternal and birth outcomes. Achieving an infant birth weight of 3000-4000 g was the primary goal, but when updating the IOM guidelines in 2009, strong associations were also found regarding the mode of delivery and PPWR [10]. To date, large cohort studies from several countries have linked non-optimal GWG with multiple maternal and offspring complications (Figure 1). A recent meta-analysis [8], covering 23 studies and more than one million pregnant women, reported that suboptimal GWG was associated with the birth of low birth weight infants, small for gestational age (SGA) and preterm births, whereas excessive GWG was linked with birth outcomes like cesarean sections, large for gestational age infants (LGA) and macrosomia. Greater risks of maternal outcomes like GDM and hypertensive orders have also been associated with excessive GWG [7, 50, 51]. The risk of high weight gain may vary by trimesters, with recent findings showing the strongest effects for high weight gain during the first trimester [7, 50].

Excessive GWG has been associated with short- and long-term outcomes

Maternal health: Exc GWG associated with hypertensive disorders and gestational diabetes

Long-term: Exc GWG is the strongest predictor of maternal overweight or obesity following pregnancy

Offspring long-term health: Exc GWG associated with offspring BMI and obesity later in life. Cardio-metabolic risk factors?

Birth outcomes: Exc GWG associated with caesarean section, stillbirth, miscarriage, malformation, macrosomia

Figure 1. Excessive GWG has been associated with short- and long-term outcomes

2.3.3 Sustained weight retention after pregnancy

High GWG and PPWR also appears to set the stage for future weight gain that can affect the risk of complications in subsequent pregnancies and maternal risk of obesity later in life [9, 52-54]. A recent meta-analysis of observational studies showed an association between excessive GWG and higher mean PPWR (Δ3.21kg; 95% CI: 2.79, 3.62) when compared to women with optimal GWG. Interestingly, the net change in PPWR (excessive vs. optimal GWG) declined during the first year postpartum, but increased in the periods spanning 12-36 months and ≥ 15 years postpartum [9], which indicates long-term influence on the weight gain trajectory. In terms of interpregnancy weight gain, studies have found strong associations between weight gain in-between pregnancies and pregnancy outcomes in the second pregnancy, for example, increased risk of preeclampsia, GDM, and preterm birth [55-57]. In a nationwide Swedish cohort (including more than 450,000 women) a weight

gain of more than four BMI units from first to second pregnancy was positively related to higher risk of stillbirth (RR=1.55; 95% CI: 1.23, 1.96) and infant mortality (RR=1.29; 95% CI: 1.00, 1.67) in the second-born offspring, when compared to women with a more stable BMI between pregnancies (-1 to <1 BMI units) [58]. Interestingly, the associations seem more pronounced in women of normal weight before pregnancy than in women who start off overweight or obese in their first pregnancy [55-58].

2.4 Offspring long-term outcomes

2.4.1 GWG and offspring later BMI

The recommendations of public health authorities regarding GWG aim primarily at minimizing the risk are of various adverse pregnancy and birth outcomes [10]. However, more information is needed on the role of excessive GWG on offspring long-term health. Recent meta-analyses have shown that excessive GWG during pregnancy (according to the IOM recommendations), is a potential risk factor for offspring obesity in childhood, adolescence, and adulthood, independent of maternal pre-pregnancy BMI [11-13]. The meta-analysis by Tie et al. (12 studies) showed that excessive GWG was associated with higher odds of childhood overweight (OR=1.21; 95% CI: 1.05, 1.40) [11]. Mamun included 12 studies focusing on older offspring, and the combined RR of excessive GWG and offspring obesity (age 5-18 years) was 1.40 (95% CI: 1.23, 1.59), compared to women with suboptimal GWG [12]. Participants in the studies included had a mean pre-pregnancy BMI between 21.3-26.3 kg/m^2.

A recent prospective study of more than 100,000 mother-child pairs from two provinces in southern China reported that the highest risk of childhood overweight was in children whose mothers were overweight or obese before pregnancy and had excess GWG (OR= 2.22; 95% CI: 1.79, 2.76), compared to offspring of women with normal weight and GWG in accordance with the recommendations [59]. High maternal pre-pregnancy BMI is, however, a strong independent risk factor for offspring's later overweight and obesity [60, 61], with meta-analysis reporting a three-fold higher risk of offspring childhood obesity when the mother was obese before pregnancy [62]. Maternal pre-pregnancy overweight or obesity may therefore potentially mask the potential association of GWG with offspring's later weight. Therefore, examining this relation among normal weight women can give valuable information and lowers the risk of confounding related to shared genetics and suboptimal family lifestyle [63, 64].

2.4.2 GWG and offspring cardio-metabolic biomarkers

Few observational studies have included data on offspring cardio-metabolic biomarkers, i.e., weight-regulating hormones (e.g., leptin and adiponectin), markers for sugar metabolism, blood lipid profile, and inflammatory factors. A few studies among children [65-67] (6 -10 years old), as well as a recent study among 17-year-old offspring [68], have reported an association between GWG and cardio-metabolic biomarkers. In the Avon Longitudinal Study of Parents and Children (ALSPAC), nine-year-old offspring (n=3457) of women with excessive GWG had higher levels of leptin, C-reactive protein (CRP) and interleukin (IL)-6 as well as lower high-density lipoprotein cholesterol (HDL) and apolipoprotein A levels [66]. Similarly, Perng et al. reported that each 5-kg increase in GWG predicted greater offspring adiposity (0.33 kg total fat (0.11, 0.54)) and higher leptin levels (6% (0,13)) in 6-10-year-old children (n=1090) [67]. Gaillard et al. also recently published results from an Australian birth cohort (n=1392). They found that higher maternal early–pregnancy weight gain was associated with higher adolescent BMI, waist circumference and increased risk of a high-metabolic risk cluster [68]. Studies have also reported a modest association or trend between GWG, offspring adiposity traits, and blood pressure levels [69, 70] in older offspring (aged 21 to 32). Across these different studies, birth weight did not explain the associations with cardio-metabolic risk factors, but they seem to be largely mediated by offspring BMI status.

2.4.3 Maternal diet and offspring cardio-metabolic health

In addition to weight gain, some evidence supports that the diet quality and macronutrient intake may have an independent effect on the development of offspring metabolic health [15, 31, 71]. A large body of evidence has accumulated on the effects of maternal protein restriction in experimental settings, but a maternal malnutrition/low-protein diet induces an adverse metabolic profile in the offspring [15]. High-protein diets have gained considerable popularity in recent years, especially as a nutritional intervention to aid weight loss [72, 73]. Concerns regarding possible deleterious effects of maternal diets with high protein density have recently been raised [74] but data on humans are scarce. In the Harlem trial in 1976, a high-protein supplement, leading to total protein intake of ~20% of energy intake (E%), was associated with a higher rate of very early premature births and neonatal deaths [75]. Animal studies have found that maternal high protein intake may influence offspring's long-term fat metabolism and adiposity [15, 76, 77], but studies on humans have shown conflicting results [78-80].

One of the focus points of this thesis is maternal protein intake and offspring's blood pressure development. Evidence from two studies [81, 82] from Scotland, i.e., the Aberdeen study (recruitment between 1948-1954) and the Motherwell Study (1967-1968), found indications that high protein diet during pregnancy was associated with higher offspring blood pressure in adulthood. Lifestyle habits, as well as obstetric care, have changed since the 1940s1960s. This calls for examining these past findings in a more recent cohort.

To sum up this chapter, the association between maternal GWG and offspring BMI is quite consistent. However, most studies to date have examined offspring BMI assessed in childhood or adolescence. It is important to know whether this association persists into adulthood, and whether it results in adverse cardio-metabolic health. Moreover, maternal macronutrient intake may also play a role in offspring programming. However, the evidence on the association between high protein intake during pregnancy and offspring cardio-metabolic health is scarce.

2.5 Inflammation – a possible underlying mechanism for early programming?

Several pathways could explain the association seen between maternal nutrition and weight gain, and offspring's later metabolic health. Shared genetics, an unhealthy family lifestyle, and the tracking of birth weight may all be important factors. However, in animal models, overnutrition during pregnancy programs metabolic disturbances, independent of other environmental factors and postnatal diet [83, 84].

Epigenetic mechanisms, i.e., DNA methylation, histone modification, and microRNA, that induce a change in phenotype without a change in genotype are marks providing a mechanistic link between early environment, nutrition, and later disease in offspring [85, 86]. These changes may contribute to an intergenerational effect of programmed obesity and a clustering of metabolic risk factors through mother–offspring inheritance. These epigenetic marks may be directly affected by GWG [87] and dietary factors [88], or indirectly through modifications in the gut microbiota [89].

In recent years, interest has increased in the role of inflammation in relation to this aspect, i.e., as a mediator or marker of early programming of metabolic imprinting in the offspring [85, 90]. Acute phase proteins and cytokines could be important mediators as these inflammatory factors are

transferable to fetal circulation and can modulate placental function and nutrient transfer [16, 91]. To further elucidate the mechanisms involved, additional studies will be needed. However, inflammation during pregnancy may be one piece of the multivariable puzzle of offspring programming and risk of developing diseases in adulthood. The chapters below present discussions of inflammatory activity during pregnancy and weight and dietary-induced inflammation.

2.5.1 Inflammatory activity during pregnancy

The inflammatory response is part of the natural defense system that protects the host from infection, injury, and other insults. For example, the response recognizes pathogens and initiates their killing and tissue repair and works to restore homeostasis to maintain health [19]. These processes induce markedly elevated concentrations of inflammatory markers. Low-grade inflammation is, however, characterized by modest elevation, around twice what is observed in controls [19]. During gestation, mild, but significant, inflammation is a normal part of a successful pregnancy, and the placenta produces a range of immunomodulatory factors at different levels depending on the stage of pregnancy [92, 93]. Other potential sources of inflammation during pregnancy, are maternal adipose tissue and vascular endothelium [94] (Figure 2). Inflammatory processes are carefully controlled during a normal pregnancy, but untimely inflammation, characterized by abnormal production and activation of pro-inflammatory signaling pathways, has been linked to adverse maternal and birth outcomes [17, 94, 95].
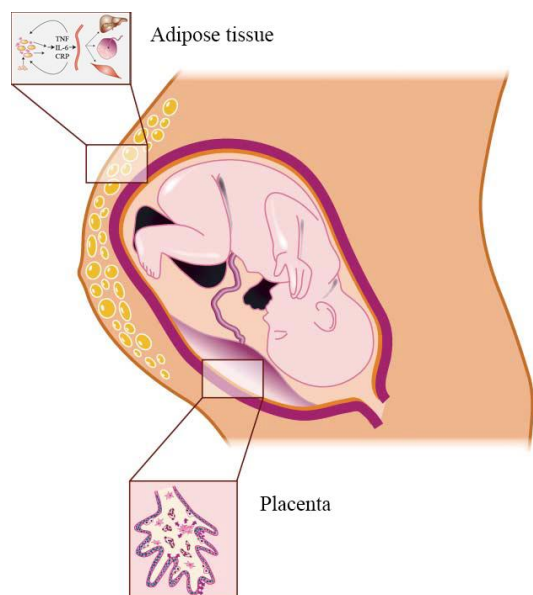
Figure 2. The main potential sources of inflammatory mediators during pregnancy, i.e., maternal adipose tissue, vascular endothelium and the placenta. Adapted from Denison FC, Roberts KA, Barr SM, and Norman JE. Obesity, pregnancy, inflammation, and vascular function. Reproduction, 2010. 140(3): p. 373-85

Currently, CRP is one of the most studied inflammatory markers. Conventional methods of CRP measurement had a sensitivity of about 5 mg/L and therefore did not reflect low-grade inflammation. However, high-sensitivity C-reactive protein (hsCRP) assays can detect levels less than 3mg/L [96]. hsCRP is a nonspecific acute-phase reactant and a marker of low-grade systemic inflammation and a downstream marker of pro-inflammatory cytokines. Several prospective observational studies have demonstrated that CRP is a strong independent predictor of adverse cardiovascular events [96]. During pregnancy, increased inflammation characterizes complicated pregnancies, but elevated maternal CRP concentrations have for example been associated with pregnancy-induced hypertension, preeclampsia, and preterm delivery [92, 97, 98]. Moreover, proinflammatory cytokines like tumor necrosis factor (TNF)-α and IL-6, as well as hsCRP, are up-regulated in women with GDM [95].

Experimental animal studies have shown that maternal inflammation during pregnancy is associated with increased adiposity levels and adverse cardio-metabolic outcomes in offspring [99, 100]. However, data for humans are scarce and inconsistent [101-103], but a recent study by Gaillard et al. on 1,116 mother-child pairs, found that maternal second-trimester CRP levels were associated with a higher fat mass index and trunk fat mass index, but not fat-free mass index for six-year-old offspring [101]. A better understanding of factors that may modulate inflammatory responses during pregnancy may provide evidence of a potential underlying causal mechanism regarding offspring programming.

2.5.2 Weight- and dietary-induced inflammation

Studies have shown that excessive GWG results in increased adipose tissue rather than lean body mass [104]. It is well known that obesity and weight gain in a non-pregnant population is associated with low-grade inflammation and oxidative stress (Figure 3) [18, 105, 106]. However, few studies have addressed this association in women during pregnancy.

High pre-pregnancy BMI and a high level of inflammation in young adulthood have been found to be predictors of inflammation during pregnancy [107-109]. When looking specifically at weight gain during pregnancy, the data are scarce and inconsistent. Studies on pregnant women considered at higher risk of metabolic complications have reported no relation between GWG and inflammation [107, 108]. However, a former

study among non-obese pregnant women demonstrated that levels of CRP were related to glucose intolerance and weight gain in late second and early third trimester [110]. Interestingly, low-grade inflammation has also been found to precede weight gain, both in non-pregnant populations, as well as during pregnancy [111-113]. Recently, Perng et al. reported that higher mid-gestation CRP in US women was associated with a slightly higher subsequent GWG rate [113].
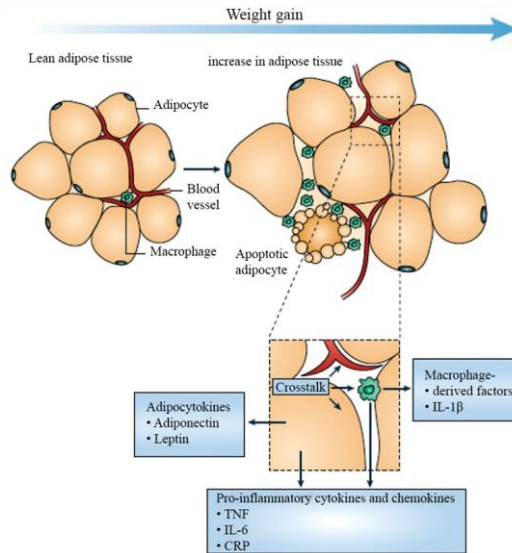
Figure 3. The interaction between adipocytes, macrophages and inflammatory factors. Adapted from: Calder PC et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. Br J Nutr, 2011. 106 Suppl 3: p. S5-78.

Diet may play a central role in the regulation of low-grade inflammation. Diets high in whole grains vegetables, fruits, nuts, and fish are related to anti-inflammation, whereas the "Western diet," characterized by high intakes of refined grains, sweets, French fries and red and processed meats, has been associated with pro-inflammation in the nonpregnant population [19, 114]. Scholl et al. were among the first and few to report findings indicating that maternal inflammatory levels during pregnancy, i.e., hsCRP, were related to maternal diet and adverse maternal outcomes [97]. They reported that for lean pregnant women, higher intakes of protein, cholesterol and low carbohydrate intake were associated with higher hsCRP levels in early pregnancy. Interestingly, two recent randomized intervention studies (nonpregnant obese individuals) found evidence

suggesting that protein intake influences inflammation status [73, 115]. The authors in one of these trials (The RESMENA project) noted that intake of animal protein (especially meat protein) was associated with a higher inflammatory score [73].

Compared to data from non-pregnant populations, data regarding weight- and dietary-induced inflammation during pregnancy are scarce to non-existent. GWG and maternal macronutrient intake might be associated with low-grade inflammation during gestation; however, this relation may perhaps depend on pre-pregnancy weight status and the food source.

2.6 Identifying pregnant women at risk
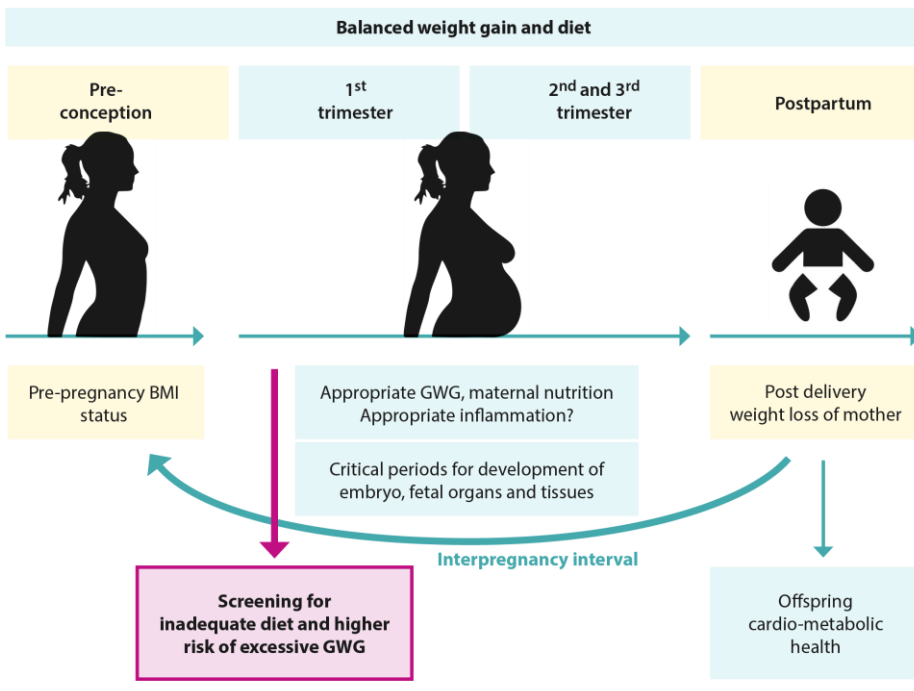
Figure 4. Early pregnancy – one important time point for screening and intervention

As mentioned in previous chapters, non-optimal nutritional status and excessive GWG have been associated with higher risk of adverse short- and long-term health outcomes for both the mother and the child [31, 116]. However, a substantial percentage of pregnant women does not comply with current weight gain recommendations, with about 30-70% gaining weight above the recommended levels [8, 20, 21]. Results from a recent register-based twin study showed that the degree of GWG may be attributed, to some extent, to genetic factors [117]. Heritability was found to explain 30-40% of the variation in GWG. These results suggest that environmental factors must therefore explain a large part of the variation [117]. This chapter focuses on modifiable predictors of excessive GWG as this knowledge may help in identifying women early in pregnancy who may be at higher risk of excessive GWG (Figure 4).

2.6.1 Currently known modifiable risk factors of excessive gestational weight gain

High GWG early in pregnancy strongly predicts total excessive GWG [118]. This suggests that women with early excessive GWG might need to be prioritized for interventions. On the other hand, experiencing nausea in the first trimester has been related to lower risk [119]. Having an obese partner [7] and more "limiting behavior," for example restricting usual level of activity, avoiding physical exercise, going to bed during the daytime, and in general putting their life on hold, has been associated with higher risk of excessive GWG [120, 121]. Moreover, other possible modifiable factors include smoking and dietary behavior. Cessation of smoking during gestation has been associated with higher GWG, compared to non-smokers [120, 122, 123]. The reason for this could be that women who smoke less or stop smoking may experience a withdrawal of the appetite suppressant effect of nicotine [124] and choose a less healthy diet [123]. Finally, total energy intake and the dietary composition may also contribute to the risk of excessive GWG. More detailed discussion of this topic appears in the next chapter.

2.6.2 Diet associated with higher risk of excessive GWG

Several intervention and observational studies examining dietary predictors of GWG have reported an association between energy intake and GWG [125, 126]. The cumulative energy cost for women starting their pregnancy at normal weight with a ~12 kg mean GWG has been estimated to be ~375, 1200, 1950 kJ day per day, for the first, second and third trimesters, respectively. However, a recent meta-analysis of energy intake and weight gain during pregnancy

found that women who gained weight in accordance with the recommendations did not have such a high energy intake, and the authors concluded that the current recommendation to increase energy intake on average by 1000 kJ/day (~239 kcal) might therefore actually encourage high GWG [127]. More emphasis should therefor perhaps be put on better diet quality rather than on increasing food intake during gestation. However, it is important to keep in mind that underreporting of food intake may possibly explain these findings [128, 129].

There have been inconsistent findings on whether the composition of the diet or macronutrient composition may influence the risk of weight gain outside the recommendations. In terms of macronutrient intake, a Cochrane review found high-protein supplements during pregnancy to be associated with a marginal increase in GWG [130]. However, Maslova et al. found that a high protein/carbohydrate ratio was associated with reduced GWG, but this association was mainly driven by a decrease in the intake of added sugar rather than an increase in protein intake [131]. Likewise, carbohydrate quality has been associated with GWG in randomized intervention studies [132-134]. Results regarding maternal fat intake have also been inconsistent, and specific subgroups might be more vulnerable (e.g., overweight women), or the effect might be dependent on specific types of fat consumed (e.g., saturated fat) [125]. However, as individuals consume combined macronutrients in foods but not individual macronutrients, the effect is likely to be dependent on the food source and interaction with other nutrients in food products. Studies using posterior-derived dietary pattern analyses have found indications that higher adherence to a "margarine, sugar and snacks" pattern [135] and "sweets, fast food, and snacks" pattern [136] is associated with a higher risk of gaining excessive GWG and higher weekly GWG, respectively. In the Norwegian MoBa cohort, adherence to the New Nordic Diet, including fruits, vegetables, whole grains, potatoes, dairy, and fish, was associated with lower risk of gaining excessive GWG for women with pre-pregnancy BMI<25 kg/m^2 [137].

To summarize, during pregnancy, modifiable lifestyle factors, such as restricting or avoiding physical activity and smoking cessation, have been linked with higher risk of excessive GWG. Moreover, excessive GWG is likely associated with high energy intake, but the dietary composition/pattern may also contribute to the risk of excessive GWG. Women with excessive GWG during the first trimester have been found to be at higher risk, which underlines the importance of education about nutrition and weight management as early in pregnancy as possible.

2.6.3 Pregnancy, a "teachable moment"?

Evidence suggests that knowledge regarding a healthy maternal diet is often not sufficient, and that the standard information pregnant women receive about nutrition and GWG does not promote meaningful understanding of what constitutes a healthy diet and weight gain [138, 139]. However, pregnancy has been described as a "teachable moment," where women are interested in optimizing fetal health, and frequent visits to the healthcare center offer the opportunity to provide good personalized education from the beginning of pregnancy [52].

But what intervention is most appropriate? Weighing alone as an intervention to change weight gain during pregnancy or reduce risk for excessive weight gain has not been found to be successful for heavier women [140, 141]. However, Ronnberg et al. [142] recently reported a significant reduction in GWG, using personalized weight graphs and regular weight monitoring in an intervention where most of the women analyzed were at normal weight (72%). Lifestyle advice during pregnancy has been found to improve knowledge without negatively impacting the emotional well-being of the mother-to-be [143]. Moreover, interventions aimed at lowering GWG during pregnancy have not resulted in adverse outcomes for the mother or the offspring [52, 144] although severe reduction in energy intake or extreme forms of dieting are never recommended [144]. Results from intervention studies, aimed at improving diet, physical activity or both, have reported that it is possible to reduce GWG by behavioral interventions, but only modestly [126, 145]. A meta-analysis of Muktabhant et al. [126], covering 49 randomized studies (n=11444), reported that high-quality evidence indicates that diet or exercise, or both, reduced the risk of excessive GWG on average by 20% overall (RR=0.8; 95% CI: 0.73, 0.87, n=7096). Recent participant data meta-analysis [145] (33 studies; n=9320) confirms that intervention, based diet and physical activity, reduced GWG compared to the control group, but only by ~0.70 kg (95% CI: −0.92, −0.48) on average. The summary effect estimates from this analysis favored the interventions. However, the reductions in maternal and offspring composite outcomes were not statistically significant. The effect on PPWR and long-term outcomes is still unclear [126, 145, 146].

One reason for these rather disappointing results from intervention studies might be related to the fact that the most commonly used approach in nutrition intervention trials is "one size fits all", i.e., recruiting and testing specific dietary interventions, like low sugar, low-calorie or low-fat diets, independent of the background diet. Moreover, a common practice in many previous intervention studies has been to recruit women, based on pre-

pregnancy weight status [132, 147-149]. Recruiting women into a <u>nutrition intervention</u> based on weight alone is perhaps not the best approach, as not all overweight or obese women have suboptimal diets [150]. In other words, if they do not have unhealthy dietary habits, it must be deemed unlikely that they would benefit from a nutrition intervention. Interestingly, one of the main results from an Icelandic study was that women who were above normal weight but had a healthy diet were not at higher risk of GDM in comparison to women at normal weight, and there seemed to be an insignificant difference in the diet of women of normal weight and women who were overweight or obese before pregnancy [150]. This suggests that it is not valid to select participants for lifestyle interventions during pregnancy, just based on weight alone, and the selection process ought to focus more on quality of the diet.

Insight into the importance of nutrition during pregnancy has increased considerably during the past two decades, and evidence from observational and intervention studies strongly suggests that a healthy diet is important for short- and long-term health of the mother and child [3]. Still, the incorporation of existing knowledge into clinical practice has been slow. One of the reasons for this is the lack of practical methods to assess dietary intake in the clinical setting. The most common way to assess diet in large epidemiological studies is by using an FFQ [33, 34]. To get accurate information on total energy consumption and the different proportions of energy-providing nutrients (fat, proteins, and carbohydrates), as well as the consumption of various other nutrients, such a list needs to be very detailed (a minimum of 10 pages with detailed questions on the consumption of various foods). Other methods used to research diet are even more time-consuming and more expensive. Such methods include the recording of a food diary over several days (usually three to seven days) [150], a 24-hour recall of diet, and a dietary history recorded through an interview with a dietitian. These methods are all time-consuming, and other advanced methods require a biopsy and are performed with expensive equipment [151]. There is a need for simpler ways to measure the healthiness of food in clinical practice.

It might be more purposeful and cost-effective to target vulnerable groups for dietary counseling, based on the background diet. It is therefore vital to find effective strategies in clinical practice to identify the women who would most benefit from a nutritional intervention.

3 Aims

The aim of this Ph.D. thesis is to examine the link between diet and maternal weight gain during pregnancy and offspring cardio-metabolic health. Moreover, the objective is to identify dietary predictors for excessive GWG.

More specifically, the aims are to:

- ✓ Examine the association between excessive GWG and maternal macronutrient intake on the offspring's long-term health, particularly with respect to the risk of overweight and cardio-metabolic risk factors (papers I and II).

- ✓ Explore the association between maternal diet, GWG, and biomarkers of inflammation during pregnancy (paper III).

- ✓ Examine whether a short dietary screening questionnaire, answered by pregnant women in their first trimester of pregnancy can reliably indicate the risk of excessive GWG and macrosomia (paper IV).

4 Methods

This thesis comprises four studies from two prospective observational cohorts. Papers I-III were based on data from the Danish DaFO88 cohort, whereas paper IV was based on recently collected data from an Icelandic pregnancy cohort, the PREWICE cohort. This chapter will provide details of the two data sources, as well as a description of the offspring follow-up in the DaFO88. The chapter includes maternal exposures and outcomes examined in the four papers (Figure 5) as well as a description of the analytical and statistical approaches applied. A detailed comparison of the papers is in Appendix 1.
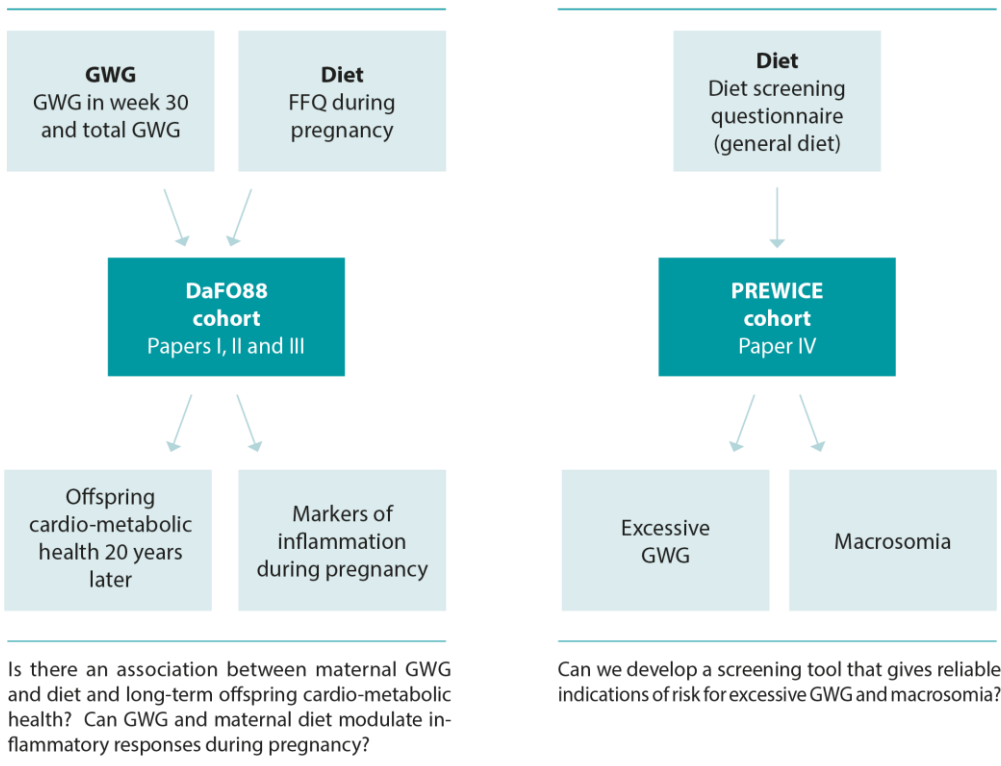
Figure 5. Overview of the two cohorts, exposures and outcomes

4.1 The study population – pregnancy cohorts

4.1.1 The DaFO88 cohort

The DaFO88 cohort was established in 1988-1989 in Aarhus, Denmark with the principal aim of exploring the impact of maternal diet during pregnancy on offspring's short- and long-term health [152, 153]. A total of 965 out of 1212 eligible women participated in the study (80%), and were recruited from April '88 to January '89. These women all had a singleton pregnancy and were scheduled to attend a routine midwife visit in gestational week 30. The questionnaire sent out covered a great range of topics concerning dietary habits, anthropometric status, lifestyle, and socioeconomic factors. During the midwife visit the questionnaires were returned, and a face to face interview was conducted providing additional information. After the interview, maternal blood samples were collected, processed, and stored at -20°C degrees. Information about the women's birth outcomes, medical history, and anthropometry during pregnancy was extracted from hospital records and from the Danish Medical Birth Registry as well as from the records kept by the midwives and general practitioners. Participants provided written informed consent at recruitment. The studies were approved by the Danish Data Protection Agency and the Central Denmark Region Committees on Biomedical Research Ethics (reference no. 20070157).

4.1.2 The PREWICE cohort

Between October 1st, 2015, and September 31st, 2016, pregnant women in their 11th-14th week of pregnancy who went to have an ultrasound at the prenatal diagnostic unit at Landspitali University Hospital were invited to participate in a population-based observational study. In total, 2113 (77%) answered a short electronic questionnaire (dietary screening questionnaire) regarding their general diet, anthropometry, education, smoking status, relationship status, and parity. Information regarding GWG and maternal/birth outcomes was extracted from maternal hospital records. The ethics committee of Landspitali University Hospital approved the study protocol (21/2015), and written consent was obtained from all participants.

4.2 Maternal exposures assessment

4.2.1 GWG – papers I and III (DaFO88 cohort)

Information on weight measurements was retrieved from records from antenatal visits. Maternal weight in week 30 and the highest recorded weight (recorded ~37th week of gestation) were used in the analyses of the DaFO88 cohort. The difference between the highest recorded weight and pre-pregnancy weight (self-reported at enrollment) was used to calculate total GWG. Total GWG was strongly correlated with GWG in week 30 (GWG30) (r=0.85). Excessive GWG was determined according to the IOM guidelines for each pre-pregnancy BMI category, for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg, and obesity >9 kg total GWG [10].

4.2.2 Maternal diet – papers II, III and IV

Dietary assessment in the DaFO88 cohort

The dietary assessment method used in the DaFO88 cohort was a self-administered semiquantitative FFQ (handed in during the midwife visit in gestational week 30), combined with a 15-minute face to face dietary interview. The questionnaire mainly covered snacks, breakfast, and lunch meals, while the interview focused on quantifying the main ingredients of cooked meals and completing the information from the questionnaire to estimate quantities of selected food items. Trained staff corroborated the responses by checking and correcting the questionnaire for any possible misunderstandings. The women were systematically asked about different categories of food items, and the answers provided information on how often per week or per day the food item was consumed and the size of portions. Photographs modeling portion sizes were used in the quantification procedure. In both the questionnaire and the structured interview, the women were asked to let the reported intakes represent the latest three months, corresponding approximately to the second trimester of pregnancy. The combination of an FFQ and a structured interview was used to assess macronutrient and energy intake more accurately, but the FFQ has been validated against biomarkers of n-3 fatty acids only [152]. Nutrient intake was quantified using the 1996 (4th) version of the Danish food composition table.

Dietary assessment in the PREWICE cohort

The 40-item dietary screening questionnaire used in the Icelandic cohort was based on the Nordic [42] and Icelandic recommendations [43]. The questionnaire was designed to give a picture of a participant 's general diet, but at the same time to detect whether the intake of key nutrients for fetal development was low (such as omega-3 fatty acids, vitamin D, and iodine). The women were asked about their diet during the previous four weeks, corresponding to the first trimester of pregnancy (enrolled in the 11th-14th week of pregnancy). It took the women 5 to 10 minutes to answer this short questionnaire. The dietary screening questionnaire was pilot tested in a group of 25 pregnant women in 2014 and compared with results from a four-day weighed food record. Acceptable correlation (>0.3) was found for most food groups/items (unpublished data).

4.3 Outcomes assessment

In the first two papers (I-II), offspring long-term cardio-metabolic factors were the main outcomes (clinical examination in 2008; the DaFO88 cohort). However, in papers III (DaFO88 cohort) and IV (PREWICE cohort), we looked at short-term outcomes, i.e., low-grade chronic inflammation (paper III), excessive GWG (papers III and IV) and macrosomia (paper IV). An overview of the outcomes examined in the four papers can be found in Appendix 1.

4.3.1 Offspring follow-up in the DaFO88 cohort – papers I and II

At the time of the follow-up study (in 2008–2009), the offspring were 19–20 years old. In total, 915 (95% of the original cohort) mother-offspring pairs were alive and living in Denmark at that time (information from the Civil Registration System). The mothers were contacted by mail and their offspring invited to participate in the study. First, the offspring had to complete a web-based self-administered questionnaire, including questions regarding their current anthropometry, health, and lifestyle. Then, the offspring were invited to attend a clinical examination. Of the 688 offspring who agreed to participate in the follow-up, 443 offspring attended the clinical examination (48%). In paper I, we decided to restrict our analysis to normal weight women as the original cohort was a lean population (81% of the original cohort were women of normal weight). The final data in paper 1, therefore, consisted of 308 mother-offspring pairs (34%) - (135 excluded because of their pre-pregnancy weight status or had missing values on the mother's weight gain during pregnancy) (Figure 6). In paper II, offspring of mothers with missing dietary data or total energy intake

<4000 kJ/day were excluded because of incomplete registration (n=9 excluded), leaving 434 for the final analyses (47%) (Figure 6).

There were no significant differences in any of the exposure variables (i.e., maternal GWG/macronutrient intake) between mothers of offspring who did not attend the clinical examination, compared to the offspring attending the examination. However, mothers of offspring who did not attend the clinical examination had lower energy intake and had lower educational levels, compared to mothers of offspring attending the examination (Table 2). Moreover, as overweight and obesity was an exclusion criterion in paper I, we also noted that primi-/multiparity were, in addition, more common in mothers of offspring not attending the clinical examination in this study population.

Figure 6. Flowchart of the enrollment process for papers I and II (DaFO88)

Table 2. Comparison of mothers whose offspring attended the clinical examination to mothers whose offspring did not attend

	Offspring attendet the examination[1] (n= 434)	Offspring did not attend the examination[1] (n= 518)	P[2]
Anthropometric and demographic characteristics			
Age (y)	29 ± 4	29 ± 4	0.45
Prepregnacy BMI (kg/m^2)	21 ± 3	21 ± 3	0.57
Weight gain at 30 wk (kg)	10 ± 4	10 ± 4	0.99
Total gestational weight gain (kg)	14 ± 5	14 ± 5	0.88
Primi- and multiparity (%)	40	44	0.22
University education (%)	56	45	<0.01
Smoking during pregnancy (%)	40	45	0.10
Dietary characteristics			
Total energy intake (MJ/d)	8.7 ± 2.4	8.4 ± 2.3	0.04
Protein (g/day)[3]	79 ± 11	77 ± 13	0.08
Carbohydrate (g/day)[3]	269 ± 27	268 ± 26	0.69
Fat (g/day)[3]	70 ± 11	70 ± 12	0.33
Pregnancy characteristics			
Gestational age (d)	283 ± 12	282 ± 11	0.93
Hospitalized during pregnancy (%)	13	14	0.74
Gestational diabetes (%)	2	2	0.99
Preeclampsia (%)	2	1	0.28

[1] Values are means ± SD's for continuous variables and percentages for categorical variables.
[2] P values were evaluated by using F test (type III) for continuous covariates and Chi-square for categorical variables.
[3] Energy-adjusted by residual model.

The clinical examination took place at Aarhus University Hospital. A trained nurse and a trained medical laboratory technician performed it. The participants were examined between 8:00 AM and 12:30 PM after an overnight fast. The examination included standard anthropometric and blood pressure measurements, and collection of a fasting (10 h) blood sample. The blood samples were centrifuged and frozen at −80 °C. The study was conducted according to the guidelines given in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local ethics committee (Journal no. 20070157) and the Danish Data Protection Agency (Journal no. 2006-41–6257). Details about the cardio-metabolic outcomes examined in this study are in the following subchapters.

Anthropometric measurements

Height, weight, and waist circumference were measured during the examination. Overweight was defined as BMI ≥25 and high waist circumference as >88 cm for women and >102 cm for men [154].

Blood pressure

The clinical examination also included three readings of systolic blood pressure (SBP), diastolic blood pressure (DBP), and resting pulse after seven minutes of rest. The measurement was done in the horizontal position, using a validated handheld device (automatic blood pressure measurement device (OMRON M6 Comfort (HEM-7000-E)) [155]. The mean of the three measurements was used in the analyses.

Blood samples

Blood glucose was measured using bedside equipment (Accu-chek; Roche Diagnostics) immediately after blood sampling. Plasma insulin concentrations were determined using a commercial ELISA kit (DAKO). Insulin resistance was estimated using homeostatic model assessment (HOMA-IR). HOMA-IR was calculated as follows: fasting glucose (mmol L^{-1}) *fasting insulin (mU L^{-1})/22.5.

Serum adiponectin and leptin concentrations were determined at the Medical Research Laboratories in Aarhus, Denmark, by in-house validated assays. Adiponectin was measured by a time-resolved immunofluorometric assay based on two antibodies and recombinant human adiponectin (R&D Systems). Serum leptin was determined by a time-resolved immunofluorometric assay, based on commercially available reagents (R&D Systems) and recombinant human leptin as the standard, and carried out essentially the same way as for adiponectin.

Serum triglycerides, total cholesterol, low-density lipoprotein (LDL) and HDL were measured according to standard methods on a Modular P analyzer (Roche Diagnostics).

4.3.2 Maternal levels of inflammatory markers in the DaFO88 cohort – paper III

Inflammatory responses have been implicated as possible mechanisms behind adverse programming of offspring disease. In paper III (DaFO88 cohort), our objective was to examine whether GWG and maternal diet may modulate inflammatory responses in blood during pregnancy. The main outcome in this paper therefore concerned maternal levels of inflammatory markers specific

for low-grade inflammation, e.g., hsCRP, serum amyloid A (SAA), IL -6, IL-8, IL-1β, and TNF-α. The proteins hsCRP and SAA are acute-phase reactants that accompany both acute and chronic inflammation. Although largely produced by hepatocytes, hsCRP and SAA are also produced by adipocytes [19]. IL-8, IL-6, IL-1β, and TNF-α are proinflammatory cytokines. Potential sources of these markers during pregnancy include adiposity tissue, vascular endothelium and the placenta [94].

After the maternal interview in gestational week 30, maternal blood samples were obtained. The samples were kept on ice and separated into serum, plasma, and erythrocytes within an hour. An analysis of the inflammatory biomarkers was conducted after 20 years of storage at -20°C. The analyses were performed in a central laboratory at the Department of Internal Medicine and Cardiovascular Research Institute Maastricht, Maastricht University Medical Centre (by C.G.S). Meso Scale Discovery's electrochemilumine-scence detection technology (MesoScaleDiscovery, Gaithersburg, MD, USA) was used for these analyses. The laboratory methods are described elsewhere [102].

Because of the long storage time (i.e., 20 years), the possibility of measuring these biomarkers was first tested in a pilot study [102]. It showed that the median serum levels of the inflammatory markers of interest were comparable to measurements done on samples that had been stored for 10 years at -80°C (data from The Amsterdam Growth and Health Longitudinal Study used as reference value).

Blood samples were available for 922 women, but those with hsCRP values >10 µg/ml (n=105) were excluded due to possible cases of unrecognized infections [156, 157], resulting in 817 women with data on inflammatory markers. Of these, 675 had information on GWG recorded in week 30. Furthermore, women with total energy intake under 4,000 kJ/d (n=4) were excluded due to probable incomplete registration. The final data set, therefore, consisted of 671 women (73%) (Figure 7).
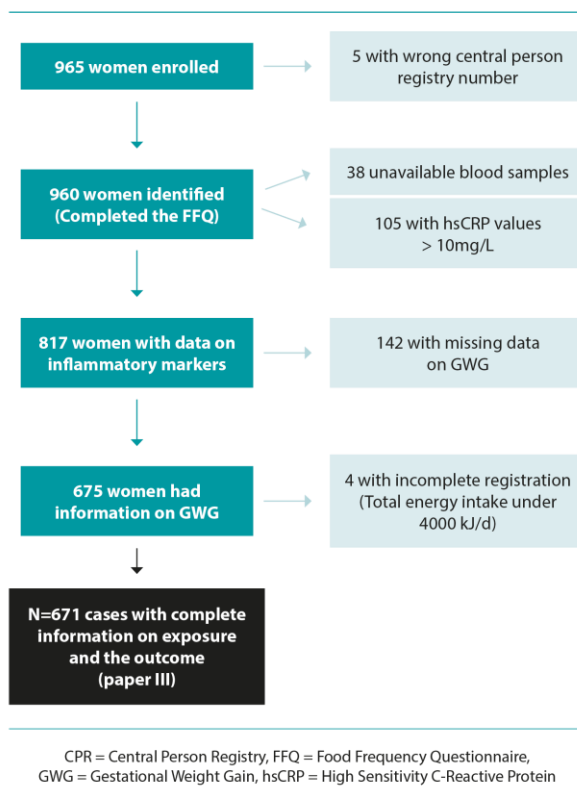
Figure 7. Flowchart of the enrollment process for paper III (DaFO88)

4.3.3 Excessive GWG – papers III and IV

Relatively detailed information about maternal dietary intake in the DaFO88 cohort during pregnancy and data regarding maternal lifestyle and socio-demographic variables (assessed in week 30 of gestation by FFQ and face-to-face interview), gave us the opportunity to investigate the association between the maternal diet and excessive GWG (paper III-supplemental analyses). Our focus in paper IV (PREWICE cohort) was also on the relation between diet and GWG, but here we wanted to test another approach. We used a short electronic food frequency questionnaire, i.e., the dietary screening questionnaire, to try to capture the mother's general diet, and the goal was to identify a dietary risk score associated with excessive GWG. In the DaFO88 cohort the IOM recommendations were used [10], however, the Icelandic guidelines [41, 48, 49] for GWG during pregnancy were used when analyzing the Icelandic data. We defined total GWG as the difference between maternal self-reported pre-pregnancy weight and the highest recorded weight (~week 37 in the DaFO88 cohort and ≥week 36 in the PREWICE cohort - data from maternal records).

4.4 Statistical analyses

4.4.1 General statistical considerations

Throughout all the papers, assumptions on the normality of model residuals were checked using histograms and QQ plots. Normally distributed variables were described by their mean and standard deviation (SD), non-normally distributed continuous variables by their median and interquartile range (IQR: 75-25th percentile), and categorical variables using frequencies (percentages). Student's t-test was used to compare normally distributed continuous variables, whereas, for skewed and categorical variables, Mann-Whitney U test and Chi-square tests were used, respectively. The statistical tests were performed using SPSS versions 20, 21, and 24 (IBM Corp., Armonk, NY, USA). Statistical significance was accepted at P<0.05.

In all the papers, we used appropriate regression models, depending on the outcome, i.e., linear regression, logistic and Poisson log-linear regression, to analyze our data (see Appendix 1). From the linear regression models, we estimated mean differences expressed as β coefficients and 95% CI. From the Poisson log-linear regression and logistic regression models, we estimated relative risk (RR) and odds ratios (OR) and 95% CI. We identified potential confounders based on previous literature. Detailed descriptions regarding the covariates can be found in the following subchapters for each cohort. The proportion of missing data was low in both cohorts, ranging from 0-8% for individual covariates. In general, we assumed that the missing values were "missing at random" [158]. Multiple imputations, as implemented in SPSS (24), were used to impute missing covariates in all four papers. When using imputed values, we compared the results to results from complete case analysis to check for inconsistencies and to evaluate the stability of our findings. Further stability analyses were conducted to explore significant associations in more detail. The following chapters describe specific analytical approaches applied in the four papers.

4.4.2 Studies based on the DaFO88 cohort (paper I-III)

Covariates assessment

We used an a priori approach, so covariates included in our adjusted models were selected, based on former studies. The following confounders were included in the main models: pre-pregnancy BMI (in quartiles), mother's age from hospital records (in quartiles), parity (0, 1, ⩾ 2), smoking status

(nonsmoker, <10, ≥10 cigarettes/day), and educational level (elementary schooling, high school/technical schooling, university education). This information (except mother's age) was retrieved from the lifestyle questionnaire that the mother answered during pregnancy (i.e., handed in at 30 weeks of pregnancy). When examining the association between maternal diet and maternal levels of inflammatory factors (paper III), we furthermore adjusted for maternal total energy (in quartiles).

In paper I, when looking at offspring's anthropometric and cardiometabolic outcomes at 20 years of age, we included in the main model an adjustment for whether offspring thought their father was overweight at that time point to consider shared familial lifestyle characteristics. In papers I and II, we also adjusted for offspring sex and examined potentially differential programming effects for male and female offspring by conducting sex-specific analyses. In further sensitivity analyses, we adjusted for possible intermediary factors, such as birth weight, gestational age, maternal GWG, and pregnancy complications (retrieved from clinical records and records from antenatal visits). Moreover, when looking at long-term outcomes, additional adjustments were made for offspring's BMI (in papers I and II) and lifestyle (paper I) at that time point, i.e., offspring smoking status (non-smoker, occasional, daily), offspring's alcohol consumption (⩾1, 2–3, 4–6, ⩾7 times per month), and whether offspring reported being on a diet (yes/no) or exercising (yes/no) to lose weight (retrieved from the web-based self-administered questionnaire).

Macronutrient analyses (papers II and III)

The macronutrient variables used in papers II and III were energy-adjusted using the residual model [159, 160]. Here, the calorie-adjusted intake is the residual for subjects from a regression model in which total energy intake is the independent variable, and the macronutrient intake is the dependent variable. For a given energy intake, these residuals therefore reflect the deviation from the expected energy intake for each subject. The underlying assumption is that the expected energy intake, to a large extent, reflects mean energy requirements. To reflect actual intake values, the energy-adjusted nutrients were then scaled by adding the mean, for each adjusted variable.

In paper III, we used a substitutional model, i.e., a model resembling an isocaloric situation. This model gives insights into the impact on health outcomes when changing diet composition versus analyzing the absolute intake. In the main model, carbohydrates were substituted with protein by allowing all energy-contributing nutrients into the model except carbohydrate

[161]. When fat and total energy intake are kept constant, a decrease in the intake of carbohydrates an increase in protein intake must be accompanied by a decrease in intake of carbohydrates.

4.4.3 The PREWICE cohort (paper IV)

The dietary risk score assessment

The dietary data collected from the 40-item questionnaire was converted to frequency per week for all food groups, which was then transformed into 13 predefined dietary risk factors for inadequate diet (Table 3). Stepwise backward elimination was used to identify a reduced set of variables with the highest maximum likelihood that best predicted excessive GWG. This set of variables was then used to calculate a combined dietary risk score (range 0-5) (Figure 8).

Table 3. The predefined dietary risk factors for inadequate diet

Risk factors for inadequate diet	
1. Not eating a varied diet	8. Sugar and artificially sweetened beverages ≥5 times/week
2. Vegetables and fruits <5 times/day	9. Sweets, ice cream, cakes, cookies ≥2.5 times/week
3. Fish intake <2 times/week	10. French fries ≥1 time/week
4. Dairy intake <2 times/day	12. High dairy intake ≥5 times/day
5. Wholegrain products <2 times/day	11. Processed meat products ≥1 time/week
6. Beans, nuts, seeds <3.5 times/week	13. Quality of fat - using butter (or other unsaturated fat sources) rather than oil (≥50%)
7. Vitamin-D <5 times/week	

The risk factors were mainly based on the Icelandic Food Based Dietary Recommendations [43], which are based on the Nordic Nutrition Recommendations [42]. The cutoff for sugar/artificially sweetened beverages and high dairy intake was set in line with Nordic studies showing that high intake of these products is associated with high GWG [20, 134], and adverse birth outcomes [162, 163].

The following six dietary risk factors (predictors) were included in the final model: not eating a varied diet, fruits/vegetables <5 times per day, dairy <2 times per day, whole grain products <2 times per day, sugar/artificially sweetened beverages ≥5 times per week, dairy ≥5 times per day. To construct a total dietary risk score, each participant got 1 for fulfilling the risk criteria, and 0 for not fulfilling the risk criteria. The scores of the six dietary risk factors were then summed up, ranging from 0 to 5 scores as it is not possible to be in both dairy risk groups (too low/too high).

Covariates assessment

Covariates in the PREWICE cohort included in our adjusted models, as in DaFO88, were, selected a priori. When examining the association between the dietary risk score and GWG, we included the following potential confounders in our adjusted models: maternal pre-pregnancy BMI, maternal age, parity, maternal smoking during pregnancy, gestational length (when highest weight in pregnancy was recorded) and experience of nausea during this pregnancy. On the other hand, when examining the association between the dietary risk score and macrosomia, we included: maternal pre-pregnancy BMI, maternal age, parity, maternal smoking during pregnancy, total gestational length and offspring sex.
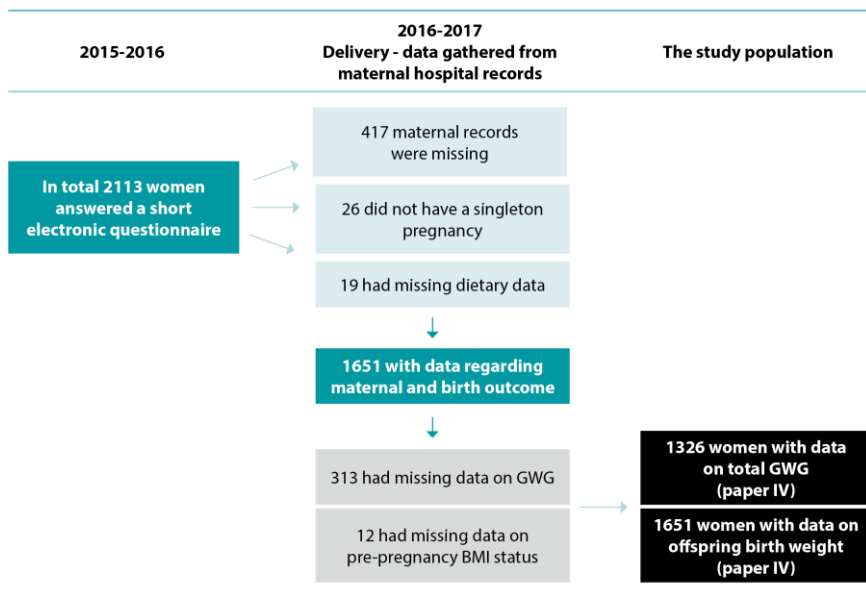
Figure 8. Flowchart for the PREWICE study (paper IV)

5 Results and discussion

The main findings from the four papers are presented and discussed below. The main descriptive results for the study populations are presented first. Small differences between the number of participants and the number of participants in the papers are due to variations in sample size according to the completeness of exposure and outcome measurements. The results from all four papers are then presented and discussed.

5.1 Descriptive results

In the DaFO88 cohort, the mean maternal pre-pregnancy BMI of the participating women (n=952) was 21 kg/m^2 (SD 3 kg/m^2), and the mean age was 29 years (SD 4 years). Overall, most mothers had a BMI within the normal range (81%), and, of note, around 40% of women reported smoking during pregnancy; 58% were nulliparous, and 15% had a higher academic degree at the time of pregnancy. The prevalence of offspring overweight or obesity was 19% in males and 17% in females.

In the PREWICE cohort (1651 mother-child pairs), the median maternal pre-pregnancy BMI was 26 kg/m^2 (IQR 7 kg/m^2) and the mean age 30 years (SD 5 years). In total, 1326 women had data on both pre-pregnancy BMI and total GWG (GWG recorded at ≥36th week of gestation), which included 46 underweight (4%), 726 normal weight (55%), 320 overweight (24%), and 234 obese mothers (18%). Most mothers were non-smokers (16% smoked before pregnancy and 7% reported smoking during pregnancy), 39% were nulliparous, and 24% of the women had a higher academic degree at the time of pregnancy.

5.2 GWG and offspring cardio-metabolic health (paper I)

For the 308 mother-offspring pairs included in the study (DaFO88 cohort), a weak positive association between GWG and offspring BMI at 20 years was found. After adjustment, each 1-kg increase in maternal GWG30 was associated with 0.1 kg/m^2 higher (95% CI: 0.0, 0.2) offspring BMI and 1.1 (95% CI: 1.01, 1.2) higher odds of offspring being overweight at age 20. The observed association between GWG30 and offspring BMI was similar for male

(β=0.1 kg/m^2; 95% CI: -0.0, 0.3) and female offspring (β=0.1 kg/m^2; 95% CI: -0.0, 0.2). A positive, non-significant trend was also observed when examining the association between GWG and offspring waist circumference (Table 4).

Table 4. Associations of maternal gestational weight gain with offspring BMI and waist circumference at follow-up

	Offspring BMI (kg/m2)		Offspring overweight	
	β[1]	95% CI	OR[2]	95% CI
GWG in week 30 (per 1 kg increase)[3]	0.10	(0.01, 0.20)	1.10	(1.00, 1.20)
IOM categories:				
Suboptimal (<11.5 kg)	-0.4	(-1.2, 0.4)	0.6	(0.2, 1.4)
Optimal (11.5-16 kg)	ref.	ref.	ref.	ref.
Excessive (>16 kg)	0.6	(-0.2, 1.4)	1.8	(0.9, 3.8)
p for trend[4]	0.02		0.01	
	Waist circ. (cm)		High waist	
	β[1]	95% CI	OR[2]	95% CI
GWG in week 30 (per 1 kg increase)[3]	0.12	(-0.14, 0.38)	1.02	(0.90, 1.14)
IOM categories:				
Suboptimal (<11.5 kg)	-1.1	(-3.3, 1.2)	0.6	(0.2, 2.0)
Optimal (11.5-16 kg)	ref.	ref.	ref.	ref.
Excessive (>16 kg)	1.3	(-0.9, 3.5)	1.6	(0.6, 4.2)
p for trend[4]	0.05		0.09	

Abbreviations: BMI, body mass index; CI, confidence interval; GWG, gestational weight gain; IOM, Institute of Medicine guidelines; OR, odds ratio.
[1]Linear regression model, adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, and educational level, offspring's sex and whether offspring considered their father overweight.
[2]Logistic regression model, reflecting the odds of offspring being overweight (BMI ⩾ 25) at ~ 20 years or having waist circumference >88 cm for females and >102 cm for males, adjusted for same covariates as in.[1]
[3]Showing increase in the outcome variable per 1-kg increase in gestational weight gain (continuous) or by the IOM categories.
[4]T-test for linear regression, Chi-square test for logistic regression.

The results for the association between GWG and offspring anthropometry was of lower magnitude, compared to previous studies [12, 14], which may be related to the young age of offspring at the time of follow-up [164]. The timing of the measurement of GWG may also play a role. We collected our data in gestational week 30 (GWG30) and at the end of pregnancy (GWG total), but we noted a stronger association with GWG30. This agrees with previous studies with more detailed measurements of GWG [65, 66, 68, 165], Those results suggest that greater weight gain in early or mid-pregnancy is more strongly associated with offspring BMI and adverse cardio-metabolic profile than weight gain late in pregnancy. It is not known whether it is the fat accumulation early in pregnancy that is especially important, or

whether weight gain just becomes an increasingly poor measure of maternal fat accumulation later in gestation.

In addition to measurements of offspring's anthropometry, we had information on their biomarkers, i.e., weight-regulating hormones, sugar metabolism, lipid profile, and blood pressure measurements. The distribution of offspring cardio-metabolic risk factors can be found in table 5. The difference in offspring cardio-metabolic risk factors at follow-up by GWG is in table 6. Compared to the offspring of mothers with optimal GWG, the children of the mothers with suboptimal GWG tended to have more favorable cardio-metabolic outcomes. For example, the offspring of mothers with suboptimal GWG tended to have 10% lower insulin levels (95% CI: -20, 1) and 7% lower leptin levels (95% CI: -25, 15), compared to the offspring of mothers with optimal weight gain (p for trend 0.05 for both outcomes). These results are in line with the meta-analysis by Mamun et al. that demonstrated a 14% decreased risk of offspring obesity among women gaining suboptimal GWG, compared to women gaining optimal GWG [12].

Table 5. Distribution of offspring cardio-metabolic risk factors at follow-up

	Men (n=121)		Women (n=187)	
	Mean	s.d	Mean	s.d
BMI (kg/m^2)	22.6	2.9	22.2	3.1
Waist (cm)	82.9	7.7	80.2	8.4
Systolic BP (mm Hg)	118.1	9.2	105.2	7.8
Diastolic BP (mm Hg)	64.8	6.9	66.0	5.7
Resting pulse (bpm)	60.9	10.6	62.8	8.7
Fasting plasma glucose (mmol/L)	5.2	0.4	4.8	0.4
HbA1c (%)	5.2	0.2	5.2	0.2
	Median	IQR	Median	IQR
Total cholesterol (mmol/L)	4.0	1.0	4.5	1.0
Triglyceride (mmol/L)	0.8	0.5	1.0	0.6
LDL (mmol/L)	2.3	0.9	2.4	0.9
HDL (mmol/L)	1.3	0.4	1.5	0.3
Insulin (µg/l)	38.0	23.8	42.5	22.0
HOMA-IR	1.5	0.9	1.5	0.8
Leptin (µg/l)	2.1	3.1	13.1	11.5
Adiponectin (µg/l)	6.9	3.8	9.2	4.7

Abbreviations: BP, blood pressure; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein, Homa-IR, homeostasis model assessment-estimated insulin resistance; LDL, low-density lipoprotein.

Table 6. Mean difference (95% CI) in offspring cardio-metabolic risk factors at follow-up by Institute of Medicine's categories of maternal GWG

Outcome	Weight gain[1]	All (n=308)[2]	Men (n=121)[3]	Women (n=187)[3]
Insulin (%)	*Suboptimal*	-10 (-20, 1)	-16 (-31, 3)	-10 (-23, 5)
	Optimal	ref.	ref.	ref.
	Excessive	3 (-8, 15)	6 (-14, 29)	-0.4 (-14, 15)
P for trend[4]		0.05	0.05	0.23
Leptin (%)	*Suboptimal*	-7 (-25, 15)	-18 (-45, 20)	-2 (-24, 26)
	Optimal	ref.	ref.	ref.
	Excessive	**<u>42 (15, 75)</u>**	**<u>93 (30, 186)</u>**	23 (-3, 57)
P for trend[4]		<0.01	<0.01	0.08
Total cholesterol (%)	*Suboptimal*	0.6 (-4, 6)	1 (-6, 9)	1 (-6, 9)
	Optimal	ref.	ref.	ref.
	Excessive	-5 (-9, -0.1)	2 (-5, 10)	**<u>-8 (-14, -1)</u>**
P for trend[4]		0.05	0.76	0.02
LDL (%)	*Suboptimal*	3 (-5, 11)	0.0 (-11, 12)	5 (-6, 18)
	Optimal	ref.	ref.	ref.
	Excessive	-7 (-14, 0.0)	3 (-8, 16)	**<u>-11 (-20, -1)</u>**
P for trend[4]		0.02	0.62	<0.01
HDL (%)	*Suboptimal*	-1 (-7, 5)	7 (-2, 17)	-5 (-12, 3)
	Optimal	ref.	ref.	ref.
	Excessive	-3 (-8, 3)	-0.9 (-9, 9)	-4 (-10, 4)
P for trend[4]		0.52	0.14	0.73
Triglyceride (%)	*Suboptimal*	0.3 (-11, 13)	-4 (-19, 14)	3 (-12, 20)
	Optimal	ref.	ref.	ref.
	Excessive	4 (-7,16)	13 (-5, 35)	-2 (-16, 13)
P for trend[4]		0.60	0.11	0.56
Resting pulse (bpm)	*Suboptimal*	-2 (-4, 1)	-4 (-9, 1)	0.1 (-3, 3)
	Optimal	ref.	ref.	ref.
	Excessive	-0.8 (-3, 2)	1 (-4, 6)	-2 (-5, 1)
P for trend[4]		0.53	0.07	0.26

Abbreviations: DBP, diastolic blood pressure; GWG, gestational weight gain; HDL, high-density lipoprotein, Homa-IR, homeostasis model assessment-estimated insulin resistance; LDL, low-density lipoprotein; SBP, systolic blood pressure.

Bold indicates that the associations showed statistical significance.

[1] Increase in the outcome variable by the Institute of Medicine guidelines; suboptimal (<11.5 kg), optimal (11.5-16) and excessive (>16 kg) weight gain.
[2] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, and educational level, offspring's sex and whether offspring considered their father overweight.
[3] Same covariates as in [2] but without offspring sex.
[4] T-test with gestational weight gain entered as a dichotomous variable.

Interestingly, a specific gender difference was observed when examining offspring biomarkers of cardio-metabolic health (interaction by sex <0.05). When taking multiple testing into account, the results were significant regarding offspring insulin and leptin levels among males. However, GWG was inversely associated with levels of total cholesterol and LDL levels among females (Table 7). For leptin, we found that each 1-kg increase in maternal GWG was associated with 10.7% (95% CI: 5.7, 15.9) higher leptin levels for male offspring compared to 0.4% (95% CI: -2.4, 3.3) for female offspring (Table 7). For mothers who gained excessive weight during pregnancy, this increment corresponded to 93% (95% CI: 30, 186) higher leptin levels among male offspring (Table 7). Although we only found a weak association between high GWG and offspring BMI, higher offspring leptin levels do indicate increased adiposity [166, 167]. Moreover, higher levels of leptin have been reported to predict BMI and waist gain in nonobese children [168] and visceral fat accumulation in healthy individuals at adult age [169].

Previous studies have not reported similar sex-dependent results regarding offspring cardio-metabolic biomarkers. In support of sex-dependent fetal programming, experimental studies have found that molecular and phenotypic outcomes of adverse in utero conditions are often more prominent in male than female offspring [170]. We, however, noted differences in behavioral responses among the genders which may also explain these different associations, e.g., female offspring were twice as likely to report that they thought their weight was too high, and that they were trying to lose weight by dieting and exercising (Table 8). Strengthening these speculations, maternal weight gain was associated with higher resting pulse among male offspring, whereas a non-significant inverse association was observed for females (Table 7). The potential programming effects because of maternal excessive GWG may, therefore, potentially be modifiable by offspring behavioral responses later in life.

Our results indicate that high GWG may influence offspring leptin and insulin regulation at young adult age. Previous findings from cohort studies, examining offspring at a younger age (i.e., 6-10 years of age) have reported associations in accordance with our results although their results for offspring leptin values were somewhat lower [66, 67]. Studies in animal models provide support for direct intrauterine mechanisms, as early overnutrition has been found to permanently affect energy homeostasis and increase susceptibility to obesity, leptin and insulin resistance later in life [171-174]. For example, Férézou-Viala et al. [174] found that in Wistar rats, maternal overfeeding,

induced by high-fat and high-energy diet, was related to altered hypothalamic leptin signaling, i.e., leptin resistance. However, these animal models have mainly explored the combined effect of maternal obesity and overfeeding during pregnancy, very few studies have focused on excess GWG with normal pre-pregnancy BMI. This was tested in a swine model, where excessive GWG promoted early indications of metabolic syndrome in the offspring, and offspring's high-energy post-weaning diet further promoted this effect [84].

Table 7. Associations of maternal GWG during the first 30 weeks of gestation with offspring cardio-metabolic risk factors at follow up

Outcome[3]	All (n=308)[1]			Men (n=121)[2]			Women (n=187)[2]		
	β	95% CI	P	β	95% CI	P	β	95% CI	P
Insulin (%)[4]	1.2	(-0.2, 2.6)	0.09	3.7	(1.4, 6.2)	<0.01	-0.2	(-1.9, 1.5)	0.82
HOMA_IR (%)[4]	1.1	(-0.3, 2.5)	0.14	3.4	(0.8, 6.0)	0.01	-0.1	(-1.8, 1.7)	0.94
Fasting glucose (mmol/liter)	0.0	(-0.01, 0.01)	0.95	-0.01	(-0.04, 0.01)	0.32	0.01	(-0.00, 0.02)	0.35
Leptin (%)[4]	3.7	(1.2, 6.4)	<0.01	10.7	(5.7, 15.9)	<0.01	0.4	(-2.4, 3.3)	0.76
Adiponectin (%)[4]	0.5	(-0.8, 1.7)	0.45	-0.6	(-2.8, 1.6)	0.58	1.5	(0.0, 2.9)	0.04
Total cholesterol (%)[4]	-0.9	(-1.5, -0.3)	<0.01	-0.1	(-1.0, 0.8)	0.81	-1.3	(-2.1, -0.6)	<0.01
LDL (%)[4]	-1.3	(-2.2, -0.4)	<0.01	0.1	(-1.2, 1.5)	0.85	-2.2	(-3.4, -0.9)	<0.01
HDL (%)[4]	-0.5	(-1.2, 0.1)	0.12	-1.1	(-2.1, -0.0)	0.05	-0.1	(-1.0, 0.8)	0.80
Triglyceride (%)[4]	-0.2	(-1.6, 1.2)	0.80	1.7	(-0.5, 3.9)	0.12	-1.1	(-2.8, 0.6)	0.21
SBP (mm Hg)	0.3	(0.0, 0.6)	0.03	0.4	(-0.1, 0.9)	0.09	0.2	(-0.2, 0.5)	0.30
DBP (mm Hg)	0.2	(-0.0, 0.4)	0.12	0.4	(0.0, 0.8)	0.03	0.0	(-0.2, 0.3)	0.92
Resting pulse (bpm)	0.2	(-0.1, 0.5)	0.25	0.9	(0.3, 1.5)	<0.01	-0.3	(-0.6, 0.1)	0.17

Abbreviations: DBP, diastolic blood pressure; GWG, gestational weight gain; HDL, high density lipoprotein, Homa-IR, homeostasis model assessment-estimated insulin resistance; LDL, low density lipoprotein; SBP, systolic blood pressure.

[1] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status and educational level, offspring's sex and whether offspring thinks their father is overweight.
[2] Same covariates as in [1] but without offspring sex.
[3] Showing increase in the outcome variable per 1-kg increase in gestational weight gain and the 95% confidence interval.
[4] Levels were log transformed in the analyses and back-transformed via exponentiation to facilitate interpretation as a percent change.

Table 8. Characteristics of offspring at follow-up by sex

Offspring characteristics[1]	Man (n=120)	Women (n=188)
Self-perceived weight: too high (%)	17	45
On a diet to lose weight (%)	8	16
Exercise to lose weight (%)	26	41
Smoking (%)	52	37
Alcohol consumption (%)		
≥7 times per month	25	13

[1] Offspring characteristics, questions answered in the web-based

Pathways by which GWG might influence offspring growth and development include high concentrations of nutrients [175] and changes in the regulation of mitogenic hormones, adipokines, and inflammation [16, 110, 176, 177]. High GWG has for example been linked with higher levels of cord blood hormones like insulin, insulin-like growth factor (IGF)-1, IGF-2, insulin-like growth factor-binding protein (IGFBP)-3, and leptin levels, as well as lower adiponectin values, i.e., growth promoting hormonal milieu [176]. Inflammation may also affect placental function that may alter the nutrient flow to the fetus [16, 177, 178]. These changes may impair fetal development and induce metabolic dysfunction later in life [16, 176].

The results observed in our study could also be related to inherited genetic traits or maternal-offspring-shared environment and lifestyle. Andersson et al. reported that the heritability of GWG is 30-40% [117]. A recent genome-wide association study showed that common genetic variants could explain approximately half of this [179]. We were not able to account for genetics, but we were able to consider confounding by important familial dietary and offspring lifestyle habits. This adjustment did not appreciably alter the effect estimates (Table 9). Recent data from a postnatal follow-up in the UK Pregnancies Better Eating and Activity Trial (UPBEAT) suggest that reduced adiposity in infants at six months is causally mediated by changes in antenatal maternal diet and GWG [180]. Dietary interventions in pregnancy [126, 144, 145] resulting in modest reduction in GWG will provide further insights into the causality of these observed associations in long-term follow-up of offspring [146].

Table 9. Associations of maternal GWG (per 1-kg increase) during the first 30 weeks of gestation, with offspring cardio-metabolic risk factors at follow-up (additional adjustment for offspring smoking and alcohol consumption)

Outcome[2]	Adjusted I[1]	
	Men (n=121)	Women (n=187)
BMI (kg/m^2)	0.2 (0.0, 0.3)	0.1 (-0.0, 0.2)
HOMA-IR (%)	3.1 (0.5, 5.9)	-0.4 (-2.2, 1.5)
Insulin (%)	3.5 (1.0, 6.0)	-0.4 (-2.2, 1.3)
Leptin (%)	10.8 (5.6, 16.3)	0.4 (-2,5, 3.4)
Adiponectin (%)	-0.9 (-3.1, 1.3)	1.7 (0.2, 3.1)
Total cholesterol (%)	0.0 (-0.9, 0.9)	-1.5 (-2.3, -0.6)
LDL (%)	0.3 (-1.0, 1.7)	-2.2 (-3.5, -1.0)
HDL (%)	-1.2 (-2.2, -0.0)	-0.2 (-1.1, 0.7)
Triglyceride (%)	1.9 (-0.3, 4.2)	-1.3 (-3.0, 0.5)
Systolic BP (mm Hg)	0.4 (-0.1, 1.0)	0.3 (-0.1, 0.6)
Diastolic BP (mm Hg)	0.4 (0.1, 0.8)	0.1 (-0.2, 0.3)
Resting pulse (bpm)	1.0 (0.4, 1.6)	-0.2 (-0.6, 0.2)

Abbreviations: BMI, body mass index; BP, blood pressure; GWG, gestational weight gain; HDL, high-density lipoprotein, Homa-IR, homeostasis model assessment-estimated insulin resistance; LDL, low-density lipoprotein.

[1] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, and educational level, offspring's sex, whether offspring considers their father to be overweight, offspring smoking and alcohol consumption.

[2] Showing increase in the outcome variable per 1-kg increase in gestational weight gain and the 95% confidence interval.

The results from this prospective cohort provide evidence that high maternal GWG of lean mothers may affect offspring cardio-metabolic health at a young adult age. A weak positive association was observed between GWG and offspring anthropometry. In addition, our results suggest that GWG is adversely associated with offspring biomarkers of cardio-metabolic health in male but not female offspring. More frequent reports of dieting and physical exercise at follow-up by female offspring may potentially explain this. Although the observed associations were modest, we cannot exclude that these modest shifts may become more apparent later in life [168, 169].

5.3 Maternal macronutrient intake and offspring blood pressure (paper II)

Results from two studies from the 1940s-1960s in Scottish populations suggested that high maternal protein intake, especially from animal sources, might adversely affect offspring blood pressure in adulthood [81, 82]. As lifestyle habits, as well as obstetric care, have changed since the 1940s-1960s, this study aimed to test whether these findings could be replicated in a more recent cohort.

Table 10. Dietary characteristics of the study population

| | All participants[1] (n= 434) | | Protein intake[2] | | |
			Quintile 1 (n=86)	Quintile 5 (n=87)	P[3]
Total energy intake (MJ/d)	8.7	± 2.3	8.8 ± 3.1	8.6 ± 2.0	0.54
Protein (g/kg)[2]	1.3	0.2	1.1 ± 0.1	1.6 ± 0.3	<0.01
Protein (g/day)[2]	79	± 11	63 ± 6	94 ± 6	<0.01
Dairy protein (g/d)[2]	34	± 12	20 ± 8	48 ± 10	<0.01
Non dairy animal protein (g/d)[2,]	19	± 8	14 ± 6	23 ± 8	<0.01
Plant protein (g/d)[2,5]	26	± 5	28 ± 5	24 ± 4	<0.01
Carbohydrate (g/day)[2]	269	± 27	278 ± 31	260 ± 25	<0.01
Sugar (g/day)[2]	37	± 22	45 ± 29	26 ± 12	<0.01
Fiber (g/day)[2]	24	± 5	25 ± 7	23 ± 4	0.01
Fat (g/day)[2]	75	± 31	80 ± 42	70 ± 24	0.05
SFA (g/day)[2]	31	± 7	32 ± 8	29 ± 7	0.03
MUFA (g/day)[2]	19	± 4	20 ± 4	19 ± 3	0.05
PUFA (g/day)[2]	9	± 3	9 ± 3	8 ± 2	<0.01

Abreviations: MUFA, monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; SFA, saturated fatty acids.

[1]Values are means ± SD's
[2]Energy-adjusted by residual model.
[3]P values were evaluated by using F test (type III)
[4]Animal protein included protein from milk/milk products, cheese, ice cream, meat, fish, eggs and related products.
[5]Plant protein included protein from cereals, vegetables, fruits and related products.

We included 434 mother-offspring pairs from the DaFO88 cohort in the analyses who had available data regarding maternal macronutrient intake and offspring blood pressure measurements. Mean absolute maternal macronutrient intakes (percentages of energy) were 79 g protein (16%), 75 g fat (31%), and 269 g carbohydrate (51%). For women with high protein intake (quintile 5, mean 20E%), 15E% came from animal protein and 5E% from plant protein, whereas for women with low intake (quintile 1, mean 13 E%), 7E% came from animal protein and 6E% from plant protein. Table 10 shows maternal dietary characteristics.

In our main analysis, we found that when comparing the highest to lowest quintile of protein intake, higher maternal protein intake at the expense of carbohydrates was associated with a 2.4 mm Hg (95% CI: 0.4, 4.4) increase in offspring DBP in adjusted models (Table 11). Similar differences, although not significant, were found for SBP (mean 2.6 mm Hg, 95% CI: 0.0, 5.3). Additional adjustment for offspring BMI at age 20 did not appreciably alter effect estimates; mean Δ for DBP went from 2.4 mm Hg (95% CI: 0.4, 4.4) to 2.1 mm Hg (95% CI: 0.1, 4.1) when comparing highest and lowest quintiles. Sensitivity analyses also showed no sex difference and additional adjustment for potential mediators, i.e., birth weight, GWG in week 30, gestational age, and pregnancy complications did not substantially alter the results.

Studies have reported that even a minor decrease in a population's average blood pressure levels may substantially reduce the burden of blood pressure-related diseases. For example, a 2 mm Hg reduction in the population mean of DBP could decrease the prevalence of hypertension by as much as 17% and coronary heart disease by 6% [181]. In relation to our study results, this suggest that even modest dietary changes during pregnancy could affect population health considerably.

Table 11. **Maternal protein intake and offspring blood pressure***

	n	Crude model[1]		Adjusted model[2]	
		SBP	DBP	SBP	DBP
Total protein intake (per 10g-change/d)[3]	434	0.7 (-0.2,1.7)	0.6 (0.0, 1.2)	0.6 (-0.1, 1.4)	0.6 (0.0, 1.1)
Protein intake (mean ± SD g/d)					
quintile 1 (63 ± 6)	86	ref	ref		
quintile 2 (73 ± 2)	86	2.5 (-0.7, 5.7)	0.7 (-1.3, 2.7)	2.5 (-0.1, 5.1)	1.3 (-0.7, 3.3)
quintile 3 (78 ± 1)	87	1.7 (-1.6, 4.9)	1.6 (-0.4, 3.6)	2.0 (-0.6, 4.6)	1.5 (-0.5, 3.5)
quintile 4 (84 ± 2)	88	2.8 (-0.5, 6.0)	1.1 (-0.9, 3.1)	2.5 (-0.2, 5.2)	1.4 (-0.6, 3.4)
quintile 5 (94 ± 6)	87	2.8 (-0.5, 6.0)	2.4 (0.4, 4.5)	2.6 (-0.0, 5.3)	2.4 (0.4, 4.4)
p for trend[4]		0.12	0.02	0.08	0.03
Dairy protein (per 10g-change/d)[3,5]	434	1.3 (0.3, 2.4)	0.7 (0.0, 1.3)	1.1 (0.3, 2.0)	0.8 (0.1, 1.4)
Non-dairy animal protein (per 10g-change/d)[3,6]	434	0.0 (-1.5, 1.5)	0.4 (-0.5, 1.3)	-0.2 (-1.4, 1.0)	0.3 (-0.6, 1.3)
Plant protein (per 10g-change/d)[3,7]	434	2.6 (-0.4, 5.6)	0.8 (-1.0, 2.7)	2.0 (-0.5, 4.5)	1.6 (-0.3, 3.5)

Abreviations: DBP, diastolic blood pressure; SBP, systolic blood pressure.

*In all models, we examine the association between higher protein intake during pregnancy at the expense of carbohydrates (isocaloric substitution) and offspring blood pressure at 20 years of age.

[1] Protein source, fat, and total energy intake entered simultaneously into the model.
[2] Adjusted for maternal pre-pregnancy body mass index, maternal age, parity, smoking status during pregnancy, maternal educational level, and offspring sex.
[3] The effect estimates can be interpreted as the effect of increasing intake of the protein source (per 10-g change) at the expense of carbohydrates while keeping energy intake constant. [4] The t-test with maternal protein intake entered as categorical variable. [5] Protein from dairy (total protein from milk, milk products, and cheese), protein from other sources, fat, and total energy intake entered simultaneously into the model. [6] Protein from non-dairy animal sources (total protein from meat, fish, and eggs and related products), protein from other sources, fat, and total energy intake entered simultaneously into the model.[7] Protein from plant sources (cereals, vegetables, fruits, and related products), protein from other sources, fat, and total energy intake entered simultaneously into the model

When comparing our results with those of the Scottish studies [81, 82], our effect estimates were lower in magnitude. This difference may be related to differences in study methodology, offspring age and variation in lifestyle factors that may have changed over time. In the Aberdeen study, 253 offspring had mothers with a mean total protein intake of 73 g/day (12.2 E %). These mothers participated in a survey of diet around the 30th week of pregnancy. A followup on their offspring 40 years later reported different associations between protein intake and offspring blood pressure, depending on the protein-carbohydrate ratio. When the mothers had animal protein intake that was less than 50 g per day, a higher carbohydrate intake was associated with higher blood pressure in the offspring. A 100-g increase in carbohydrate intake associated with a 3 mmHg increase in SBP. When the mother's intake of animal protein intake was, however, high (>50 g/day), each 100-g decrease in carbohydrate was related to an 11-mm Hg rise in SBP and an 8-mm Hg rise in DBP. These increases in blood pressure were also associated with decreased placental size. In the Motherwell study in Scotland [82], women (n=626) attending the maternity hospital were advised to eat high animal protein and a low carbohydrate diet, i.e., 450 g of red meat per day and other sources of animal proteins in moderate quantities but to avoid carbohydrate-rich foods during pregnancy (mean total protein intake was estimated to be 88 g/day (~24E%)). They reported that higher maternal intake of meat and fish in late pregnancy was associated with 0.19 mm Hg (per portion of meat or fish per week) higher offspring blood pressure three decades later. The authors of this study speculated that this association might be related to metabolic stress and cortisol release because of the high protein diet, but glucocorticoids have been found to play an important role in the nutritional programming of blood pressure [182].

We used the substitution model rather than an absolute increase because the former Scottish studies indicated that the protein-carbohydrate balance might be important [76, 81, 82]. Moreover, it is important to keep in mind in observational settings that the perceived impact of increasing intake of one macronutrient or specific foods may also be related to lower intake of the other macronutrients or other factors in foods for which it is substituted. Taking this into consideration in nutrition studies gives better insights into the association between diet and health [161, 183]. The difference in blood pressure among offspring whose mothers had higher versus lower protein intake might therefore also be related to differences in carbohydrate intake but not protein. However, our secondary analyses showed that the results did not substantially change when we relaxed the substitution condition (i.e., protein could be

replaced by either carbohydrates or fat), indicating that the association observed in our study is most likely driven mainly by protein intake alone.

Glucocorticoids are necessary for normal fetal development and are particularly important for maturation of the brain, lungs, and kidneys. However, overexposure to glucocorticoids in utero leads to long-term programming of the function of the hypothalamic–pituitary–adrenal (HPA) axis that can lead to metabolic abnormalities and hypertension in the offspring [184, 185]. In relation to maternal diet, this has mainly been linked to maternal protein restriction which down-regulates the expression of the placental enzyme 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), that protects the fetus from overexposure to maternal glucocorticoids [182]. A recent experimental study in pigs, however, showed that both low protein (approx. 7E%) and high protein (30E%) diets, compared to adequate (12E%) protein levels, fed throughout gestation, was associated with disruptions of the circadian rhythm in maternal plasma cortisol, higher daily response of maternal cortisol, and disturbances in the central setting of the fetal cortisol regulation, which has been shown to affect blood pressure regulation and salt and water balance [186]. Interestingly, the Motherwell study group also reported findings from the same cohort, showing that the blood-pressure-raising effect of a high protein and low-carbohydrate dietary pattern during pregnancy might have been mediated through hypercortisolemia in the offspring [187].

Notably, we found a more pronounced association between maternal protein intake from milk and milk products and offspring blood pressure (Table 12). This is in keeping with findings from the animal study by Thone-Reineke et al, where a high casein diet (40E% protein) at the expense of carbohydrates during pregnancy and lactation, programmed blood pressure, food efficiency, and body weight of the rat offspring in a sex-dependent manner [76]. However, as milk consumption was the largest component of protein in our population (mean 829±369 g/day), and 51% of the total protein intake came from dairy protein for the women with the highest protein intake (quintile 5, mean milk intake 1173±350 g), the observed association might just reflect the relation between protein intake per se and offspring blood pressure. Further research is needed to examine whether the association between maternal milk intake and offspring blood pressure risk in our population is due to dairy protein itself, other components in dairy or total protein content.

The results from this prospective cohort support previous studies indicating that maternal high protein diet may influence offspring's adult blood pressure. As our study population consisted of young offspring with normal

blood pressure values, we acknowledge that the clinical relevance of a 2.4-2.6 mm Hg higher DBP or SBP values as a result of high maternal protein intake remains uncertain. However, as mentioned earlier, modestly higher blood pressure levels present already in early adulthood have been found to track into later adulthood and increase the risk of developing hypertension and its sequelae later in life [181, 188]. Dietary changes during pregnancy, like avoiding excessive protein intake, need to be compared and weighed against other health risks [74, 131, 189]. In this respect, both the quantity and quality of the macronutrients that are substituted must be considered.

5.4 Maternal diet, GWG, and inflammatory markers during pregnancy (paper III)

Effective regulation of inflammation is an important part of a healthy pregnancy; inflammatory processes are, therefore, important determinants of optimal pregnancy and birth outcomes [93]. However, untimely and abnormal inflammation characterizes complicated pregnancies [17, 98, 190]. Studies have also indicated that maternal inflammation might contribute to fetal programming of obesity [16, 101], allergic diseases [191] and psychiatric disorders [192]. Understanding the mechanisms of untimely triggering of inflammation during pregnancy may be important for preventative measures. In this part of the project, we examined the association between maternal GWG, diet, and levels of inflammatory markers among 671 pregnant women in the DaFO88 cohort, for whom we had available data on exposures and blood measurement.

5.4.1 Pre-pregnancy weight status, GWG, and inflammation (paper III)

The concentrations of the inflammatory markers were comparable to values observed among other pregnant populations (Table 12) [113, 193, 194]. When comparing the levels by pre-pregnancy BMI, higher levels of hsCRP, IL-6, IL-6, and IL-1β were observed in women who were overweight or obese (Table 12). This is in accordance with previous findings [101, 113, 195]. Friis et al. analyzed cytokine levels over the course of pregnancy and reported that women who were obese before pregnancy displayed elevated levels of circulating pro-inflammatory factors (Monocyte Chemoattractant Protein 1 (MCP-1), IL-6 and CRP) in early pregnancy, but this pro-inflammatory upregulation was not as evident towards the end of pregnancy [109]. The authors suggested that inflammation induced by pre-pregnancy adiposity is

perhaps restrained late in gestation. Inflammatory diseases, for example rheumatoid arthritis, have been found to improve during pregnancy, and this suppressed inflammatory effect might therefore also work on obesity-associated low-grade inflammation [16]. As we only had one blood measurement during pregnancy and a limited number of overweight/obese women, we could not evaluate this matter in our data.

Table 12. Maternal inflammatory biomarkers in week 30 by pre-pregnancy BMI

	Underw. (n=71; 11%)[1]		Normalw. (n=564; 84%)[2]		Overw. (n=36; 5%)[3]		
	Median	10-90 percentile	Median	10-90 percentile	Median	10-90 percentile	P value[4]
hsCRP (µg/ml)	2.0	0.7-6.5	2.6	0.7-6.3	4.7	1.5-7.8	<0.01
SAA(ng/ml)	667	237-1679	788	300-1775	666	331-2057	0.19
Il8 (pg/ml)	3.0	1.9-9.8	3.4	1.8-8.7	3.0	2.1-13.9	0.48
IL-6 (pg/ml)	1.0	0.6-2.1	1.1	0.7-2.3	1.2	0.9-3.2	0.04
Il-1β (pg/ml)	0.5	0.2-1.4	0.5	0.2-1.3	0.6	0.3-2.5	0.04
TNF-α (pg/ml)	5.1	3.4-11.1	5.5	3.9-10.5	6.0	4.2-11.9	0.17

Abbreviations: BMI, body mass index; hsCRP, a high-sensitivity C-reactive protein; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; SAA, Serum amyloid A; TNF-α, Tumor necrosis factor alpha.

[1] Underweight, BMI <18.5 kg/m^2
[2] Normal weight, BMI 18.5 – 24.99 kg/m^2
[3] Overweight and obesity, BMI ≥25 kg/m^2
[4] The Kruskal-Wallis test

Studies looking specifically at weight gain and inflammation during pregnancy are inconsistent [107, 108, 110, 113]. In our adjusted analyses, we found that each 1-kg increase in maternal GWG during the first 30 weeks of gestation was associated with 3% (95% CI: 1, 5) higher hsCRP and 3% (95% CI: 1, 4) higher SAA. This corresponded to 18% to 25% increase in hsCRP and SAA among those with excessive GWG, compared to women with non-excessive GWG (Table 13). GWG was also inversely associated with IL-8, while no associations were found for the other inflammatory markers (IL-6, IL-1β and TNF-α). Table 14 shows absolute median concentrations of the inflammatory markers hsCRP, SAA, and IL-8 in relation to GWG.

Table 13. Association between both GWG during the first 30 weeks of gestation and excessive GWG with maternal inflammatory biomarkers

	Unadjusted			Adjusted[1]			
Outcome (%)[2]	β(%)	95% CI	P	β(%)	95% CI	P	Exc. vs non exc. GWG (95% CI)[3]
hsCRP	3	1, 5	<0.01	3	1, 5	<0.01	25 (9, 44)
SAA	2	1, 4	<0.01	3	1, 4	<0.001	18 (5, 33)
IL-8	-2	-3, -0	0.02	-2	-4, -1	<0.01	-14 (-24, -3)
IL-6	0	-1, 2	0.36	0	-1, 1	0.71	2 (-6, 11)
Il-1β	-1	-2, 1	0.25	-1	-3, 0	0.16	-8 (-19, 3)
TNF-α	1	-0, 1	0.19	1	-0, 2	0.11	0 (-7, 7)

Abbreviations: GWG: gestational weight gain; hsCRP, a high-sensitivity C-reactive protein; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; SAA, Serum amyloid A; TNF-α, Tumor necrosis factor alpha.

[1] Adjusted for maternal age, pre-pregnancy BMI, parity, smoking status, and educational level.
[2] The percent increase in the outcome variable per 1-kg increase in gestational weight gain during the first 30 weeks.
[3] The percent difference between women gaining excessive gestational weight, compared to women gaining optimal or suboptimal gestational weight gain. Excessive GWG was determined in accordance with the IOM recommendations for each pre-pregnancy BMI category, for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg and obese >9 kg total GWG.

Table 14. Absolute median levels (IQR) of maternal CRP, SAA, and IL-8 by gestational weight gain

		Median (IQR)														
	n	hsCRP (µg/ml)	p[1]	SAA (ng/ml)	p[1]	IL-8 (pg/ml)	p[1]	IL-6 (pg/ml)	p[1]	Il-1β (pg/ml)	p[1]	TNF-α (pg/ml)	p[1]			
Gestational weight gain[2]																
Optimal GWG	283	2.4 (2.8)	ref	715 (611)	ref	3.3 (2.6)	ref	1.1 (0.7)	ref	0.51 (0.47)	ref	5.5 (3.1)	ref			
Suboptimal GWG[3]	179	2.4 (2.9)	0.92	672 (874)	0.94	3.5 (2.7)	0.24	1.1 (0.6)	0.61	0.46 (0.47)	0.35	5.4 (2.7)	0.26			
Excessive GWG[4]	209	3.2 (3.5)	<0.01	866 (646)	<0.01	3.3 (1.8)	0.50	1.1 (0.7)	0.27	0.52 (0.52)	0.58	5.5 (2.6)	0.98			

Abbreviations: GWG, gestational weight gain; hsCRP, a high-sensitivity C-reactive protein; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; SAA, Serum amyloid A; TNF-α, Tumor necrosis factor alpha.

[1] Mann–Whitney U test.
[2] The weight gain categories were determined in accordance with the IOM recommendations for each pre-pregnancy BMI category (see reference no. 12 in the main manuscript.
[3] Suboptimal GWG, i.e. for underweight women <12.5 kg, normal weight <11.5, overweight <7 kg and obese <5 kg total GWG.
[4] Excessive GWG, for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg and obese >9 kg total GWG.

Two recent studies on women considered at higher risk of metabolic complications, i.e., obese women [108] or women with high waist-hip ratio [107], have reported no relation between GWG and inflammation. However, Rota et al. reported an association between weight gain during pregnancy, glycemic parameters and CRP levels in gestation weeks 26-28 in a study among non-obese pregnant women [110]. Indicating that perhaps lean women may be more vulnerable to high weight gain in terms of adiposity-related inflammation. Interestingly, low-grade inflammation has also been found to precede weight gain in both non-pregnant populations [111, 112] and during pregnancy [113]. Perng et al. reported that higher mid-gestation CRP in US women was associated with a slightly higher subsequent GWG rate, whereas no association was found for TNF-α and IL-6 [113]. The authors speculated that this could perhaps be related to disturbances in the gut microbiota, which has been implicated in the development of obesity through processes like microbial energy extraction, regulation of appetite, and microbiota induced-inflammation [196, 197].

5.4.2 Maternal diet and inflammation (paper III)

In the DaFO88 cohort, the inflammatory markers associated with GWG, hsCRP, SAA, and IL-8, were further explored in relation to maternal diet (n=671). After adjusting for possible confounders, we noted an association between maternal protein intake with both hsCRP and SAA. These associations were of similar magnitude as for GWG. Each 10-g change in protein intake was associated with 6% higher levels of both hsCRP (95% CI: 1, 11) and SAA (95% CI: 1, 10). Concerning other nutrients, fiber intake was inversely associated with IL-8, but no association was found for other nutrients (Table 15).

The importance of the protein source (of either animal or plant origin) was also examined (Table 16). Women in the highest quintile of animal protein intake had 25% higher hsCRP levels (95% CI: 2, 53) and 23% higher SAA level (95% CI: 4, 47) than women in the lowest quintile. Intake of plant protein intake was, however, inversely associated with hsCRP levels (p for trend <0.001) and SAA levels (p for trend 0.013). Table 17 shows the mean protein and fiber intake by quintiles of intake (n=671).

Table 15. Multivariable associations of maternal diet with maternal inflammatory biomarkers in week 30 of gestation

	hsCRP (%)	SAA (%)	IL-8 (%)
	β (95% CI)[1]	β (95% CI)[1]	β (95% CI)[1]
Total energy intake (MJ/d)[2]	2.4 (-0.6, 5.4)	2.5 (-0.0, 5.2)	0.3 (-2.5, 3.1)
Protein (g/day)[3,4]	6 (1, 11)	6 (1, 10)	3 (-2, 8)
Glycemic index[3,4]	-9 (-18, 1)	-1 (-9, 8)	1 (-8, 9)
Glycemic load[3,4]	0 (-2, 2)	0 (-2, 1)	0 (-2, 2)
Fiber (g/day)[3,4]	-10 (-23, 3)	-6 (-17, 5)	-13 (-24, -1)
SFA (g/day)[3,4]	-2 (-11, 7)	1 (-6, 9)	3 (-5, 11)
MUFA (g/day)[3,4]	6 (-12, 25)	11 (-5, 27)	8 (-8, 25)
PUFA (g/day)[3,4]	-10 (-39, 19)	6 (-19, 32)	-19 (-45, 7)

Abbreviations: hsCRP, a high-sensitivity C-reactive protein; IL-8, Interleukin 8; MUFA, monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; SAA, Serum amyloid A; SFA, saturated fatty acids.

[1] The percent increase in the outcome variable per 1-MJ increase in total energy intake, otherwise per 10-unit increase in nutrients and glycemic index/glycemic load (95% confidence interval).
[2] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, and educational level.
[3] Same covariates as in[2] but also adjusted for total energy.
[4] Energy-adjusted by the residual model.

Table 16. Multivariable associations of quintiles of maternal protein intake with maternal inflammatory biomarkers in week 30 of gestation

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	*p for trend*[1]
		% change (95% CI)				
hsCRP[2]						
Protein[3]	Ref	9 (-11, 33)	11 (-10, 35)	22 (-0, 50)	26 (3, 54)	0.026
Animal protein[3,4]	Ref	-3 (-21, 19)	5 (-14, 29)	19 (-3, 46)	25 (2, 53)	0.004
Plant protein[3,5]	Ref	-5 (-23, 16)	-22 (-36, -4)	-19 (-34, 0.5)	-24 (-38, -6)	<0.001
SAA[2]						
Protein[3]	Ref	11 (-6, 32)	16 (-3, 37)	16 (-3, 37)	31 (10, 55)	0.011
Animal protein[3,4]	Ref	3 (-13, 23)	13 (-5, 34)	21 (2, 43)	23 (4, 47)	0.003
Plant protein[3,5]	Ref	3 (-13, 22)	-2 (-18, 17)	-11 (-26, 6)	-10 (-25, 8)	0.013

Abbreviations: hsCRP, a high-sensitivity C-reactive protein; SAA, Serum amyloid A.

[1] T-test with maternal protein intake entered as continuous variable.
[2] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, educational level and total energy
[3] Energy-adjusted by the residual model.
[4] Animal protein included protein from milk/milk products, cheese, ice cream, meat, fish, eggs and related products.
[5] Plant protein included protein from cereals, vegetables, fruits and related products.

Table 17. Mean protein and fiber intake by quintiles

	Quintile 1[1] (n=134)	Quintile 2[1] (n=134)	Quintile 3[1] (n=135)	Quintile 4[1] (n=134)	Quintile 5[1] (n=134)
Total protein (g/day) [2]	61 ± 6	72 ± 2	79 ± 2	85 ± 2	95 ± 5
Animal protein (g/day)[2,3]	33 ± 7	46 ± 3	54 ± 2	61 ± 2	73 ± 6
Plant protein (g/day)[2,4]	16 ± 2	19 ± 1	22 ± 1	24 ± 1	29 ± 3
Fiber (g/day)[2]	17 ± 2	21 ± 1	23 ± 1	26 ± 1	31 ± 3

[1] Mean intake ± standard deviation.
[2] Energy-adjusted by the residual model.
[3] Animal protein included protein from milk, cheese, ice cream, meat, fish, eggs and related products.
[4] Plant protein included protein from cereals, vegetables, fruits and related products.

Studies show that both specific diets and nutrients may modulate the inflammatory status [19, 115, 198]. Dietary-induced inflammation has consequently emerged as an important research topic. In nonpregnant populations, a healthy diet, rich in fruits, vegetables, whole grains, seafood, and low-glycemic diets have been associated with lower concentrations of circulating inflammatory markers [19, 115, 198]. However, data from pregnant populations are scarce. In accordance with our results, Scholl et al. found a significant linear trend for higher intakes of protein only to correlate with higher hsCRP in women lean before pregnancy, but not overweight or obese women [97]. These data support the idea that high pre-pregnancy CRP levels in heavy women may possibly swamp the increase in CRP levels during pregnancy related to high weight gain or adverse dietary behavior during pregnancy.

Two 8-week intervention studies among nonpregnant groups of overweight and obese participants have also reported results in agreement with our observations [73, 115]. The DIOGenes intervention found that a low-glycemic-index diet and, to a lesser extent, low-protein diet (10–15E%) may specifically reduce hsCRP levels [115]. In the RESMENA project, adults were recruited to follow a reduced calorie diet consisting of either 30E% or 15E% protein for 8 weeks. There was no difference in the weight loss between the groups. However, the endpoint inflammation score, which included hsCRP, IL-6, and TNF-α, was significantly higher in the high protein group. Animal protein, i.e., meat protein, was associated with higher plasma levels of inflammatory markers [73]. The authors noted that this association could be related to factors like advanced glycation end products (AGEs), saturated fat or iron. However,

excessive protein intake [199] and intake of different protein sources [200] may also influence microbial diversity, which may affect inflammatory levels.

Our stability analyses showed that when analyzing protein intake from different food groups, i.e., milk, cheese, ice cream, meat and meat products, fish, eggs, cereals, vegetables, fruits and related products, significant inverse association with hsCRP and SAA were observed for protein intake from cereals (for each 10g-increase in protein intake from cereals: β= -26%; 95% CI: -12, -40). However, increase in intake of protein from all the animal sources tended to be associated with higher levels of hsCRP and SAA. Interestingly the association between high milk intake and hsCRP levels was significant (for each 10g-increase in protein from milk and milk products: β= 6%; 95% CI: 1, 12). This relation may simply be related to the fact that milk was the main protein source in this cohort, as noted in paper II [201]. A recent systematic review of 52 clinical trials investigating inflammatory markers in relation to the consumption of dairy products suggests that dairy products, particularly fermented products (for example, soured milk and yogurt), have, anti-inflammatory properties in humans, possibly because of modulation of the gut microbiota [202]. Women in our cohort were, on the other hand, mainly drinking milk rather than eating fermented products (72% of the total dairy intake came from milk, mostly low-fat milk (39%)), and in total 86% of the women had intake higher than the recommended two portions of milk and milk products per day. Interestingly, oxidative stress and higher levels of inflammatory biomarkers have been linked with high intakes of D-galactose in experimental and observational studies [203, 204]. This sugar is mainly found in milk. It is though important to keep in mind that separating the potential role of individual foods or macronutrients in observational settings is difficult, as many nutrients are highly correlated and have synergistic or interactive effects. Our interpretation of these results is therefore that a high intake of animal versus plant-based foods may have different relations with inflammatory levels during pregnancy.

5.4.3 Inflammation and maternal/birth complications (paper III)

We were underpowered (n=922) to examine meaningfully different obstetric complications (prevalence of GDM 2.4%, preeclampsia 1.3% and low-birth weight births 2.9%), but we noted that the median concentrations of markers of inflammation tended to be higher in women exposed to various obstetric complications, compared to women with uncomplicated pregnancies (Table 18). For GDM (hsCRP), hypertension (hsCRP and TNF-α) and preeclampsia (IL-6 and TNF-α), these differences were statistically significant. In addition, women giving birth to low-birth-weight neonates (<2.500 g) were found to have

higher hsCRP levels (6.5 vs. 2.9 mg/mL), SAA levels (1213 vs. 842 ng/mL) and IL-1β levels (0.8 vs 0.5 pg/ml) in week 30, than women giving births to full-term neonates of normal birth weight. Exclusion of participants that had been hospitalized during pregnancy (15%) or had any pregnancy complications (23%), or additionally adjusting for each obstetric complication during pregnancy did not appreciably alter the result for the observed associations between GWG, diet, and inflammation during pregnancy. These associations seem to be independent of pregnancy and birth complications.

To conclude, our results from the DaFO88 cohort indicate that both GWG and diet are related to the inflammatory status of pregnant women. We found evidence that excess GWG and high intake of animal-based diets during pregnancy may be proinflammatory, whereas plant-based intake may be anti-inflammatory. These associations seem to be independent of pregnancy and birth complications. The clinical relevance of the ~25% increase in hsCRP and SAA in a lean, healthy population, found to be associated with excessive GWG or high animal protein intake is uncertain

Table 18. Absolute median levels (IQR) of inflammatory markers by different obstetric and birth complications (examined for all participants having blood measurements[1])

		Median (IQR)												
	n	hsCRP (µg/ml)	p^2	SAA (ng/ml)	p^2	IL-8 (pg/ml)	p^2	IL-6 (pg/ml)	p^2	Il-1β (pg/ml)	p^2	TNF-α (pg/ml)	p^2
Obstetric complications													
No complication	720	3.0 (3.7)	ref	818 (833)	ref	3.5 (2.4)	ref	1.1 (0.7)	ref	0.5 (0.5)	ref	5.6 (3.2)	ref
Diabetes	22	4.4 (9.8)	0.02	949 (1115)	0.26	3.4 (1.7)	0.99	1.2 (1.2)	0.86	0.8 (1.3)	0.08	6.8 (3.1)	0.22
Hypertension	32	4.9 (4.3)	<0.01	891 (1101)	0.26	3.4 (2.7)	0.36	1.4 (0.9)	0.10	0.5 (0.6)	0.32	6.8 (3.2)	<0.01
Preeclampsia	12	4.5 (4.8)	0.06	1215 (881)	0.19	3.2 (3.3)	0.71	1.5 (1.1)	0.03	0.6 (0.7)	0.61	7.9 (6.5)	0.01
Bleeding	40	2.3 (4.3)	0.12	844 (703)	0.82	3.5 (3.7)	0.43	1.1 (1.1)	0.98	0.5 (0.6)	0.26	5.3 (3.1)	0.59
Other complications[3]	110	2.9 (3.7)	0.67	954 (958)	0.16	3.3 (2.4)	0.60	1.1 (0.7)	0.78	0.5 (0.6)	0.93	5.3 (2.9)	0.24
Birth outcomes													
Non-preterm non-low birth weight	766	2.9 (3.7)	ref	842 (840)	ref	3.3 (2.4)	ref	1.1 (0.8)	ref	0.5 (0.5)	ref	5.7 (3.2)	ref
Pre-term birth [4,5]	30	4.1 (3.7)	0.17	1103 (1596)	0.06	3.3 (4.1)	0.94	1.2 (0.8)	0.20	0.5 (0.7)	0.50	5.9 (3.2)	0.31
Low birth weight [6]	27	6.5 (6.3)	<0.01	1213 (1488)	0.02	4.8 (4.5)	0.08	1.4 (0.7)	0.06	0.8 (0.8)	<0.01	6.3 (3.5)	0.10

Abbreviations: hsCRP, a high-sensitivity C-reactive protein; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; SAA, Serum amyloid A; TNF-α, Tumor necrosis factor alpha.

[1] As the prevalence of obstetric complications was low in this cohort, these analyses included all individuals that had blood measurement (n=922). However, the main analyses were restricted to GWG measurements and subjects with hsCRP values <10 mg/L (due to possible cases of unrecognized infections with high CRP values).
[2] Mann-Whitney U test.
[3] Excluding diabetes, hypertension, preeclampsia and bleeding.
[4] Birth before 37th week of gestation.
[5] 12 preterm cases also had low birth weight.
[6] Birth weight of an infant of less than 2500g, regardless of gestational age.

5.5 Maternal diet and GWG (paper III and IV)

Recommendations regarding GWG naturally focus on the weight without offering dietary recommendations on how to lower the risk of excessive GWG. Examining this issue is of great importance in terms of preventative measures. There is uncertainty regarding which specific components of the diet, e.g., foods or nutrients, might have the greatest potential to contribute to the risk or prevention of excessive GWG. This association was examined in both the DaFO88 cohort (n=671, paper III) and the PREWICE cohort (n=1326, paper IV).

5.5.1 The DaFO88 cohort (paper III)

In this paper, we focused primarily on dietary factors identified in earlier reports [20, 119, 205]. For the 671 women included in the analyses, we found that each 1 MJ increase in energy intake was associated with 11% (95% CI: 2, 20) higher odds of excessive GWG. We noted that substituting 1E% of animal protein intake with plant protein intake was associated with 32% lower odds of excessive GWG. We also found that intake of milk/milk products, meat/meat products, and sweets were associated with higher odds of excessive GWG. Interestingly, these associations were independent of total reported energy intake (Table 19).

Our results, as well as results from previous studies [134, 135, 137], show that specific foods/food patterns may be associated with higher GWG beyond energy intake. These associations could though be related to underreporting of energy intake [128, 129], or perhaps reflect other attributes of the mother's lifestyle, for example, daily physical activity. However, recent data also indicate that the diet, specifically dietary diversity, is associated with the richness of the microbiota [206], which may influence weight gain. We speculate whether the underlying mechanisms linking GWG, diet, and inflammation together in the DaFO88 cohort could be related to the gut microbiota composition. The microbiota has been shown to influence metabolic pathways by modulating extraction of calories, satiety control and inflammation [196, 197]. Individuals eating a typical Western diet, high in animal protein and fat, have for example been found to have a *Bacteroides*-dominated enterotype. This has been associated with inflammation [207]. Interestingly, high presence of *Bacteroides* has been linked with excessive weight gain in pregnancy [208]. Plant-based diets, on the other hand, have been linked with a more diverse microbiota in the non-pregnant population, reduced abundance of pathogens and greater

abundance of *Prevotella* enterotype, and species like *F. prausnitzii*, which can produce anti-inflammatory factors [207].

Table 19. Multivariable associations of maternal diet with gestational weight gain in week 30 of gestation

Variable	Intake level		Multivariate adjusted odds of excessive GWG[1]
	Mean or median	SD or [IQR]	OR (95 CI)[2]
Total energy intake (MJ/d)[3]	8.5	2.2	1.11 (1.02, 1.20)
Protein intake (E%)[3]			
Protein	16	2	1.12 (0.78, 1.59)[4]
Plant protein[5]	5	1	0.68 (0.55, 0.84)[6]
Carbohydrate quality[7]			
Fiber (g/day)	24	5	0.84 (0.70, 1.01)
Glycemic index (u/d)	71	7	1.13 (0.87, 1.47)
Glycemic load (u/d)	174	[143, 209]	1.04 (0.99, 1.10)
Food groups and diet[8]			
Milk and milk products (glasses/day)[9]	4	2	1.11 (1.00, 1.22)
Cheese (g/day)	20	[14, 40]	0.90 (0.82, 0.99)
Meat and meat products (g/day)	73	42	1.34 (1.08, 1,66)
Fish and fish products (g/day)	17	[9, 26]	0.90 (0.71, 1.15)
Fruits, vegetables and related products (g/day)	493	[400, 622]	1.03 (0.93, 1.14)
Wholegrain bread (g/day)	130	[100, 165]	0.91 (0.79, 1.05)
Vegetarian meal (g/day)	0	[0,80]	0.88 (0.78, 1.00)
Sweets (g/day)	29	[14, 51]	1.20 (1.04, 1.39)
Sugar-sweetened soda and fruit beverages (glasses/day)[9]	1	[0, 3]	1.07 (0.94, 1.22)

Abbreviations: GWG, gestational weight gain*;* OR, odds ratio.

[1] Total GWG was calculated as the difference between the greatest obtained weight in pregnancy and pre-pregnancy weight. This GWG was used to classify women's weight gain according to the IOM guidelines. Excessive GWG was determined in accordance with the IOM recommendations for each pre-pregnancy BMI category, for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg and obese >9 kg.
[2] Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, educational level, gestational age and total energy. [3] We present OR per 1MJ/d for total energy intake, per 5E% for total protein intake and per 1E% for plant protein intake. [4] Multivariate nutrient density model. Total energy intake and percent energy from protein and fat are entered simultaneously into the model. [5] Plant protein included protein from cereals, vegetables, fruits and related products. Animal protein included protein from milk, cheese, ice cream, meat, fish, eggs and related products.
[6] Multivariate nutrient density model. Total energy intake and percent energy from plant protein, carbohydrate and fat are entered simultaneously into the model. [7] We present OR for fiber intake per 5g/day, glycemic index per 10 u/day, glycemic load per 50 u/day. [8] We present OR for milk/milk products per glass/day, cheese per 10g/day, meat/meat products per 50g/day, fish and fish products per 20g/day, fruits, vegetables and related products per 100g/day, wholegrain bread per 40g, vegetarian meal per 50g, sweets per 30g/day and sugar-sweetened beverages per glass/day. [9] One glass is equal to 2 dL.

5.5.2 The PREWICE cohort (paper IV)

Our results from the DaFO88 cohort [209], as well as results from studies using predefined scores and data-driven methods indicate that dietary habits characterized by high consumption of fruits and vegetables, wholegrain, fish, and unsaturated fat and low consumption of food with little nutritional value, is associated with lower risk of excessive GWG and various pregnancy complications [33, 34, 36, 37, 134-137, 150, 210]. Moreover, a recent follow-up of a randomized controlled trial, the UBEAT trial, showed that improved maternal diet and modestly reduced GWG has the potential to reduce infant adiposity [180]. However, translating these results into clinical practice for preventative measures is complicated as the dietary assessments used in these studies are very detailed and time-consuming [151]. In this paper, our aim was, therefore, to examine in the PREWICE cohort whether a short dietary screening questionnaire on food choices and a specific dietary risk score calculated in the first trimester of pregnancy could give indications of risk for excessive GWG and macrosomia.

Table 20. The percentage of women gaining suboptimal, optimal and excessive weight during pregnancy (The Icelandic recommendations)

	GWG (kg) mean ± std.	Suboptimal	Optimal	Excessive
All (n=1326)	14.0 ± 6.3	25%	39%	36%
		Suboptimal (≤12.0 kg)	Optimal (12.1-18.0 kg)	Excessive (> 18.0 kg)
Pre-prengnancy BMI < 25 (n=772)	15.4 ± 5.1	25%	48%	26%
		Suboptimal (≤7.0 kg)	Optimal (7.1-12.0 kg)	Excessive (> 12.0 kg)
Pre-pregnancy BMI ≥ 25 (n=554)	12.0 ± 7.1	25%	26%	49%

Abbreviations: BMI, body mass index; GWG, gestational weight gain.

In total, 1651 women had data regarding offspring birth weight and 1326 had data regarding total GWG (≥36th week of gestation). The mean (SD) total GWG was 14.0 (6.3) kg. Table 20 presents the percentage of women gaining optimal, suboptimal and excessive weight during pregnancy, by pre-pregnancy BMI. We found that, relative to the Icelandic recommendations, 36% gained excessive weight in pregnancy. This proportion is in line with numbers (30-

70%) reported in the literature [8, 20, 21]. Mothers gaining excessive weight were more likely to be overweight or obese. This is in accordance with previous studies showing that high pre-pregnancy and early pregnancy BMI status are strong predictors of excessive GWG, despite absolute weight gains being lower compared to women of normal-weight [7, 20, 119, 120].

When comparing women gaining optimal vs. excessive GWG, more frequent intake of sugar and artificially sweetened beverages (p=0.02) and not as frequent intake of fatty fish (p=0.02), whole grain products (p=0.02), and butter/saturated fat when cooking (0.01), was found in the latter group (Figure 9).

The dietary data were transformed into the 13 predefined dietary risk factors for inadequate diet (See Table 3 in methods). We found that a risk score, including: non-varied diet, non-adequate intake of fruits/vegetables, dairy and whole grain, as well as excessive intake of sugar/artificially sweetened beverages and dairy, was associated with higher risk of excessive GWG and macrosomia. Women with poor dietary habits, i.e., those with the highest dietary risk scores (≥4 scores), had higher risk of excessive GWG (RR=1.24; 95%CI: 1.01, 1.52) and higher odds of offspring being born macrosomic (OR=2.28; 95% CI:1.18, 4.38) than women with the lowest scores (≤2 scores). The association between the dietary risk score and excessive GWG tended to be stronger for women obese before pregnancy, while the association with macrosomia was stronger for women of normal-weight (Table 21).

Being born macrosomic (birthweight ≥4500g) is associated with increased risk of type 2 diabetes mellitus, hypertension, and obesity later in life [211-213]. It is well known that pre-pregnancy obesity and GDM are risk factors for offspring macrosomia [214]. Excessive GWG is also an independent risk factor [8, 214-217], with recent studies indicating that women who are lean before pregnancy should receive more attention in this matter [8, 217]. Our data also support this as more than 40% of the macrosomic cases were among women with normal BMI before pregnancy. Among these women, both excessive GWG (>18 vs. ≤18 kg, OR=2.65; 95% CI:1.21, 5.81) (Table 22) and poor dietary habits (OR=1.43; 95% CI:1.11, 1.86) (Table 21) were associated with higher odds of macrosomia.

Frequency of foods (per week) by gestational weight gain (n=1326)
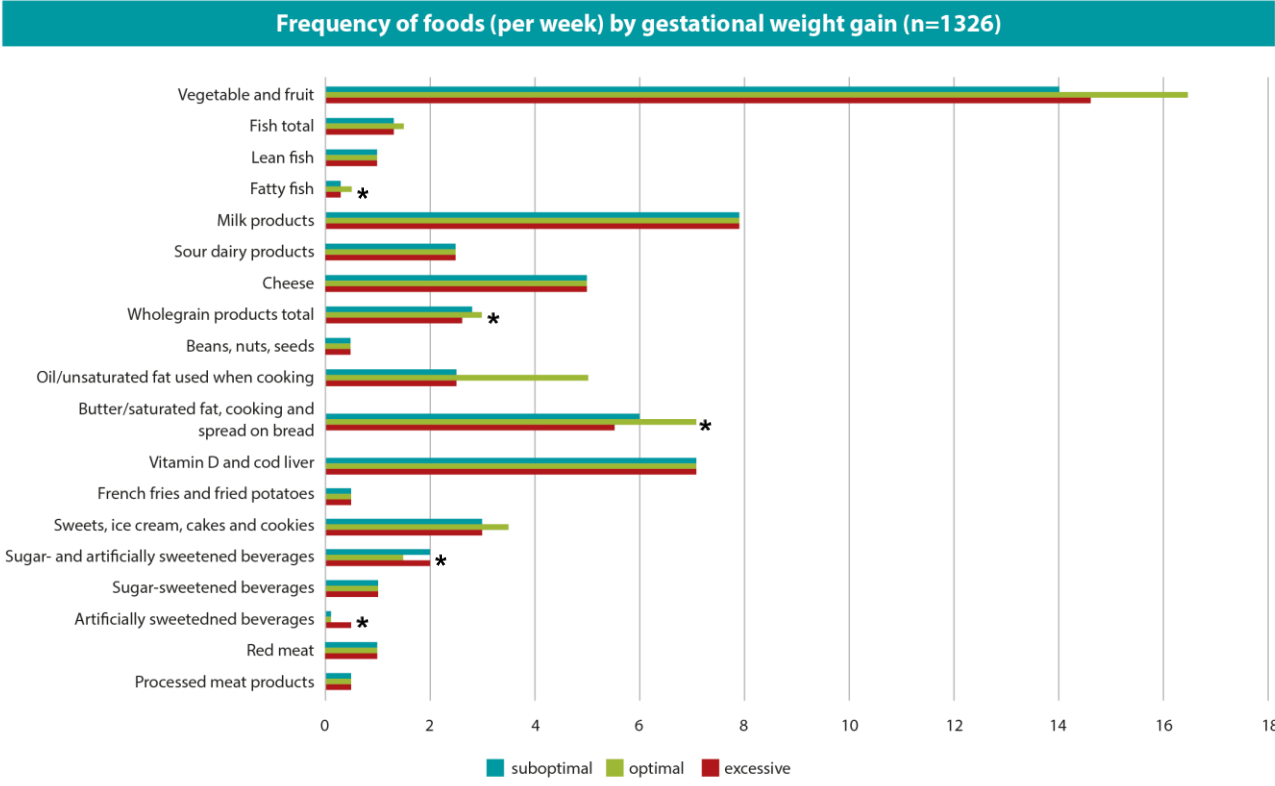
✱ *Indicating significant differences between women gaining optimal vs. excessive gestational weight gain (*Mann-Whitney U test).

Figure 9. Median frequency per week in intake of different foods by adherence to the gestational weight gain recommendation

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Table 21. The association between dietary risk score, GWG and macrosomia

	Excessive GWG			Macrosomia		
	RR (95%CI)[1]			OR (95%CI)[2]		
	cases (%)/n	crude	adjusted[3]	cases (%)/n	Crude	adjusted[4]
Low scores (≤2)	99 (32%)/305	ref	ref	14 (4%)/ 377	Ref	ref
Medium scores (3)	217 (34%)/632	1.06 (0.87, 1.28)	1.04 (0.86, 1.27)	40 (5%)/766	1.43 (0.77, 2.66)	1.40 (0.74, 2.64)
High scores (≥4)	160 (41%)/389	1.27 (1.04, 1.55)	1.24 (1.01, 1.52)	37 (7%)/ 508	2.04 (1.09, 3.83)	2.28 (1.18, 4.38)
Stratified analyses (continuous)[5]						
All women	476 (36%)/1326	1.12 (1.03, 1.22)	1.10 (1.01, 1.19)	91 (6%)/1651	1.36 (1.06, 1.73)	1.43 (1.11, 1.86)
BMI < 25 kg/m2	202 (26%)/772	1.13 (0.99, 1.29)	1.07 (0.94, 1.22)	38 (4%)/950	1.56 (1.07, 2.28)	1.62 (1.09, 2.38)
BMI 25-30 kg/m2	206 (64%)/320	1.05 (0.96, 1.16)	1.05 (0.94, 1.15)	33 (8%)/395	1.45 (0.96, 2.19)	1.53 (0.94, 2.47)
BMI ≥ 30 kg/m2	68 (29%)/234	1.21 (0.93, 1.57)	1.26 (0.96, 1.64)	20 (7%)/306	0.78 (0.45, 1.36)	0.88 (0.46, 1.66)
Early GWG < 2 kg[6]	220 (26%)/840	1.20 (1.05, 1.37)	1.18 (1.03, 1.35)	48 (5%)/1001	1.32 (0.94, 1.86)	1.35 (0.93, 1.96)
Early GWG ≥ 2 kg[6]	222 (57%)/389	1.11 (1.01, 1.22)	1.08 (0.98, 1.19)	29 (6%)/490	1.37 (0.89, 2.11)	1.55 (0.96, 2.49)

Abbreviations: BMI, body mass index; GWG, gestational weight gain.

1. Poisson log-linear regression model, reflecting the risk of excessive GWG. Excessive GWG was determined in accordance with the Icelandic recommendations for underweight and normal weight women >18 kg and overweight and obese women >12 kg total GWG.
2. Logistic regression model, reflecting the odds of giving birth to a macrosomic infant (birthweight ≥4500g).
3. Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, gestational length when highest weight was recorded and nausea
4. Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, total gestational length and offspring sex.
5. When stratifying by pre-pregnancy BMI and early GWG, maternal pre-pregnancy BMI, age, and gestational length were continuous in the adjusted model.
6. In total, 97 had missing values on early GWG of the 1326 women with data regarding exc. GWG and 160 of the women had data on birth weight.

Table 22. The association between excessive GWG and macrosomia

Exc. GWG vs. non exc.GWG[2]	Macrosomia		
		OR (95%CI)[1]	
	cases (%)/n	crude	adjusted[3]
All	77 (6%)/1326	2.38 (1.50, 3.79)	1.86 (1.11, 3.12)
BMI < 25 kg/m2	31 (4%)/772	2.78 (1.34, 5.73)	2.65 (1.21, 5.81)
BMI 25-30 kg/m2	29 (9%)/320	1.25 (0.55, 2.86)	1.21 (0.48, 3.03)
BMI ≥ 30 kg/m2	17 (7%)/234	2.33 (0.86, 6.31)	2.16 (0.69, 6.77)

Abbreviations: BMI, body mass index; GWG, gestational weight gain.

1. Logistic regression model, reflecting the odds of giving birth to a macrosomic infant (birthweight ≥4500g).

2. GWG was determined in accordance to the Icelandic recommendations for each pre-pregnancy BMI category, for lean women (BMI < 25kg/m^2) >18 kg, overweight (BMI 25-30 kg/m^2) >12 kg, and obesity (BMI <30 kg/m^2) >12 kg total GWG.

3. Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, total gestational length and offspring sex.

The relation between diet, GWG and macrosomia may be related to maternal hyperinsulinemia, hyperleptinemia, hypo-adiponectinemia, and inflammation associated with high weight gain [176, 209]. This metabolic profile is associated with excessive nutrient transport at the placental level, which can induce increased fetal growth [218]. Maternal energy intake and dietary glycemic load [205], as well as milk intake [219] have previously been associated with the risk of giving birth to a macrosomic infant. A recent systematic review and meta-analysis of nine randomized controlled trials (n=7458) reported that personalized maternal nutrition guidance could significantly reduce the birth rate of fetal macrosomia (RR= 0.29; 95 % CI:0.18, 0.45) [220]. Interestingly, our study showed an inverse nonsignificant association between the dietary risk score and the odds of macrosomia among obese women. The explanation for this is likely related to the GDM treatment that around half (49%) of the obese women received, which may have resulted in improved dietary habits and lower GWG [221]. Exclusion of women with GDM (n=264) resulted in a similar trend within all the BMI groups (Table 23).

Table 23. The association between the dietary risk score, GWG, and macrosomia (GDM cases excluded *)

	GDM excluded (264 cases excluded)			
	Excessive GWG		Macrosomia	
	cases (%)/n	RR (95%CI)[1,2]	cases (%)/n	OR (95%CI)[3,4]
low scores (≤2)	89 (34%)/266	ref	11 (3%)/332	Ref
medium scores (3)	184 (35%)/533	1.01 (0.82, 1.24)	31 (5%)/645	1.37 (0.67, 2.83)
high scores (≥4)	136 (42%/ 326	1.20 (0.97, 1.49)	30 (7%)/410	2.57 (1.22, 5.40)
Stratified analyses (continuous)[5]				
All women	409 (36%)/1125	1.09 (1.00, 1.19)	72 (5%)/1387	1.51 (1.12, 2.03)
BMI < 25 kg/m2	190 (26)/732	1.07 (0.94, 1.23)	35 (4%)/899	1.72 (1.14, 2.59)
BMI 25-30 kg/m2	182 (66%)/275	1.03 (0.93, 1.14)	26 (8)/333	1.38 (0.79, 2.42)
BMI ≥ 30 kg/m2	37 (31%)/118	1.31 (0.87, 1.97)	11 (7%)/155	1.14 (0.44, 2.98)
Early GWG < 2 kg[6]	195 (27%)/712	1.13 (0.99, 1.31)	38 (5%)/843	1.54 (1.00, 2.39)
Early GWG ≥ 2 kg[6]	186 (57%)/325	1.09 (0.99, 1.22)	23 (6%)/406	1.43 (0.85, 2.42)

Abbreviations: BMI, body mass index; GDM, gestational diabetes; GWG, gestational weight gain.

* The IADPSG criteria were used [221]

1. Poisson log-linear regression model, reflecting the risk of excessive GWG. Excess GWG was determined in accordance with the Icelandic recommendations for underweight and normal weight women >18 kg and overweight and obese women >12 kg total GWG.

2. Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, gestational length when highest weight was recorded and nausea.

3. Logistic regression model, reflecting the odds of giving birth to a macrosomic infant (birthweight ≥4500g).

4. Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, total gestational length and offspring sex.

5. When stratifying by pre-pregnancy BMI and early GWG, maternal pre-pregnancy BMI, age, and gestational length were continuous in the adjusted model.

6. In total, 88 had missing values on early GWG of the 1125 women with data regarding exc. GWG and 138 of the 1651 women with data on birth weight.

As noted in the introduction, results from intervention studies, aiming at improving diet, physical activity or both, have shown that reduction in GWG can be achieved. However, the effect size reported in these studies has been modest (~0.7 kg on average), which is a concern [145]. It has been suggested that it might be too late to change the diet after conception and the focus should rather be on dietary intervention with young women of childbearing age than pregnant women [223, 224]. However, by improving the design of future intervention studies, e.g., recruiting as early in pregnancy as possible, and by screening for higher risk of adverse pregnancy outcomes, greater effects may be seen than in previous studies. Pregnant women can quite easily be reached as many countries offer free prenatal care. Moreover, pregnancy provides a positive setting for families to re-evaluate their health. At this time, the mother-to-be is perhaps more motivated to make changes in her lifestyle [52, 225]. Interestingly, recent results from two German cohorts [226] (n=6665 mother-offspring pairs) found that avoidance of excessive GWG in the third trimester was linked with lower risk of childhood overweight even in cases of excessive weight gain in the first trimester, a known risk factor of excessive GWG [118]. This indicates that implementing a screening method in the first maternal visit to the healthcare clinics (8-14 weeks of pregnancy) might be a suitable time for preventative measures.

Apart from issues relating to recruitment, the reason for the modest effect size seen in behavioral intervention studies [126, 145] may also be related to poor compliance. A commonly used approach in former trials is testing one specific dietary intervention for the whole group, e.g., low glycemic load, calorie-controlled or low-fat diets, independent of the background diet. Now, interest is growing in personalized nutrition, where dietary advice is provided, based on the background diet or the nutritional status of the individual. The main purpose of the dietary screening questionnaire used in this study was to try to develop a screening tool that could be used to identify women at higher risk of suboptimal diets and excessive GWG. However, the results could also give valuable information regarding the nutritional care that could follow. As changing dietary habits is a complicated task, prioritizing and identifying a few factors to focus the work on might be more attainable for women and more practicable in a clinical setting.

The dietary screening questionnaire could very easily be implemented into standard maternity care as it only takes 5-10 minutes to answer the questions. By focusing more on maternal diet, prioritized by urgency and expected impact, healthcare staff could avoid spending time encouraging pregnant women to make changes in dietary intake that are unlikely to result

in improved pregnancy and birth outcomes. This personalized approach, rather than a one-size fits all approach could translate into more cost-effective strategies in primary care. The combined use of a screening and personalized nutrition, based on the results from the dietary screening questionnaire needs further exploration in future studies.

In summary, our results from both the DaFO88 and the PREWICE cohorts stress that dietary interventions promoting healthy diet and adequate weight gain during pregnancy should not focus on targeting overweight and obese women only, but also women of normal weight. The results from the DaFO88 cohort showed that high energy intake was linked with higher risk of excessive GWG. Furthermore, substituting plant-based foods for animal-based foods was linked with substantially lower odds of excessive GWG in our statistical analysis. In the PREWICE cohort, using a short dietary questionnaire, a dietary risk score that included six dietary risk factors (i.e., non-adequate intake of fruits/vegetables, dairy and whole grain, as well as excessive intake of sugar/artificially sweetened beverages and dairy) was identified, and this score was associated with excessive GWG and macrosomia. Our findings indicate that by asking simple questions about women's dietary habits early in pregnancy, we may be able to identify women in more need of support and counseling to meet the GWG recommendations and to find women at higher risk of giving birth to a macrosomic infant. Using the background diet in future interventions and later within primary care, could translate to more cost-effective strategies.

6 Methodological considerations

The strength of our data is the prospective data collection, information from medical records (papers I-IV), and long-term follow-up period with clinical measurements of offspring anthropometry and markers of cardiometabolic health (papers I and II). Observational studies have their pros and cons, and this section provide a methodological discussion covering some of the core epidemiologic concepts relevant to the studies in this thesis.

6.1 Selection bias

The overall participation rates in both the original DaFO88 cohort and the PREWICE cohort were 80%. Participation in birth cohorts has previously been associated with healthier lifestyle behavior [227]. As the participation rate was high, it is less likely that bias because of selection in the original cohort affected our results. The loss to follow-up in papers I and II was however high, with 47% of the original cohort attending the clinical examination. This is an inevitable feature of cohort studies with long-term follow-up. Attrition at the end may introduce bias and reduce the study's power, affecting the generalizability, validity, and reliability of the results [228]. We, however, found no evidence that offspring participation in the follow-up examinations was affected by the maternal characteristics; i.e., there were no significant differences in maternal GWG or macronutrient intake between mothers of offspring who did not attend the clinical examination and the offspring attending the examination at follow-up. Moreover, maternal pre-pregnancy BMI, age, parity, smoking status, and gestational age did not differ between mothers whose offspring later did or did not participate in the follow-up study. However, mothers of offspring who did not attend the clinical examination at follow-up had lower energy intake and lower educational levels.

6.2 Information bias

Information bias occurs when the information about the exposure, covariate or outcome is erroneously recorded, leading to misclassification of participants [229]. Some misclassification is likely to occur in epidemiologic studies and may have influenced our results to some degree.

6.2.1 Weight and GWG measurements

Pre-pregnancy weight and height were self-reported, possibly leading to bias due to misreporting. It is well known that overweight and obese women tend to underreport their weight [139, 230]. Although difficult to predict, some underreporting of weight among normal weight women is more likely than over-reporting [230, 231]. We suspect that potential bias regarding GWG would, therefore, more likely lead to underestimation of the true effect.

Our measure of offspring weight and maternal GWG was based on clinically measured weight. In the DaFO88 cohort maternal weight was recorded in both week 30 (GWG30) and week ~37 (total GWG). Examining GWG to week 30 has the advantage that weight gain at that time should be minimally influenced by fetal weight and maternal edema, compared to using total GWG [232].

6.2.2 Food frequency questionnaire

FFQs have become the method of choice in large epidemiologic studies as they are inexpensive and can assess long-term habitual intake [151]. FFQs may tend to overestimate the amount of consumed foods among women, compared to other methods like dietary recalls and weighed food records [233, 234] We are, therefore, aware that data obtained using an FFQ is useful for ranking individuals but does not necessarily permit confident assessment of the absolute intake. The quantified intake levels are therefore approximate and should be interpreted cautiously. In the main analyses of the DaFO88 cohort we used quintiles of distribution, to report "high vs. low intake", nondifferential misclassification of dietary intake should therefore not have affected the results. The women in the DaFO88 cohort were only asked once about their diet (week 30) and were asked to report it for 12 weeks back in time. Accounting for change in dietary intake was therefore not possible. Nevertheless, dietary intake, pattern and macronutrient composition has not been shown to change largely during pregnancy [235, 236]. The FFQ used in the DaFO88 cohort has been validated against marine foods and biomarkers of n-3 fatty acids (correlation coefficient 0.46), but the list has not been validated against another dietary method [152]. However, the dietary assessment, i.e., combination of an FFQ and structured interview, was designed to assess macronutrient and energy intake more accurately, compared to using an FFQ alone. As we see in Table 19, total energy intake was found to be associated with GWG, indicating that the combination of these two dietary recording methods assessed macronutrient intake relatively accurately. Validation of the short dietary screening questionnaire used in the PREWICE cohort against biochemical analysis is part of an

ongoing project. Its ability to rank subjects according to consumption will be assessed, as well as its ability to detect the risk of predefined nutrient deficiency.

6.2.3 Markers of inflammation and cardio-metabolic health.

The variation and normal fluctuation in inflammatory factors throughout pregnancy are not well established [92, 93, 109]. In the DaFO88 cohort, we had data available about maternal inflammatory markers from a single blood sample drawn in week 30 of gestation. A Statement for Healthcare Professionals from the Centers for Disease Control and Prevention and the American Heart Association recommends an average of two assays for CRP measurements in the non-pregnant population [237]. This procedure may provide a more stable estimate of the level of this marker. However, for at least non-pregnant women, a single measurement has been shown to have a acceptable reliability for epidemiological studies [157].

Although adipose tissue mass is a major factor influencing individual leptin concentrations, other factors like sex steroids, infections, stress and long-term fasting may affect individual leptin levels as well [167]. Leptin concentrations have also been shown to vary during the menstrual cycle [238, 239], which may bias the results for females. It would, therefore, have been preferable to have repeated measurements of offspring leptin levels. However, a within-subject variation of serum leptin concentrations has been shown to be relatively small compared to the variation between individuals [240], and repeated measurements are expensive and not always feasible for epidemiology studies.

Using an automatic blood pressure device, like the OMRON M6 Comfort used in this study, has been reported to be appropriate to reduce observer bias and digit preference, compared to measurements with mercury sphygmomanometer [241]. However, automated blood pressure monitors have been found to slightly underestimate DBP [155, 241]. In our study, the focus was, however, on the difference in blood pressure between the protein intake groups (quintiles of protein intake) rather than examining absolute levels.

6.3 Covariates and confounding

As with all observational studies, causality cannot be inferred from this study and the possibility of residual confounding or confounding by unmeasured

factors cannot be excluded. We employed an a priori approach to handle confounding in both cohorts, i.e., we decided which variables should be included as potential confounders in our multivariable models before the analyses were conducted. We based this decision based on the existing literature. The advantage of this approach is that explorative analyses to identify potential confounders were avoided.

As previously noted, we were not able to account for genetics when looking at offspring long-term outcomes, but we were able to consider confounding by important familial dietary and offspring lifestyle habits. For example, in paper I, offspring were asked at the time of the follow-up whether they thought their father was overweight. This information was used as a proxy for unmeasured confounding by shared familial factors. Since the obesity epidemic became virulent, studies have focused more on risk groups, i.e., overweight and obese women, while women of normal weight have received less attention. In the DaFO88 cohort, we had the opportunity to work with data collected before the obesity epidemic (1988-1989), when most women of fertile age were normal weight (81% of the total cohort). Examining the relation focusing on women of normal weight before pregnancy lowers the risk of confounding related to shared genetics and suboptimal family lifestyle resulting in obesity. When we compared the effect estimates from our crude models to those from adjusted multivariable models in papers I-III, no marked changes were observed, suggesting limited confounding in our analyses. However, we cannot exclude the possibility that we left out potential confounders. For example, in paper II, higher protein intake during early childhood significantly influences the child's growth pattern [242], but accelerated weight gain in early life has been associated with the risk of adult hypertension. We were unable to account for offspring's own protein intake in our analyses. Interestingly, higher protein intake in adulthood may have an opposite influence on blood pressure regulation than early life exposure, as studies on adult individuals have found that replacing dietary carbohydrate with protein in adulthood is associated with a reduction in blood pressure [243]. Moreover, more detailed data regarding physical activity (both maternal and offspring physical activity) would have been valuable when looking at outcomes related to maternal inflammation and offspring's later health.

6.4 Multiple testing

In this thesis, we examined several outcomes. The more inferences are made, the more false inferences are likely to occur (i.e., type I error) [244]. When the same test is repeated in a subsample, for example when conducting stratified

analyses, like in our case by sex in paper I, adjustment for multiple comparisons is appropriate [245]. To counter this problem, we used the Bonferroni correction. Using this method in paper I, the associations for HOMA-IR and blood pressure would not be considered significant, whereas the associations for serum leptin, insulin levels, and blood lipids (i.e., total cholesterol and LDL cholesterol) were robust after the correction ($p<0.0013$).

In our analyses, we found that the reported associations were not just found in the overall analyses but also in subgroup analyses, which indicates decreased risk of chance finding. The Bonferroni adjustment is considered to be overly conservative and has been criticized as it increases the risk of type II error – false acceptance of the null hypothesis, and this has to be taken into account when deciding whether this correction is appropriate or not [245].

6.5 Generalizability

It is important to note the time discrepancies between the Danish and the Icelandic study. The recruitment to the DaFO88 cohort went on in 1988 and 1989. The pregnant women included in the DaFO88 cohort may therefore not resemble pregnant women in the general population today with respect to health behaviors, which may affect the generalizability of our results. The Danish population was white, fairly well educated and mostly of normal-weight. Whether the results from the three first papers presented in this thesis could be applied to other populations today, perhaps with higher BMI or at higher cardiovascular risk is unclear. However, they show associations important to report. Likewise, the methodology used in the PREWICE data was based on both predefined (dietary recommendations) and data-driven (stepwise backward elimination) methods, tested in an Icelandic population. The results might therefore not be mirrored in other populations with different dietary patterns. However, the same methodology could be used to identify dietary predictors for excessive GWG in other populations.

7 Conclusions

This thesis addressed relevant questions on maternal diet and weight gain during pregnancy. Together, the studies highlight the importance of maternal nutrition in terms of short- and long-term outcomes;

The results showed a weak positive association between gestational weight gain and offspring BMI at 20 years. The relationship between GWG and offspring BMI was similar for both male and female offspring, whereas, for biomarkers of cardio-metabolic health, maternal GWG was associated with higher leptin and insulin levels in male offspring only. GWG was, however, inversely associated with levels of total cholesterol and LDL levels in females. Differences in lifestyle habits may have accounted for these sex-specific differences.

Higher intake of protein during pregnancy was found to be associated with slightly higher offspring blood pressure when substituted for carbohydrates. When examining the protein source, comparable results were found for animal versus plant protein intake, although the associations were slightly stronger for the former. Interestingly, a more pronounced association was noted between maternal protein intake from milk and milk products and offspring blood pressure; however, as milk consumption was the main protein source in our cohort, we are unable to infer whether the association between maternal protein intake and offspring blood pressure may be due to the dairy protein or just to protein per se.

Our results indicate that both GWG and diet are related to inflammatory status in pregnant women. Our results indicate that high consumption of animal protein during pregnancy may be pro-inflammatory, whereas plant-based protein may be anti-inflammatory. These associations seem to be independent of maternal complications during pregnancy.

In the DaFO88 cohort, we noted that eating more plant-based protein sources at the expense of animal protein was associated with lower odds of excessive GWG. Total energy intake and intake of milk/milk products, meat/meat products, and sweets were also associated with higher risk of excessive GWG. In support of this finding, we found in the PREWICE cohort that a dietary risk score (characterized by: a non-varied diet, not-adequate frequency of consumption of fruits/vegetables, milk and whole grain intake, as well as

excessive intake of sugar/artificially sweetened beverages and milk) was associated with higher risk of excessive GWG and macrosomia. Our results indicate that it is possible to find women potentially at increased risk of excessive GWG by asking simple questions about their dietary habits early in pregnancy.

From a public health point of view, the scientific value of this project is high. An increasing number of women are gaining more weight during pregnancy than is currently recommended, followed by increased prevalence of pregnancy/ birth complications and obesity. The results from this project gave important information seen from different angles, not only regarding the possible long-term consequences of suboptimal diet and excessive GWG on offspring metabolic health but also in terms of short-term outcomes. The clinical implications of our results indicate that interventions promoting healthy GWG and diet during pregnancy should not focus on targeting only overweight and obese women, but also women of normal weight, who today get limited attention regarding this matter in the primary health care setting. The results indicate that using the background diet as a recruitment tool in lifestyle intervention studies might be of value. The dietary screening questionnaire could be used to aim dietary counseling towards more vulnerable groups. Doing this could translate into more cost-effective strategies within primary care.
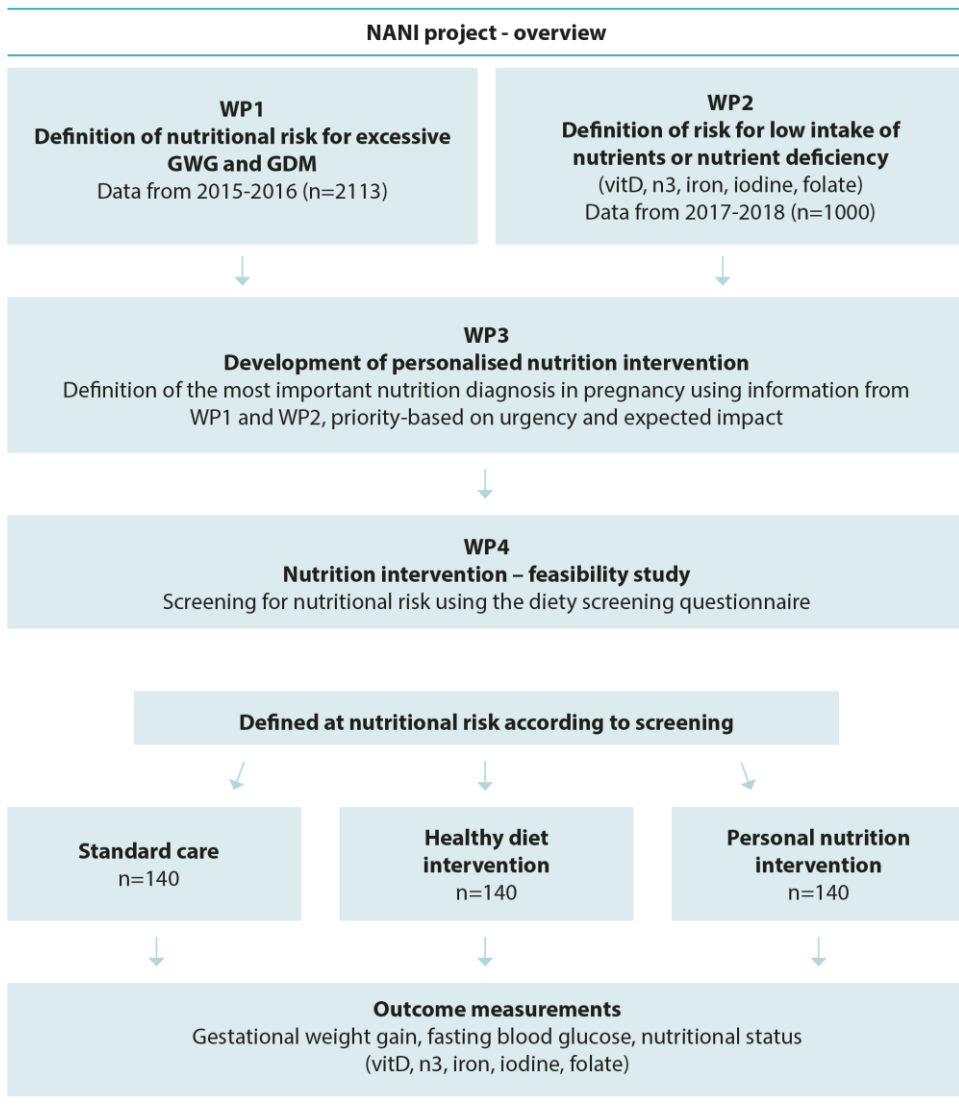
8 Future perspectives

The US Institute of Medicine has acknowledged that more information is needed on the role of excessive GWG in offspring long-term health. Presently, few studies have data regarding offspring adult cardio-metabolic health. Interestingly, the results from paper I as well as from previous studies [13, 66] indicate that offspring of women with suboptimal GWG tend to have lower BMI and more favorable cardio-metabolic outcomes, compared to the offspring of mothers with optimal GWG. Establishing optimal GWG for both short- and long-term outcomes may, therefore, be complex, and there is a need to examine whether the relatively modest shifts observed in our study (paper I) and in those of others [12, 13, 66] become more clinically relevant later in adulthood. Using information from pharmaco-registries could, for example, present the opportunity to analyze the relationship between GWG and offspring use of drugs related to cardiovascular and metabolic disturbances later in life. Further research is also important to establish if previous findings are truly caused by GWG or confounded by familial postnatal diet and lifestyle. Long-term follow-up of offspring of mothers that participated in recent maternal dietary interventions that led to a modest reduction in GWG will provide further insights into the causality of these observed associations [132, 146, 246]. Future studies may also gain from incorporating maternal body composition assessment in addition to GWG, as excess fat mass rather than GWG may give more valuable information, in terms of both pregnancy complications and long-term offspring outcomes.

Our results showed that maternal dietary protein intake was linked with a modest increase in offspring blood pressure in young adulthood. Moreover, the origin of macronutrients, i.e., plant vs. animal-based protein sources, may also be important regarding levels of inflammation during pregnancy. Because of the increased popularity of high-protein diets, a better understanding of the health consequences (short-and long-term) of such regimens during pregnancy and possible underlying mechanisms is needed. Moreover, the importance of the timing and duration of exposure to high protein foods needs further exploration. Studies in animal models are important to examine the underlying mechanisms and to determine whether these relations are truly caused by the protein component per se or related to other factors found in protein-rich foods, such as salt, saturated fat, iron, AGEs or D-galactose.

Furthermore, the perceived impact of increasing the intake of one macronutrient or specific foods may also be related to lower intake of the other macronutrients or other factors in foods that people substitute. It is vital that observational studies take this into consideration when looking at diet-disease associations [161, 183].

Furthermore, suboptimal diet and excessive GWG are potentially modifiable risk factors for several adverse pregnancy/neonatal outcomes and later obesity in mothers and their offspring. In addition, most dietary and lifestyle intervention studies have not yet found clear results and initiatives in terms of restricting excessive GWG. Thus, thinking outside the box in future research is needed. Our results indicate that it might be more logical to recruit women into dietary intervention studies, based on their background diet. However, testing the short dietary screening questionnaire in other datasets will allow drawing more rigorous conclusions. The next step is to validate the dietary screening questionnaire against a biochemical analysis. This is part of an ongoing project to test the questionnaire's ability to rank subjects according to consumption and to detect the risk of nutrient deficiency. Moreover, the plan is to recruit women for a feasibility intervention study. There we will test whether recruiting women for an intervention early in pregnancy, based on their "nutritional risk" (i.e., based on their background diet), and by using different nutritional counseling methods, may result in better outcomes (GWG, fasting blood glucose and nutritional status), than standard care (Figure 10). The abbreviation for this feasibility study is **NANI**: Towards a **N**ew **A**pproach of recruiting for and executing **N**utrition **I**nterventions during pregnancy.

NANI project - overview

WP1
Definition of nutritional risk for excessive GWG and GDM
Data from 2015-2016 (n=2113)

WP2
Definition of risk for low intake of nutrients or nutrient deficiency
(vitD, n3, iron, iodine, folate)
Data from 2017-2018 (n=1000)

WP3
Development of personalised nutrition intervention
Definition of the most important nutrition diagnosis in pregnancy using information from WP1 and WP2, priority-based on urgency and expected impact

WP4
Nutrition intervention – feasibility study
Screening for nutritional risk using the diety screening questionnaire

Defined at nutritional risk according to screening

Standard care
n=140

Healthy diet intervention
n=140

Personal nutrition intervention
n=140

Outcome measurements
Gestational weight gain, fasting blood glucose, nutritional status
(vitD, n3, iron, iodine, folate)

Abbreviations: GDM, Gestational diabetes mellitus; GWG, gestational weight gain; n3, Omega-3 fatty acids; vitD, Vitamin D; WP, work package.

Figure 10. Future perspectives - Overview of the NANI project

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Papers I-IV

Paper I

ORIGINAL ARTICLE

Gestational weight gain in normal weight women and offspring cardio-metabolic risk factors at 20 years of age

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OBJECTIVE: Limited knowledge exists on the long-term implications of maternal gestational weight gain (GWG) on offspring health. Our objective was to examine whether high GWG in normal weight women is associated with adult offspring cardio-metabolic risk factors.

METHODS: We used a cohort of 308 Danish women who gave birth in 1988–89 and whose offspring participated in a clinical examination at 20 years of age. Main outcome measures were offspring body mass index (BMI), waist circumference, weight-regulating hormones, blood lipids and glucose metabolism. Associations were assessed using multivariable linear and logistic regression models.

RESULTS: A weak positive association was observed between GWG during the first 30 weeks and offspring anthropometry. Each 1-kg increase in maternal GWG was associated with 0.1-kg m^{-2} higher (95% confidence interval (CI): 0.01, 0.2) offspring BMI and 10% (95% CI: 0.1%, 20%) higher odds of offspring overweight at the age of 20 years, with similar associations observed in both sexes. However, sex differences were observed for the association between maternal GWG and specific cardio-metabolic risk factors. Hence, a 1-kg increase in GWG was associated with 3.4% (95% CI; 0.8, 6.0) higher homeostasis model assessment-estimated insulin resistance (HOMA-IR), 3.7% (95% CI: 1.4, 6.2) higher insulin and 10.7% (95% CI: 5.7, 15.9) higher leptin levels in male offspring. These associations were not observed in females, which may partly be explained by more frequent reports of dieting and physical exercise at follow-up among female offspring.

CONCLUSIONS: In normal-weight women, high GWG may have modest long-term implications on offspring cardio-metabolic risk factors at adult age.

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INTRODUCTION

Maternal nutrition is important for foetal health, and this presumption is supported by substantial evidence showing that the *in utero* environment is a strong determinant for later risk of disease in the offspring.[1] For example, both maternal under- and overnutrition have been linked to greater adiposity in the offspring and a number of different metabolic events later in life.[2–4] Excessive weight gain during pregnancy is primarily caused by imbalance between energy expenditure and intake, although other factors such as fluid retention (oedema) may play a role. For pregnant women, such imbalance may affect the health of the mother and the offspring, as high gestational weight gain (GWG) has been associated with unfavourable outcomes, including macrosomia, emergency caesarean delivery and postpartum weight retention.[5] As a result, the US Institute of Medicine (IOM) has issued guidelines for optimal pregnancy weight gain.[5] These guidelines vary according to pre-pregnancy body mass index (BMI) and are specifically aimed at promoting optimal pregnancy and birth outcomes. The IOM has, however, acknowledged that more information is needed on the role of excessive GWG on offspring long-term health.

Recent findings suggest that maternal GWG may be associated with offspring anthropometric measures during different life stages,[6–8] whereas other studies have found no association.[9–11] A limitation of many studies investigating the relationship between GWG and offspring adiposity is the difficulty to factor-out potential confounding by familial dietary and lifestyle habits postpartum. Association between GWG and comorbidities of adiposity such as weight-regulating hormones and other cardio-metabolic risk factors has also been minimally explored.

High maternal pre-pregnancy BMI is considered an independent risk factor for offspring overweight and obesity.[3,12,13] Although the causality of this relationship is unclear,[14] maternal overweight and obesity may mask the potential association of GWG with offspring later health. Hence, the potential influence of GWG on offspring adiposity and disease may be different in normal weight women.[15–17] The aim of this study was to examine the associations between GWG in women of normal weight and offspring anthropometry and cardio-metabolic risk factors at 20 years of age.

SUBJECTS AND METHODS

The birth cohort

Details about the cohort (DAFO88) and its dietary component have been described elsewhere.[18–20] A total of 965 out of 1212 eligible women participated in the study and were recruited from April 1988 to January

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1989 in Aarhus, Denmark. These women all had a singleton pregnancy and were scheduled to attend a routine midwife visit in gestational week 30. At the time of the follow-up study (in 2008–2009), the offspring were 19–20 years old. The study was approved by the Danish Data Protection Agency and the Central Denmark Region Committees on Biomedical Research Ethics (Reference No. 20070157). Participants provided written informed consent at recruitment.

Offspring follow-up

At follow-up, a total of 915 offspring of the 965 mothers were contacted and asked to fill out a web-based questionnaire concerning their anthropometry, current health and lifestyle. Of the 688 offspring who agreed to participate in the follow-up, 438 offspring attended a clinical examination. Of these offspring, 35 were born to mothers in underweight (pre-pregnancy BMI < 18.5), 308 to mothers in normal weight (pre-pregnancy BMI 18.5–24.9), 32 to overweight or obese mothers (pre-pregnancy BMI ⩾ 25) and 63 had missing values on their mother's weight gain during pregnancy. As the original cohort was a lean population (81% of the women had pre-pregnancy BMI 18.5–24.9 kg m^{-2}), we decided to restrict our analysis to normal weight women. The final data set therefore consisted of 308 mother–offspring pairs (45% of the follow-up cohort).

Mothers (in normal weight) of offspring not attending the clinical examination had a lower energy intake compared with mothers of offspring attending the examination (8.4 vs 8.7 MJ d^{-1}). Lower educational level and primi- and multiparity (43 vs 34%) were also more common among mothers of offspring not attending the clinical examination (data not shown).

Measurement of exposure variables and covariates

Prior to the routine midwife visit in gestational week 30, dietary and lifestyle questionnaires, including questions on: dietary habits, pre-pregnancy BMI lifestyle and socioeconomic factors, were mailed to the women. During the visit, the questionnaires were returned, the responses were corroborated by trained personnel and a dietary interview was conducted. Information on offspring birth weight was retrieved from birth certificates and weight measurements at week 30 and at the end of pregnancy were retrieved from clinical records and records from antenatal visits. The exposure variables were maternal GWG at week 30 (GWG30) and the total GWG (GWGtotal). GWGtotal was calculated as the difference between the greatest obtained weight in pregnancy and pre-pregnancy weight. GWG30 was used as a continuous term and the GWGtotal was used to classify women's weight gain according to the IOM guidelines (suboptimal (< 11.5 kg), optimal (11.5–16 kg) and excessive (> 16 kg)).[5] Examining weight gain up to week 30 has the advantage that GWG at that time should be minimally influenced by foetal weight and maternal oedema, compared with using GWGtotal. In contrast, the definition of optimal weight gain during pregnancy is based on GWGtotal and therefore facilitates comparison with current IOM recommendations.

For the 308 subjects included in our analyses, there were missing values of either GWGtotal or GWG30 (but not both) for 12 subjects. Missing GWG values for these 12 subjects were imputed based on the predicted value (GWG30 = 2.50+0.55*GWGtotal) using linear regression for the 296 women with complete data on both GWG measures.

Measurement of outcome variables

Offspring's clinical examination included standard anthropometric measurements, that is, height, weight and waist circumference, and a collection of a fasting (10 h) blood sample. The blood sample was centrifuged and frozen at − 80 °C. Serum leptin and adiponectin concentrations were determined at the Medical Research Laboratories in Aarhus, Denmark, by in house-validated assays and carried out as described previously.[23] Briefly, serum leptin concentrations were measured by time-resolved immuno-fluorometric assay based on commercially available reagents (R&D Systems, Abingdon, UK) and recombinant human leptin as standard. Adiponectin levels were also determined by a time-resolved immuno-fluorometric assay based on two antibodies and recombinant human adiponectin (R&D Systems). Plasma insulin levels were measured using a commercial ELISA kit (DAKO, Glostrup, DK). The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated as: Fasting glucose (mmol l^{-1})*fasting insulin (mU l^{-1})/22.5. Serum triglycerides, total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein were measured according to standard methods on a Modular P (Roche Diagnostics, Basel, Switzerland). Three readings of systolic blood

pressure, diastolic blood pressure (DBP) and resting pulse were recorded with an automatic blood pressure device (OMRON M6 Comfort (HEM-7000-E), OMRON HEALTHCARE CO., LTD., Kyoto, Japan) and the mean of each was used.

Statistical analysis

Multivariable regression models were used to investigate the associations between maternal GWG and offspring outcomes at follow-up, with effect estimates presented either as mean change (linear regression) or odds ratios (logistic regression) with 95% confidence intervals (CI). As a measure of an association, we employed trend tests (t-test for linear regression, chi-square test (type III test) for logistic regression) entering GWG either as a continuous variable or as dummy variables (1 = suboptimal, 2 = optimal, 3 = excessive GWG) when coding GWG according to IOM guidelines. Owing to skewed distributions, insulin, leptin, adiponectin, total cholesterol, triglyceride, LDL and high-density lipoprotein levels were transformed using the natural logarithm. The estimates from regression models were back-transformed via exponentiation to facilitate interpretation as a percent change.

The following confounders were chosen a priori: offspring's sex, mother's age (in quartiles), pre-pregnancy BMI (in quartiles), parity (0, 1, ⩾ 2), smoking status (non-smoker, < 10, ⩾ 10 cigarettes per day), educational level (elementary schooling, high school or technical schooling, university education (bachelor's degree), higher academic (master's and doctoral degrees), other education) and whether offspring thought their father was overweight (no, yes, missing category). In sensitivity analyses, adjustments were made for intermediary factors such as birth weight, gestational age, offspring's smoking status (non-smoker, occasional, daily), offspring's alcohol consumption (⩾1, 2–3, 4–6, ⩾ 7 times per month), whether offspring reported being on a diet (yes/no) or exercising (yes/no) to lose weight, offspring leptin levels and BMI (when analysing serum biomarkers) at 20 years of age.

Multiple imputations as implemented in SPSS were used to impute missing covariates (mother;s smoking status (3.2%), educational level (5.8%), gestational age (3.2%), offspring's alcohol (8.4%) and smoking habits (3.9%).

Additional analyses regarding the association between offspring dieting and exercising and cardio-metabolic outcomes were performed with Student's t test and Mann Whitney U test. All analyses were done in SPSS 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Maternal characteristics are described in Table 1. The mean total GWG was 14.4 ± 4.9 kg and the mean duration of gestation was 283 ± 10 days. According to the IOM guidelines, 27% of the mothers gained inadequate weight, 44% gained appropriate weight and 29% gained excessive weight. Women with excessive GWG had longer pregnancy duration and their offspring had, as expected a higher birth weight compared with women with optimal or suboptimal weight gain.

At ~ 20 years of age, the mean BMI of offspring attending clinical examination was 22.3 ± 3.0 kg m^{-2} and 17.2% of the offspring were overweight or obese (BMI ⩾ 25 kg m^{-2}), which is comparable to the results from The Danish Health Examination Survey 2007-2008.[21] Offspring characteristics at follow-up are shown in Table 2. Cigarette smoking among females was the only examined questionnaire-based characteristic that was associated with GWG. Distribution of offspring cardio-metabolic biomarkers by sex can be found in online supplementary information.

Multivariable association between GWG and offspring BMI and waist circumference

After adjusting for covariates, each 1-kg increase in maternal GWG30 was associated with 0.1 kg m^{-2} higher (95% CI 0.01; 0.2) offspring BMI and 1.1 (95% CI; 1.01; 1.2) higher odds of offspring being overweight at age 20 (Table 3). Furthermore, 1.8 higher odds (95% CI; 0.9; 3.8) of offspring being overweight was observed when mothers had excessive GWG compared with mothers with optimal weight gain (P for trend = 0.01). A positive, non-significant

Table 1. Birth outcomes and characteristics of mothers at baseline in relation to maternal gestational weight gain

Mothers' characteristics[a]	All	GWG according to Institute of Medicine guidelines			P-value
	(n = 308)	Suboptimal (< 11.5 kg) (n = 83)	Optimal (11.5–16 kg) (n = 136)	Excessive (> 16 kg) (n = 89)	
Pre-pregnancy BMI (kg m^{-2})	21.0 ± 1.5	21.0 ± 1.5	21.0 ± 1.5	21.2 ± 1.4	0.31[b]
Age (years)	29.0 ± 4.9	29.6 ± 4.2	28.8 ± 4.1	28.8 ± 3.7	0.31[b]
Gestational age (days)	283 ± 10	281 ± 12	282 ± 9	286 ± 9	< 0.01[b]
Weight gain 30 week (kg)	10.4 ± 3.6	7.2 ± 2.4	10.0 ± 1.8	13.9 ± 3.5	< 0.01[b]
Total energy intake (MJ per day)	8.7 ± 2.1	8.7 ± 2.6	8.6 ± 1.9	8.8 ± 2.0	0.82[b]
Birth weight (kg)	3.5 ± 0.5	3.3 ± 0.5	3.5 ± 0.4	3.7 ± 0.5	< 0.01[b]
Parity (%)					0.58[c]
0	65	71	62	65	
1	25	19	29	25	
2+	9	10	9	10	
Smoking (%)					0.14[c]
Never	61	62	65	54	
< 10 cigarettes per day	18	15	14	27	
⩾ 10 cigarettes per day	22	24	21	20	
Education (%)					0.66[c]
Elementary schooling	9	10	10	6	
High school/technical school	20	25	16	23	
University education	42	35	47	40	
Higher academic	19	21	17	20	
Other education	10	9	10	11	

Abbreviations: BMI, body mass index; GWG, gestational weight gain. [a]Values are means ± s.d.s for continuous variables and percentages for categorical variables. [b]F-test (Type III) of differences among groups. [c]Chi-square test of differences among groups.

association was also observed between maternal GWG30 and offspring waist circumference. For each 1-kg increase in GWG30 the increase in offspring BMI was similar for both sexes, 0.12 kg m^{-2} (95% CI: − 0.03, 0.27) and 0.10 kg m^{-2} (95% CI: − 0.02, 0.23) in males and females, respectively, and comparable estimates were also observed for waist circumference (data not shown).

Table 4 shows the mean increment in offspring metabolic biomarkers per 1-kg change in maternal GWG30 (mean difference by IOM categories of maternal GWG can be found in online supplementary information. In adjusted models, maternal GWG30 (each 1-kg increase) was associated with 3.7% higher offspring serum leptin levels (95% CI: 1.2, 6.4). The association was primarily driven by male offspring, where each 1-kg increase in maternal GWG was associated with 10.7% (95%CI: 5.7, 15.9) higher leptin levels among male offspring compared with 0.4% (95%CI: − 2.4, 3.3) among female offspring. For mothers who gained excessive weight during pregnancy, this increment corresponded to 93% (95%CI: 30, 186) higher leptin levels among male offspring (online supplementary data). Moreover, each 1-kg increase in GWG30 was also positively associated with DBP, resting pulse, HOMA-IR and insulin levels among the male offspring only. When taking into account multiple comparisons in Table 4, the associations for HOMA-IR and DBP would not be considered significant whereas the associations for resting pulse, serum leptin and insulin levels were robust for this correction.

Among female offspring, significant inverse associations were, however, found with offspring blood-lipid levels. Differences in behavioural responses to increased weight may account for these sex differences as more frequent reports of dieting (16 vs 8%) and physical exercise (41 vs 26%) at follow-up was observed among female offspring compared with males (Table 2).

In comparison with offspring of mothers with optimal GWG, children of those with sub-optimal GWG tended to have lower BMI (Table 3) and more favourable cardio-metabolic outcomes (online supplementary data), for example, offspring of mothers with suboptimal GWG tended to have 10% lower insulin levels (95% CI:

− 20, 1) and 7% lower leptin levels (95% CI: − 25, 15) compared with offspring of mothers with optimal weight gain (P for trend < 0.05 for both outcomes).

Additional analyses

In our sensitivity analyses, we found that taking birth weight and gestational age into account, the effect sizes were attenuated slightly for offspring BMI (β went from 0.10–0.08 per 1-kg increase during the first 30 weeks of gestation). However, these additional adjustments did not attenuate the observed associations of GWG with offspring cardio-metabolic outcomes. For example, β for serum leptin levels went from 3.7 to 3.6% per 1-kg increase in GWG during the first 30 weeks of gestation when also adjusting for birth weight and gestational age. Adjusting for offspring smoking, alcohol habits and BMI (when analysing serum biomarkers) at the age of 20 did not appreciably alter effect estimates (online supplementary information).

However, effect sizes were significantly attenuated for DBP, HOMA-IR and insulin levels when adjusting separately for offspring leptin levels (data not shown).

In our models, we adjusted for paternal overweight reported by the offspring at follow-up (yes/no) as a proxy for confounding for familial lifestyle. Information about maternal overweight at follow-up reported by the offspring (see Table 2) was not included because this variable did not affect our estimates (data not shown), most likely because this adjustment was already accounted for by pre-pregnancy BMI.

Given the sex-specific differences observed for cardio-metabolic outcomes in Table 4, we formally tested effect modification by sex by including GWG (continuous variable), sex (binary variable) and an interaction term between the two in the regression model, along with the remaining covariates. Statistically significant interactions (P < 0.05) were observed for serum leptin levels, insulin, HOMA-IR, resting pulse, total cholesterol and LDL cholesterol.

Table 2. Characteristics of offspring at follow-up in relation to maternal gestational weight gain

Offspring characteristics[a]	All	GWG according to Institute of Medicine guidelines			P-value
		Suboptimal (< 11.5 kg)	Optimal (11.5–16 kg)	Excessive (> 16 kg)	
	(n = 308)	(n = 83)	(n = 136)	(n = 89)	
Sex (%)					0.50[b]
Male	39	39	43	35	
Female	61	61	57	65	
Male					
Self-perceived weight: too high (%)	17	17	13	24	0.43[b]
On a diet to lose weight (%)	8	3	7	14	0.34[b]
Exercise to lose weight (%)	26	19	21	36	0.21[b]
Smoking (%)	52	55	41	69	0.17[b]
Alcohol consumption (%)					
⩾ 7 times per month	25	41	22	14	0.15[b]
Female					
Self-perceived weight: too high (%)	45	35	45	55	0.11[b]
On a diet to lose weight (%)	16	12	13	22	0.23[b]
Exercise to lose weight (%)	41	41	37	43	0.77[b]
Smoking (%)	37	27	27	59	0.01[b]
Alcohol consumption (%)					
⩾ 7 times per month	13	12	11	16	0.12[b]
Maternal overweight (%)[c]	17	15	13	31	< 0.01[b]
Paternal overweight (%)[d]	19	21	20	23	0.82[b]
Sibling overweight (%)[e]	7	10	6	10	0.49[b]

Abbreviations: GWG, gestational weight gain. [a]Offspring characteristics, questions answered in the web-based questionnaire. [b]Chi-square test of differences among groups. [c]The proportion of offspring who think their mother is overweight. [d]The proportion of offspring who think their father is overweight. [e]The proportion of offspring who think their sibling is overweight.

DISCUSSION

In a study of women with normal pre-pregnancy weight, we observed a weak positive association between GWG and BMI in both male and female offspring at 20 years. In addition, our results suggest that GWG is adversely associated with offspring biomarkers of cardio-metabolic health in male but not female offspring. GWG was, however, inversely associated with levels of total cholesterol and LDL levels among females. Differences in lifestyle habits may account for these differences as we observed higher prevalence of physical activity and dieting among female offspring.

Accumulating evidences indicate that GWG is associated with offspring BMI in childhood.[7] Our findings suggest that this association may extend to adulthood, which is in accordance with other recent findings.[6] Current recommendations on optimal weight gain are primarily based on limiting pregnancy complications and promoting optimal foetal growth. In accordance with previous studies,[6,7,22] we found that in comparison with offspring of mothers with optimal GWG, children of those with sub-optimal GWG tended to have lower BMI and more favourable cardio-metabolic outcomes (online supplementary data). Establishing optimal GWG for short- and long-term outcomes may therefore be complex and there is a need to examine whether the modest shifts observed in our study and by others[6,7,22] become clinically relevant later in adulthood.

In our study, the relationship between GWG and offspring BMI was similar for both male and female offspring, whereas for biomarkers of cardio-metabolic health, maternal GWG was relatively strongly associated with higher leptin and insulin levels, HOMA-IR index, DBP and resting pulse in male offspring only. The association for HOMA-IR was driven by greater insulin levels as fasting blood sugar was not related to GWG, which is not surprising given the young age of the offspring.[23] Effect sizes were significantly attenuated for DBP, HOMA-IR and insulin levels when

adjusting separately for offspring leptin levels, which indicates that these modest shifts in offspring cardio-metabolic biomarkers may be mediated through increased adiposity. A more favourable inverse association between GWG and total and LDL cholesterol was, however, observed for female offspring. Although most studies have not reported sex-specific differences, Mamun et al.[15] observed a stronger relationship between maternal GWG and offspring BMI in males compared with females at the age of 21. Furthermore, animal studies have reported that male and female offspring exhibit different programmed outcomes following insults in utero.[24] For example, disturbed glucose homeostasis has been reported in male offspring of over-nourished mothers, despite both sexes displayed elevated levels of adiposity compared with controls.[25,26]

The sex difference we observed for biomarkers of cardio-metabolic health could also be related to differences in behavioural responses to increased weight. Compared with males, female offspring of mothers gaining excessive weight during pregnancy were almost twice as likely to report that they thought their weight was too high and that they were trying to lose weight by dieting and exercising (Table 2). We also noted that although BMI levels were slightly higher among females on a diet compared with those not dieting, total cholesterol levels were significantly lower (P = 0.02) in females dieting compared with females not on a diet (data not shown). This may explain why inverse association between maternal GWG and blood lipids were observed in female but not in male offspring. These speculations are strengthened by differences observed in offspring resting pulse, that is, maternal weight gain was associated with higher resting pulse among male offspring, whereas a non-significant inverse association was observed for females (Table 4 and online supplementary data). Physical activity and fitness is known to improve biomarkers of cardio-metabolic health relatively rapidly,[27,28] while reducing weight takes longer time to achieve. However, adjusting for

offspring dieting and physical activity did not change estimates which could be related to the fact that we did not have information about the intensity of the diet. Not answering yes to 'being on a diet' does not exclude that females were not in

general still relatively more active and had more preferences for healthy foods compared with males. Being able to account for the inverse association between GWG and blood lipids had required an accurate assessment of dieting and physical activity. We therefore speculate that 'programming' by maternal GWG may be mitigated by offspring behavioural responses that are more prevalent among female offspring.

The mechanism by which maternal GWG could influence later health of offspring is currently not well understood. The association with offspring anthropometry could be mediated by the effect of GWG on birth weight and therefore reflect tracking in size across the life course.

The magnitude of the associations between GWG and offspring BMI decreased slightly after birth weight and gestational age were added to the model, which suggest that this association might be partly mediated by foetal growth. Additional adjustment for birth weight did, however, not alter associations regarding cardio-metabolic outcomes. We also had two measurements of maternal weight gain, that is, at week 30 and highest obtained GWG, but slightly stronger associations were observed with GWG at week 30 compared with total GWG, when analysed both as continuous variables (data not shown). This may indicate that our observations are likely associated with maternal fat mass accumulation rather than only with foetal growth, which makes up a larger proportion of GWG in late pregnancy. Shared familial genetic and lifestyle characteristics, like high energy diet and low levels of physical activity may also link greater GWG with greater offspring BMI and adverse cardio-metabolic profile in adulthood. We had information regarding whether offspring thought their mother, father or sibling was overweight in addition to information on offspring's smoking and alcohol habits. We were therefore able to take into account confounding by important familial dietary and lifestyle habits postpartum (online supplementary data) and this in addition to long-term follow-up is the major strength of our study. The persistent relationship observed after adjustment for these factors suggests that, at least in part, high GWG may affect offspring's weight and metabolism by modifying the intrauterine environment, possibly by influencing maternal and foetal hormonal profile, which may affect offspring appetite control and adiposity later in life.[29,30] Our results therefore indicate that interventions promoting healthy GWG should not only target overweight and obese women, but also women in normal weight.

Table 3. Associations of maternal gestational weight gain with offspring BMI and waist circumference at follow-up (n = 308)

	Offspring BMI (kg m^{-2})		Offspring overweight	
	β[a]	95% CI	OR[b]	95% CI
GWG in week 30 (per 1-kg increase)[c]	0.10	(0.01, 0.20)	1.10	(1.00, 1.20)
IOM categories:				
Suboptimal (< 11.5 kg)	− 0.4	(−1.2, 0.4)	0.6	(0.2, 1.4)
Optimal (11.5–16 kg)	ref.	ref.	ref.	ref.
Excessive (> 16 kg)	0.6	(−0.2, 1.4)	1.8	(0.9, 3.8)
P for trend[d]	0.02		0.01	
	Waist circ. (cm)		High waist	
	β[a]	95% CI	OR[b]	95% CI
GWG in week 30 (per 1-kg increase)[c]	0.12	(−0.14, 0.38)	1.02	(0.90, 1.14)
IOM categories:				
Suboptimal (< 11.5 kg)	− 1.1	(−3.3, 1.2)	0.6	(0.2, 2.0)
Optimal (11.5–16 kg)	ref.	ref.	ref.	ref.
Excessive (> 16 kg)	1.3	(−0.9, 3.5)	1.6	(0.6, 4.2)
P for trend[d]	0.05		0.09	

Abbreviations: BMI, body mass index; CI, confidence interval; GWG, gestational weight gain; IOM, Institute of Medicine guidelines; OR, odds ratio. [a]Linear regression model, adjusted for offspring's sex, maternal pre-pregnancy BMI, age, parity, smoking status, educational level and whether offspring think their father is overweight. [b]Logistic regression model, reflecting the odds of offspring being overweight (BMI ⩾ 25) at ~ 20 years or having waist circumference > 88 cm for females and > 102 cm for males, adjusted for same covariates as in[a]. [c]Showing increase in the outcome variable per 1-kg increase in gestational weight gain (continuous) or by the IOM categories. [d]T-test for linear regression, Chi-square test for logistic regression.

Table 4. Associations of maternal gestational weight gain during the first 30 weeks of gestation with offspring cardio-metabolic risk factors at follow-up (n = 308)

Outcome[a]	All (n = 308)[b]			Men (n = 121)[c]			Women (n = 187)[c]		
	β	95% CI	P	β	95% CI	P	β	95% CI	P
Insulin (%)[d]	1.2	(−0.2, 2.6)	0.09	3.7	(1.4, 6.2)	< 0.01	− 0.2	(−1.9, 1.5)	0.82
HOMA-IR (%)[d]	1.1	(−0.3, 2.5)	0.14	3.4	(0.8, 6.0)	0.01	− 0.1	(− 1.8, 1.7)	0.94
Fasting glucose (mmol l^{-1})	0.0	(−0.01, 0.01)	0.95	− 0.01	(−0.04, 0.01)	0.32	0.01	(−0.00, 0.02)	0.35
Leptin (%)[d]	3.7	(1.2, 6.4)	< 0.01	10.7	(5.7, 15.9)	< 0.01	0.4	(−2.4, 3.3)	0.76
Adiponectin (%)[d]	0.5	(−0.8, 1.7)	0.45	− 0.6	(−2.8, 1.6)	0.58	1.5	(0.0, 2.9)	0.04
Total cholesterol[d]	− 0.9	(−1.5, − 0.3)	< 0.01	− 0.1	(−1.0, 0.8)	0.81	− 1.3	(−2.1, − 0.6)	< 0.01
LDL (%)[d]	− 1.3	(−2.2, − 0.4)	< 0.01	0.1	(−1.2, 1.5)	0.85	− 2.2	(−3.4, − 0.9)	< 0.01
HDL (%)[d]	− 0.5	(−1.2, 0.1)	0.12	− 1.1	(−2.1, − 0.0)	0.05	− 0.1	(−1.0, 0.8)	0.80
Triglyceride (%)[d]	− 0.2	(−1.6, 1.2)	0.80	1.7	(−0.5, 3.9)	0.12	− 1.1	(−2.8, 0.6)	0.21
SBP (mm Hg)	0.3	(0.0, 0.6)	0.03	0.4	(−0.1, 0.9)	0.09	0.2	(−0.2, 0.5)	0.30
DBP (mm Hg)	0.2	(−0.0, 0.4)	0.12	0.4	(0.0, 0.8)	0.03	0.0	(−0.2, 0.3)	0.92
Resting pulse (bpm)	0.2	(−0.1, 0.5)	0.25	0.9	(0.3, 1.5)	< 0.01	− 0.3	(−0.6, 0.1)	0.17

Abbreviations: CI, confidence interval; DBP, diastolic blood pressure; HDL, high-density lipoprotein, HOMA-IR, homeostasis model assessment-estimated insulin resistance; LDL, low-density lipoprotein; SBP, systolic blood pressure. [a]Showing increase in the outcome variable per 1-kg increase in gestational weight gain and the 95% confidence interval. [b]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status and educational level, offspring's sex and whether offspring thinks their father is overweight. [c]Same covariates as in adjusted for same covariates as in[b] but without offspring sex. [d]Levels were log-transformed in the analyses and back-transformed via exponentiation to facilitate Interpretation as a percent change.

Concerning weaknesses, we cannot, as with all observational studies exclude residual confounding or confounding by unmeasured covariate(s). Even though our covariate adjustments had minimal influence on our effect estimates compared with unadjusted models (data not shown), the role of residual confounding particularly during long-term follow-up can never fully be excluded. Furthermore, we were unable to account for genetics. Relying on only one measure for biomarkers of cardio-metabolic health such as leptin can also be considered a limitation.[31,32] Our measure of pre-pregnancy weight was based on self-report, possibly leading to bias because of underreporting; however, we suspect that such bias should be small given our restriction to women of normal weight.[33,34] In addition, the population studied was white, fairly well educated, with normal BMI and for the most part normal GWG. Whether the result could be applied to other populations, perhaps at higher cardiovascular risk, remains to be studied.

In conclusion, our results provide evidence that maternal GWG among normal weight mothers may affect offspring cardio-metabolic health at young adult age. Although the observed associations were modest, we cannot exclude that these modest shifts may become more apparent later in life. Measurements over longer periods of time are needed to add to the current understanding of the long-term influence of non-optimal GWG on offspring's anthropometry and cardio-metabolic health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paper II

Maternal Macronutrient Intake and Offspring Blood Pressure 20 Years Later

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Background—Results from 2 cohort studies in Scotland established in the 1940s and 1950s (Aberdeen and Motherwell) suggested that a high protein diet during pregnancy might adversely influence offspring blood pressure at adult age. Our objective was to examine this association in the Danish Fetal Origins Cohort (DaFO88).

Methods and Results—This was a prospective birth cohort of 965 women who gave birth in 1988–1989 in Aarhus, Denmark, and whose offspring (n=434) participated in a clinical examination ≈20 years later. Macronutrient intake was assessed in gestational week 30. Multivariable adjusted linear regression was used to examine the relation between higher maternal protein intake, at the expense of carbohydrates, and offspring blood pressure (isocaloric substitution). Main analyses were adjusted for mother's age during pregnancy, prepregnancy body mass index, parity, smoking during pregnancy, educational level, and offspring's sex. The mean total energy intake was 8.7 MJ/day (SD 2.3 MJ/day). The mean energy from carbohydrate, fat, and protein intake was 51, 31, and 16 of total energy, respectively. The results showed that after adjustment, higher maternal protein intake was associated with slightly higher offspring diastolic blood pressure (highest compared with the lowest quintile of protein intake: Δ=2.4 mm Hg; 95% CI 0.4–4.4; *P*=0.03 for trend). Similar differences, although not significant, were found for systolic blood pressure (Δ=2.6 mm Hg; 95% CI −0.0 to 5.3; *P*=0.08 for trend).

Conclusions—Higher maternal dietary protein intake at the expense of carbohydrates was associated with a modest increase in offspring blood pressure in young adulthood. (*J Am Heart Assoc*. 2017;6:e005808. DOI: 10.1161/JAHA.117.005808.)

Maternal diet may alter offspring metabolic programming,[1] with macronutrient composition identified as a potentially important etiological factor.[2,3] Extensive evidence from animal studies shows a relation between maternal

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malnutrition or low-protein diet (≈6–12 total energy [E%]) and an adverse metabolic profile in the offspring,[4] including higher blood pressure (BP).[5,6] Less focus, however, has been directed at high-protein diets, which have gained considerable popularity in recent years, especially as a nutritional intervention to aid weight loss.[7,8] Although human data are limited, evidence from at least 2 studies suggests that the association between maternal protein intake and offspring BP may be U-shaped. A cohort study from Aberdeen, Scotland (established in 1948–1954, n=253) reported different associations between carbohydrate intake and offspring BP, depending on the protein–carbohydrate intake. In that study, low carbohydrate intake combined with high animal protein intake (>50 g/day) was associated with reduced placental size and increased offspring BP 40 years later.[9] Similar results were observed in the Motherwell study (n=626), in which pregnant women attending the same maternity hospital between 1952 and 1976 were advised to eat 450 g of red meat per day and to avoid carbohydrate-rich foods, like bread and potatoes. In that study, offspring of women who reported greater consumption of meat and fish in late pregnancy had higher BP when measured 3 decades later.[10] In line with these findings, adverse effects of a high-protein diet during

pregnancy on offspring BP, kidney morphology, and anthropometry have also been reported in experimental animals.[11]

Understanding the role of macronutrient composition during pregnancy with regard to offspring chronic disease susceptibility in early life is important to inform preventive measures early in life. Previous studies established in the 1940s and 1950s have found indications that a high-protein diet during pregnancy might influence offspring BP at adult age. Since then, awareness of and focus on the importance of diet during pregnancy has changed considerably, and factors other than protein may have accounted for previous findings. Consequently, it is relevant to examine whether these findings can be replicated in a more contemporary setting. The aim of this study was to examine the relation between dietary carbohydrate substitution with protein and offspring BP at 20 years of age.

Methods

The Study Population

We recruited 965 women with singleton pregnancies in Aarhus, Denmark, from April 1988 to January 1989 (DAFO88 cohort). This number was 80% of a consecutive sample of 1212 eligible women who were attending prenatal care during that time. Details about the cohort and its dietary component have been described previously.[12] These women were scheduled to attend a routine midwife visit in gestational week 30 at which dietary intake was assessed and information on maternal anthropometry, lifestyle, and socioeconomic factors was recorded. Obstetric outcomes were extracted from hospital records.

In the follow-up in 2008–2009, a total of 915 (95% of the original cohort) mother-and-child dyads could be identified in the central registration registry and were alive and living in Denmark. Of the 688 offspring who agreed to participate in the follow-up, 443 offspring attended clinical examination. Offspring of mothers with missing dietary intake or total energy intake <4000 kJ/day were excluded for incomplete registration (n=9). The final data set consisted of 434 mothers and their offspring.

Mothers of offspring who did not attend the clinical examination had lower energy intake (8.4 versus 8.7 MJ/day, P=0.04) compared with mothers of offspring attending the examination. There were no significant differences in maternal protein intake (77 versus 79 g/day, P=0.08), carbohydrate intake (268 versus 269 g/day, P=0.69), or fat intake (70 versus 70 g/day, P=0.33) between these groups. Moreover, age, anthropometry, smoking status during pregnancy, parity status, and gestational age did not differ between mothers whose offspring later did or did not participate in the follow-up study (data not shown).

The study was approved by the Danish Data Protection Agency and the Central Denmark Region Committees on Biomedical Research Ethics (reference no. 20070157). Participants provided written informed consent at recruitment.

Exposure Assessment and Outcomes

Details about the dietary assessment have been described previously.[12] In short, during the prenatal visit in gestational week 30, food frequency questionnaires were handed in. The response was corroborated by trained personnel. In addition, we also conducted a 15-minute face-to-face interview to more accurately assess macronutrient and energy intake. We asked the women about their diet over the previous 3 months, corresponding to the second trimester of pregnancy. The food frequency questionnaire has been validated against biomarkers of n-3 fatty acids only.[12] Nutrient intake was quantified using the Danish food composition table from 1996 (fourth version). At the follow-up in 2008–2009, the offspring were asked to fill out a Web-based lifestyle questionnaire and to attend a clinical examination. The clinical examination included 3 readings of systolic BP (SBP) and diastolic BP (DBP) after 7 minutes of rest with an automatic BP measurement device (Omron M6 Comfort [HEM-7000-E]; Omron Healthcare Co, Ltd), and the means of the SBP and DBP readings were used in our analyses.

Statistics

We used multivariable regression models to examine the association between maternal protein intake at the expense of carbohydrates and offspring BP at ≈20 years of age. The model, therefore, was a substitution model reflecting isocaloric substitution of carbohydrates with protein by allowing all energy-contributing nutrients into the model apart from carbohydrates.[13] All macronutrient variables were energy adjusted using the residual model.[14] We modeled total protein intake in this substitution model both as a continuous variable and in quintiles to account for potential nonlinearity. A test for trend across quintiles was evaluated by entering the categorical variable as a continuous measure.

Covariates included in our models were selected a priori on the basis of former studies.[3,10,11] The following confounders were included in the main model (model A): mother's age from hospital records (in quartiles), prepregnancy body mass index (BMI; in quartiles), parity (nulliparous versus multiparous), smoking status during pregnancy (nonsmoker, <10 or ≥10 cigarettes/day), educational level during pregnancy (elementary schooling, high school or technical schooling, university education) and offspring's sex. We additionally adjusted for offspring BMI (clinical measurements of height and weight at 20 years of age) at follow-up (model B) because

a previous study in this cohort observed an association between higher intake of protein and offspring risk of being overweight.[3] In sensitivity analyses, adjustments were also made for possible intermediary factors such as birth weight, gestational age, gestational weight gain, and pregnancy complications. Missing data ranged from 0% to 6% for individual covariates. Multiple imputations, as implemented in SPSS 24.0 (IBM Corp), were used to impute missing covariates.

In additional analyses, protein intake was divided into animal (from milk, cheese, ice cream, meat, fish, eggs and related products) and plant sources (from cereals, vegetables, fruits, and related products). Analyses were also done separately for men and women to evaluate potential sex-specific associations. Statistical significance was accepted at 2-sided $P<0.05$. All analyses were done in SPSS 24.0.

Results

Anthropometric, demographic, and dietary characteristics of mothers and offspring are presented in Table 1. The mean total energy intake was 8.7 MJ/day (SD 2.3 MJ/day). The mean energy percentage of carbohydrate, fat, and protein intake was 51, 31, and 16 E%, respectively. Among women with high protein intake (quintile 5, mean 20 E%), 15 E% came from animal protein and 5 E% from plant protein, whereas among women with low intake (quintile 1, mean 13 E%), 7 E% came from animal protein and 6 E% from plant protein. Women in the highest quintile of protein intake were more often nulliparous (64% versus 51%), had higher mean gestational weight gain (15 versus 11 kg), lower mean intake of added sugars (5.1 versus 8.7 E%), lower mean fiber intake (23 versus 25 g/day), and lower mean intake of saturated fatty acids (13.3 versus 14.6 E%) and polyunsaturated fatty acids (3.6 versus 4.1 E%) compared with women in the lowest quintile of protein intake. At ≈20 years of age, the mean BMI (kg/m^2) of offspring attending clinical examination was 22±3, and ≈18% had BMI ≥25 (Table 1). The mean offspring SBP and DBP at 20 years was 111±11 and 66±7 mm Hg, respectively.

Table 2 shows the association between maternal carbohydrate substitution with protein (isocaloric model) and offspring BP at 20 years of age. In the fully adjusted model (model A), each 10-g substitution of carbohydrates with protein intake was associated with 0.6 mm Hg (95% CI 0.0–1.1) higher DBP. When comparing the highest and lowest quintiles of protein intake, mean Δ for DBP was 2.4 (95% CI 0.4–4.4) mm Hg. Similar differences were found for SBP (mean 2.6 mm Hg, 95% CI −0.0 to 5.3). The difference was dose-dependent and significant for DBP (P=0.03 for trend) but not for SBP (P=0.08 for trend). Additional adjustment for

offspring BMI (model B) at age 20 years did not appreciably alter effect estimates; mean Δ for DBP went from 2.4 mm Hg (95% CI 0.4–4.4; model A) to 2.1 mm Hg (95% CI 0.1–4.1; model B) when comparing highest and lowest quintiles (Table 2).

Additional Analyses

Although our primary analyses explored the association with offspring BP as a result of higher maternal protein intake at the expense of carbohydrates (isocaloric model), we observed similar results when we relaxed this substitution condition (ie, protein could be replaced by either carbohydrates or fat). Mean Δ for DBP was 2.0 mm Hg (95% CI 0.0–4.0) when comparing highest and lowest quintiles in this model (data not shown) compared with 2.4 mm Hg (95% CI 0.4–4.4) in the fully adjusted substitution model (Table 2).

When examining the protein source, comparable results were found for animal versus plant protein intake, although the associations were slightly stronger for the former (Table 3). Analyses of protein intake from food groups showed that a higher maternal protein intake from milk and milk products was associated with higher offspring DBP. Each 10-g higher milk protein intake was associated with a 0.5-mm Hg mean increase in DBP (95% CI 0.01–1.00) and 0.7-mm Hg mean increase in SBP (95% CI 0.02–1.33).

The increase in offspring BP was similar for both sexes. When comparing quintile 5 with quintile 1, the increase in offspring mean DBP were 2.4 mm Hg for male offspring (95% CI −1.1 to 5.9) and 2.6 mm Hg for female offspring (95% CI 0.1–5.1). A similar, although nonsignificant, difference was observed for SBP.

Sensitivity analyses also showed that adjustment for potential mediators (ie, birth weight, gestational weight gain in week 30, gestational age, and pregnancy complications) did not substantially alter the results (data not shown).

Discussion

In this prospective study with 20 years of follow-up, we found that higher intake of dietary protein during pregnancy was associated with slightly higher offspring BP when substituted for carbohydrates. We observed a 2.4-mm Hg difference in DBP in offspring of mothers with the highest compared with the lowest quintile of protein intake; a similar, although insignificant, difference was also observed for SBP. Notably, this difference was dose-dependent for DBP but not for SBP.

Elevated BP is a major public health concern because of the accompanying increase in risk of cardiovascular disease and the high global prevalence.[15] In young and middle-aged adults, DBP has been found to be the strongest predictor of coronary

Table 1. Anthropometric and Demographic Characteristics of the Study Population (n=434)

		Protein Intake[†]		
	All Participants* (n=434)	Quintile 1 (n=86)	Quintile 5 (n=87)	P Value[‡]
Mothers anthropometric and demographic characteristics				
Age, y	29±4	30±4	29±4	0.16
Prepregnancy BMI, kg/m^2	21±3	21±3	22±3	0.38
Height, cm	168±6	167±7	168±6	0.41
Gestational age, day	282±11	284±12	281±10	0.14
Gestational weight gain, kg	14±5	11±4	15±5	0.05
Nulliparous, %	60	51	64	0.02
Smoking during pregnancy, %	37	39	48	0.35
University education, %	56	51	51	0.99
Mothers dietary characteristics				
Total energy intake, MJ/day	8.7±2.3	8.8±3.1	8.6±2.0	0.54
Protein, g/kg[†]	1.3±0.3	1.1±0.2	1.6±0.3	<0.01
Protein, g/day[†]	79±11	63±6	94±6	<0.01
Dairy protein, g/day[†]	34±12	20±8	48±10	<0.01
Nondairy animal protein, g/day[†,§]	19±8	14±6	23±8	<0.01
Plant protein, g/day[†,‖]	26±5	28±5	24±4	<0.01
Carbohydrate, g/day[†]	269±27	278±31	260±25	<0.01
Sugar, g/day[†]	37±22	45±29	26±12	<0.01
Fiber, g/day[†]	24±5	25±7	23±4	0.01
SFA, g/day[†]	31±7	32±8	29±7	0.03
MUFA, g/day[†]	19±4	20±4	19±3	0.05
PUFA, g/day[†]	9±2	9±3	8±2	<0.01
Offspring characteristics				
Male, %	40	38	38	0.67
Birth weight, g	3.5±5.1	3.4±5.8	3.5±4.7	0.70
Birth length, cm	51.9±2.3	51.7±2.3	51.9±1.8	0.44
Height, cm	174±9	173±9	174±10	0.49
BMI, kg/m^2	22±3	22±3	23±3	0.06
BMI ≥25, %	18	11	28	<0.01
Systolic blood pressure, mm Hg	111±11	109±12	111±11	0.17
Diastolic blood pressure, mm Hg	66±7	65±7	67±7	0.06
Current smoker, %	18	19	17	0.69
Physical activity (≥12 times per month), %	46	42	51	0.11
Alcohol consumption (≥7 times per month), %	16	27	8	<0.01
Fruit intake (≥6 times per week), %	48	48	51	0.70
Vegetable intake (≥6 times per week), %	54	49	53	0.64
Fish intake (≥5 times per month), %	13	12	21	0.13

BMI indicates body mass index; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

*Values are mean±SD for continuous variables and percentages for categorical variables.

[†]Energy-adjusted by the residual model.

[‡]P values were evaluated by using the *F* test (type III) for continuous variables and the chi-square test for categorical variables.

[§]Animal protein included protein from milk or milk products, cheese, ice cream, meat, fish, and eggs and related products.

[‖]Plant protein included protein from cereals, vegetables, fruits, and related products.

Table 2. The Association Between Maternal Protein Intake (Substituted for Carbohydrates) and Offspring BP at 20 Years of Age (n=434)*

	n	Crude Model[†] β (95% CI)	Model A[‡] β (95% CI)	Model B[§] β (95% CI)
Systolic blood pressure				
Total protein intake (per 10-g change/day)[∥]	434	0.7 (−0.2, 1.7)	0.6 (−0.1, 1.4)	0.6 (−0.1, 1.3)
Protein intake, mean±SD (g/day)				
Quintile 1 (63±6)	86	Reference	Reference	Reference
Quintile 2 (73±2)	86	2.5 (−0.7, 5.7)	2.5 (−0.1, 5.1)	2.1 (−0.4, 4.7)
Quintile 3 (78±1)	87	1.7 (−1.6, 4.9)	2.0 (−0.6, 4.6)	1.9 (−0.6, 4.4)
Quintile 4 (84±2)	88	2.8 (−0.5, 6.0)	2.5 (−0.2, 5.2)	2.4 (−0.1, 5.0)
Quintile 5 (94±6)	87	2.8 (−0.5, 6.0)	2.6 (−0.0, 5.3)	2.1 (−0.4, 4.7)
P for trend[¶]		0.12	0.08	0.12
Diastolic blood pressure				
Total protein intake (per 10-g change/day)[∥]	434	0.6 (0.0–1.2)	0.6 (0.0–1.1)	0.5 (−0.0, 1.0)
Protein intake, mean±SD (g/day)				
Quintile 1 (63±6)	86	Reference	Reference	Reference
Quintile 2 (73±2)	86	0.7 (−1.3, 2.7)	1.3 (−0.7, 3.3)	1.2 (−0.8, 3.1)
Quintile 3 (78±1)	87	1.6 (−0.4, 3.6)	1.5 (−0.5, 3.5)	1.4 (−0.6, 3.4)
Quintile 4 (84±2)	88	1.1 (−0.9, 3.1)	1.4 (−0.6, 3.4)	1.4 (−0.6, 3.4)
Quintile 5 (94±6)	87	2.4 (0.4–4.5)	2.4 (0.4–4.4)	2.1 (0.1–4.1)
P for trend[¶]		0.02	0.03	0.05

*In all models, we examine the association between higher protein intake during pregnancy at the expense of carbohydrates (isocaloric substitution) and offspring blood pressure at 20 years of age.

[†]Protein, fat, and total energy intake entered simultaneously into the model.

[‡]Adjusted for maternal prepregnancy body mass index, maternal age, parity, smoking status during pregnancy, maternal educational level, and offspring sex.

[§]Same covariates as in ‡ but also adjusted for offspring body mass index at age 20.

[∥]The effect estimates can be interpreted as the effect of increasing intake of protein (per 10-g change) at the expense of carbohydrates while keeping calories constant.

[¶]The *t* test with maternal protein intake entered as categorical variable.

heart disease risk over 20-year follow-up.[16] Even small decreases in a population's average BP levels may substantially reduce the population burden of BP-related diseases. Cook et al, for example, showed that a 2-mm Hg reduction in DBP in the mean of the population distribution may result in a 17% decrease in the prevalence of hypertension and a 6% reduction in the risk of coronary heart disease.[17]

Two former cohort studies in Scotland found results in line with ours.[9,10] The cohort study from Aberdeen, Scotland (n=253), found different associations between protein intake and offspring BP 40 years later, depending on the protein:carbohydrate ratio (mean total protein intake 12.2 E%). When the mother's intake of animal protein intake was high (>50 g/day), each 100-g decrease in carbohydrate was related to an 11-mm Hg rise in SBP and an ≈8-mm Hg rise in DBP.[9] In the Motherwell study in Scotland, women (n=626) attending the maternity hospital were advised to eat 450 g of red meat per day and other sources of animal proteins in moderate quantities but to avoid carbohydrate-rich foods during

pregnancy, resulting in a high protein diet ≈24 E%. They reported that greater consumption of meat and fish in late pregnancy was associated with 0.19 mm Hg (per portion of meat or fish per week) higher offspring BP 3 decades later.[10] The results from these previous studies indicated a dose response. In our study, however, we observed a dose-dependent relationship for DBP but not SBP. Our effect estimates were also of lower magnitude than those reported in the 2 Scottish studies, and that may relate to the younger age of the offspring in our study. Another explanation could be differences in study methodology, as we were able to adjust for total energy intake, for which previous studies did not account for. Differences in effect size could also be related to variation in a number of lifestyle factors that have changed over time, such as dietary habits, smoking, and exercise, that may act as modifiers for our observed association.

Results from animal studies also lend support for our findings. Thone-Reineke et al reported that rats eating a high protein diet (40 E%) at the expense of carbohydrates during

Table 3. The Association Between Maternal Animal and Plant Protein Intake (Substituted for Carbohydrates) and Offspring BP at 20 Years of Age (n=434)*

Protein Intake, Mean±SD (g/day)	SBP[†] β (95% CI)	DBP[†] β (95% CI)
Animal protein intake (per 10-g change/day)[‡]	0.8 (0.0–1.6)	0.7 (0.1–1.3)
Quartile 1 (34±6)	Reference	Reference
Quartile 2 (46±3)	3.3 (0.6, 6.0)	2.0 (−0.1, 4.1)
Quartile 3 (53±2)	2.1 (−0.8, 4.9)	1.5 (−0.6, 3.7)
Quartile 4 (59±2)	2.3 (−0.7, 5.3)	1.4 (−0.9, 3.7)
Quartile 5 (72±7)	4.1 (0.9–7.4)	2.8 (0.3–5.2)
P for trend[§]	0.07	0.11
Plant protein intake (per 10-g change/day)[∥]	2.0 (−0.5. 4.5)	1.6 (−0.3, 3.5)
Quartile 1 (16±2)	Reference	Reference
Quartile 2 (20±1)	2.5 (−0.3, 5.3)	1.7 (−0.4, 3.8)
Quartile 3 (22±1)	2.8 (−0.2, 5.7)	1.9 (−0.3, 4.1)
Quartile 4 (25±1)	2.5 (−0.7, 5.7)	1.3 (−1.1, 3.8)
Quartile 5 (30±3)	2.9 (−0.6, 6.4)	2.1 (−0.6, 4.7)
P for trend[§]	0.19	0.25

DBP indicates diastolic blood pressure; SBP, systolic blood pressure.

*In all models, we examine the association between higher protein intake during pregnancy at the expense of carbohydrates (isocaloric substitution) and offspring blood pressure at 20 years of age.

[†]Adjusted for maternal prepregnancy body mass index, maternal age, parity, smoking status during pregnancy, maternal educational level, and offspring sex.

[‡]Protein from animal sources (ie, total protein from milk or milk products, cheese, ice cream, meat, fish, and eggs and related products), protein from other sources, fat, and total energy intake entered simultaneously into the model.

[§]The *t* test with maternal protein intake entered as categorical variable.

[∥]Protein from plant sources (ie, cereals, vegetables, fruits, and related products), protein from other sources, fat, and total energy intake entered simultaneously into the model.

pregnancy had pups with higher offspring BP compared with the group eating a normal-protein diet (20 E%; isocaloric diets).[11] In contrast, another study, also using a rat model,[18] found no effect of maternal high-protein diet on BP, although this result may be related to differences in the study design; for example, the number of animals was about half the size of the study groups of Thone-Reineke et al, and the studies examined different developmental periods. The main supplemented protein source in the study by Thone-Reineke et al was the milk protein casein. When examining the protein source in our study, similar results were found for animal and plant protein intake, although the associations were stronger for animal protein (Table 3). Our analyses of individual food groups, however, showed more pronounced association between maternal protein intake from milk and milk products and offspring BP. Nevertheless, it is worth noting that milk consumption was very high in our cohort (mean 829±369 g/ day). Among women in the highest quintile of protein intake,

51% of the total protein intake came from milk and milk products, and this intake corresponded to 1173±350 g of milk and milk products per day in this group (Table 1). Because of this high intake, we are unable to separate whether the association between maternal protein intake and offspring BP may be due to the dairy protein or just to protein per se.

Difference in BP among offspring whose mothers had higher versus lower protein intake might be related to differences in carbohydrate intake.[9] Our additional analyses, however, showed that the results did not substantially change when we relaxed the substitution condition (ie, protein could be replaced by either carbohydrates or fat), indicating that the association is most likely driven mainly by protein intake alone. There was also limited difference in the carbohydrate intake and quality in our cohort when comparing women with relatively high (quintile 5) versus low (quintile 1) protein intake (Table 1). The main difference observed was a slightly higher intake of added sugar among women with lower protein intake, although both groups had an intake within the recommended maximum of 10 E%.[19]

Animal studies,[11,20] as well as former results in this cohort,[3] have reported that high protein exposure during pregnancy may influence offspring adiposity and risk of being overweight. Because BMI is associated with BP regulation[21] an increase in weight might be mediating this association between a maternal high protein diet and offspring BP; however, further adjustment for offspring BMI in our study did not alter effect estimates (Table 2). Other possible mechanisms for the association between high maternal dietary protein intake and offspring BP suggested in former studies include abnormal placental activity (ie, reduced placental growth), metabolic stress, and abnormalities in glucocorticoid secretion.[9,10,22–24]

The main strength of our study was the long follow-up and the 3 BP measurements taken by health professionals at the clinical visits. We also were able to adjust for a number of potential confounding factors collected during pregnancy. Regarding weaknesses, as in any observational setting, we cannot exclude residual confounding. The focus of this study was on macronutrient intake; maternal intake of other nutrients not examined in this study could also be important with regard to offspring BP, although results have been inconsistent.[25–29] We acknowledge that confounding or effect modification by postnatal dietary factors may be important in our study. According to the early protein hypothesis, higher protein intake during early childhood may induce accelerated weight gain, which has been associated with higher adult BP.[30,31] Interestingly, partially replacing dietary carbohydrate with protein in adulthood has been related to reduction in BP; therefore, higher protein intake in adulthood may have a different influence on BP regulation than early life exposure.[32] Finally, we acknowledge that the clinical relevance of 2.4- to 2.6-mm Hg higher DBP or SBP values as a result of high

maternal protein intake remains uncertain. It is important to keep in mind that our study population consisted of young offspring with normal BP levels, but even mildly raised blood pressure levels present already in adulthood have been found to track to later adulthood and increase the risk of developing hypertension and its sequelae later in life.[17,33,34]

In summary, we found higher maternal dietary protein intake at the expense of carbohydrates during the second trimester of pregnancy to be associated with slightly higher offspring BP in young adulthood. In light of the increased popularity of high-protein diets, a better understanding of long-term health consequences of such regimens during pregnancy and a possible underlying mechanism is needed.

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Disclosures

None.

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Maternal Macronutrient Intake and Offspring Blood Pressure 20 Years Later

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Paper III

Maternal Diet, Gestational Weight Gain, and Inflammatory Markers During Pregnancy

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Objective: To examine the associations of gestational weight gain (GWG) and diet with low-grade inflammation in pregnancy.

Methods: A cross-sectional analysis of 671 pregnant women was performed, and diet was assessed in gestational week 30. GWG was recorded in weeks 30 and ~37 (difference between the weight recorded at these time points and pre-pregnancy weight). Markers of inflammation, high-sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), interleukin (IL)−6, IL-8, IL-1β, and tumor necrosis factor-α were quantified in serum from week 30.

Results: After adjusting for age, pre-pregnancy BMI, parity, smoking status, and education, each 1 kg increase in GWG was associated with 3% (95% CI: 1–5) higher hsCRP and 3% (95% CI: 1–4) higher SAA concentrations, which corresponded to ~18% to 25% increase in these biomarkers among those with excessive weight gain. GWG was inversely associated with IL-8 while no associations were found for the other inflammatory markers. With respect to diet, women in the highest compared with lowest quintile of protein intake had 26% (95% CI: 3–54) higher hsCRP concentrations. This increase appeared to be driven by intake of animal protein. A similar pattern was observed for SAA.

Conclusions: Excessive GWG, as well as high intake of animal protein, was associated with higher concentrations of inflammatory factors.

Introduction

The significance of inflammation during pregnancy on maternal physiological responses and fetal programming is an important area of research. Pregnancy is considered a natural inflammatory state, as it can give rise to proinflammatory and anti-inflammatory conditions depending on the stage of gestation (1). On the other hand, a heightened maternal inflammatory response has been associated with pregnancy complications, e.g., raised maternal high-sensitivity C-reactive protein (hsCRP) concentrations have been associated with hypertension, gestational diabetes, and premature birth (2-4). Studies have also indicated that maternal inflammation might contribute to fetal programming of obesity (5).

It is well known that in nongravid individuals, weight gain and increased adipose tissue are associated with chronic, low-grade inflammation (6). However, less is known about whether similar relations exist for gestational weight gain (GWG) and to what extent lifestyle factors, such as diet, can modify inflammatory changes during gestation. In nonpregnant populations, a healthy diet, rich in seafood, fruits, vegetables, and whole grains, and low-glycemic diets have been associated with lower concentrations of circulating inflammatory markers (7-9). On the other hand, a Western-type diet, characterized by a high intake of red meat, high-fat dairy, and refined grains, has been observed to be proinflammatory (7). Interestingly, a recent intervention reported that a high protein intake

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(~30% of energy) over a period of 8 weeks adversely affected the inflammatory status (10).

Further examining which factors may modulate inflammatory responses during pregnancy may shed light on possible mechanisms behind adverse pregnancy outcomes and long-term programming of offspring disease. The aim of this study was to examine the associations of GWG and diet with low-grade inflammation in pregnant women.

Methods

The study population

A total of 965 pregnant women were recruited in Aarhus, Denmark, from April 1988 until January 1989. These women represented 80% of a consecutive sample of 1,212 women attending routine antenatal care at a specific antenatal care clinic in the city. These women all had a singleton pregnancy and were scheduled to attend a routine midwife visit in gestational week 30. The study was approved by the Danish Data Protection Agency and the Central Denmark Region Committees on Biomedical Research Ethics (Reference No. 20070157). Written consent was obtained from all participants.

Exposure variables, covariates, and outcomes

Diet was assessed in week 30 by a self-administered food frequency questionnaire (FFQ), combined with a 15-min face-to-face interview conducted by trained personnel. The combination of an FFQ and structured interview was used to assess macronutrient and energy intake more accurately (11). The women were asked about their diet in the previous 3 months, corresponding to the second trimester of pregnancy. The questions asked were how often a specific food item was consumed and what amount was consumed per portion. The FFQ focused on food items that were easily quantifiable such as breakfast and lunch meals. The interview, however, focused on quantifying main ingredients of cooked meals and completion of the FFQ in order to make a quantitative estimation on selected food items. Photographs modeling various portion sizes were showed to the participants and used in the quantification procedure. Nutrient intake was quantified using the 1996 (4th) version of the Danish food composition table. The FFQ has been validated against biomarkers of $n-3$ fatty acids only (11).

Self-reported pre-pregnancy body mass index (BMI) and information on maternal lifestyle and socioeconomic factors was also recorded in week 30. Information about gestational age, obstetric complications, and birth outcomes was retrieved from the Danish Medical Birth Registry and hospital records.

Information on GWG at week 30 and the highest recorded weight (~week 37) were retrieved from antenatal records. Examining GWG at week 30 has the advantage that weight gain at that time should be minimally influenced by fetal weight and maternal edema, compared with using total GWG (12). The definition of optimal GWG during pregnancy is, however, based on total GWG and, therefore, facilitates comparison with the 2009 Institute of Medicine guidelines (12). Total GWG was calculated as the difference between the highest recorded weight (~week 37) in pregnancy and pre-pregnancy weight; this information was used to classify women's weight gain, i.e., in suboptimal, optimal, and excessive weight gain. Excessive GWG was

determined in accordance to the Institute of Medicine guidelines for each pre-pregnancy BMI category, i.e., for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg, and obesity >9 kg total GWG (12).

Blood samples and biochemical analysis

Maternal blood samples were obtained after the interview in the 30th week of gestation. The samples were kept on ice and separated into serum, plasma, and erythrocytes within an hour. Analysis of inflammatory biomarkers, that is, hsCRP, serum amyloid A (SAA), interleukin (IL)−6, IL-8, IL-1β, and tumor necrosis factor (TNF)-α was conducted after 20 years of storage at −20°C. The effect of 20 years of storage at −20°C was tested in a pilot study which showed that serum median levels of the inflammatory markers of interest were comparable with measurements done on samples that had been stored for 10 years at −80°C (13).

Details of the biochemical analysis have been given elsewhere (13). In brief, plasma concentrations were measured by 4-plex multiarray electrochemiluminescence detection platforms of Meso Scale Discovery (MesoScaleDiscovery, Gaithersburg, MD; available at: www.mesoscale.com (14). This method uses multiarray plates fitted with multielectrodes per well with each electrode being coated with a different catching antibody. The assay procedure follows that of a classic sandwich ELISA. The captured analytes were then detected by a secondary, analyte-specific, ruthenium-conjugated antibody, which is capable of emitting light after an electrochemical stimulation. This approach minimizes nonspecific signals, as the stimulation mechanism (electricity) is decoupled from the signal (light). Each sample was analyzed in duplicate. The intra- and interassay coefficients of variation for the platform of Meso Scale Discovery for hsCRP was 4.6% and 5.8%, for SAA: 4.2% and 11.7%, for IL8: 4.5% and 7.8%, for IL-6: 6.0% and 15.6%, for IL-1β: 13.3% and 18.0%, and for TNF-α: 4.5% and 8.5%.

Subjects included in the analyses

Of the 965 eligible for this study, 960 completed the FFQ. Blood samples were available for 922, but subjects with hsCRP values >10 mg/L ($n = 105$) were excluded due to possible cases of unrecognized infections (15), resulting in 817 women with data on inflammatory markers. Of these, 675 had information on GWG recorded in week 30. Furthermore, women with total energy intake under 4,000 kJ/d ($n = 4$) were excluded due to probable incomplete registration. The final data set therefore consisted of 671 women (70% of the eligible population). As the prevalence of obstetric complications was low in this cohort, we reported absolute median concentrations of inflammatory markers among individuals exposed and unexposed to obstetric and birth complications, for all individuals that had blood samples available ($n = 922$) (Supporting Information).

Statistics

Multivariable regression models were used to examine the associations for maternal GWG and diet, respectively, with inflammatory markers at gestational week 30. Due to skewed distributions, all inflammatory markers were log transformed. The estimates from regression models were then back-transformed to reflect percent change in the biomarker per unit increase in the explanatory

TABLE 1 Anthropometric, demographic, and dietary characteristics of the study population

	All participants (n = 671)[a]	Excess GWG (n = 209)[b]	Non-exc. GWG (n = 462)[c]	P
Anthropometric and demographic characteristics				
Age (yr)	29 ± 4	29 ± 4	29 ± 4	0.62[d]
Pre-pregnancy BMI (kg/m^2)	21 ± 2	22 ± 3	21 ± 3	<0.01[d]
Normal weight (%)[e]	84	83	84	0.68[f]
Gestational age (d)	283 ± 11	284 ± 10	282 ± 11	0.04[d]
Weight gain at 30 wk (kg)	10 ± 4	14 ± 3	9 ± 3	<0.01[d]
Birth weight (g)	3,517 ± 531	3,724 ± 483	3,425 ± 512	<0.01[d]
Parity (%)				0.19[f]
Nulliparous	61	61	61	
Primiparous	29	27	31	
Multiparous	9	12	8	
Smoking during pregnancy (%)				0.39[f]
Never	60	56	62	
<10 cigarettes/d	18	19	17	
≥10 cigarettes/d	22	25	21	
Education (%)				0.84[f]
Elementary schooling	13	11	13	
High school/technical school	25	25	25	
University education	38	39	38	
Higher academic	17	16	17	
Other education	7	9	7	
Dietary characteristics				
Total energy intake (MJ/d)	8.5 ± 2.2	8.7 ± 2.1	8.4 ± 2.2	0.04[d]
Protein (g/d)[g]	78 ± 12	79 ± 13	78 ± 12	0.45[d]
Animal protein (g/d)[g,h]	53 ± 14	55 ± 15	53 ± 14	0.10[d]
Plant protein (g/d)[g,i]	25 ± 5	24 ± 4	25 ± 5	<0.01[d]
Carbohydrate (g/d)[g]	246 ± 24	247 ± 27	246 ± 23	0.57[d]
Sugar (g/d)[g]	39 ± 23	41 ± 23	38 ± 22	0.07[d]
Fiber (g/d)[g]	24 ± 5	23 ± 4	24 ± 5	0.07[d]
Fat (g/d)[g]	69 ± 12	69 ± 13	69 ± 11	0.95[d]
SFA (g/d)[g]	31 ± 7	30 ± 8	31 ± 7	0.45[d]
MUFA (g/d)[g]	20 ± 4	20 ± 4	20 ± 3	0.68[d]
PUFA (g/d)[g]	8 ± 2	8 ± 2	8 ± 2	0.44[d]

[a]Values are means ± SDs for continuous variables and percentages for categorical variables.
[b]Excess GWG was determined in accordance to the institute of Medicine recommendations for each pre-pregnancy BMI category, i.e., for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg, and obesity >9 kg total GWG.
[c]Suboptimal and optimal GWG groups combined.
[d]F-test (type III) of differences among GWG groups.
[e]Normal weight, i.e., BMI 18.5–24.99 kg/m^2.
[f]χ^2 test of differences among GWG groups.
[g]Energy-adjusted by the residual model.
[h]Animal protein included protein from milk/milk products, cheese, ice cream, meat, fish, eggs, and related products.
[i]Plant protein included protein from cereals, vegetables, fruits, and related products.
exc., excessive; GWG, gestational weight gain; BMI, body mass index; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

variable. Assumptions of normality of model residuals were checked, using histograms and QQ plots. Statistical significance was accepted at $P < 0.05$. All analyses were done in SPSS 21.0.

Macronutrient variables were energy-adjusted using the residual model (16). Total protein intake was divided into animal (from milk, cheese, ice cream, meat, fish, eggs, and related products) and plant sources (from cereals, vegetables, fruits, and related products) and analyzed as continuous variables, and in quintiles to account for

potential nonlinearity. P for trend was calculated by entering maternal protein intake as a continuous variable.

Covariates included in our adjusted models were selected *a priori* and were chosen on the basis of their potential influence on dietary habits, GWG, and markers of inflammation (17,18). When examining the association between GWG and inflammation we included the following potential confounders in our adjusted models: pre-pregnancy BMI (in quartiles), age (in quartiles), parity (0, 1, ≥2),

TABLE 2 Associations of GWG during the first 30 weeks of gestation and excessive GWG with maternal inflammatory biomarkers (*n* = 671)

Outcome (%)[b]	Unadjusted			Adjusted[a]			
	β (%)	95% CI	*P*	β (%)	95% CI	*P*	Exc. vs. non-exc. GWG (95% CI)[c]
hsCRP	3	1 to 5	<0.01	3	1 to 5	<0.01	25 (9 to 44)
SAA	2	1 to 4	<0.01	3	1 to 4	<0.001	18 (5 to 33)
IL-8	−2	−3 to −0	0.02	−2	−4 to −1	<0.01	−14 (−24 to −3)
IL-6	0	−1 to 2	0.36	0	−1 to 1	0.71	2 (−6 to 11)
Il-1β	−1	−2 to 1	0.25	−1	−3 to 0	0.16	−8 (−19 to 3)
TNF-α	1	−0 to 1	0.19	1	−0 to 2	0.11	0 (−7 to 7)

[a]Adjusted for maternal age, pre-pregnancy BMI, parity, smoking status, and educational level.
[b]The percent increase in the outcome variable per 1 kg increase in GWG during the first 30 weeks.
[c]The percent difference between women with excessive GWG compared with women with optimal or suboptimal GWG. Excess GWG was determined in accordance to the Institute of Medicine recommendations for each pre-pregnancy BMI category, i.e., for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg, and obesity >9 kg total GWG.
GWG, gestational weight gain; exc., excessive; hsCRP, high-sensitivity C-reactive protein; IL-8, interleukin 8; IL-6, interleukin 6; IL-1β, interleukin-1β; SAA, serum amyloid A; TNF-α, tumor necrosis factor-α.

smoking status (nonsmoker, <10, ≥10 cigarettes/day), educational level (elementary schooling, high school or technical schooling, university education). When examining the association between maternal diet and inflammation, maternal energy intake (in quartiles) was also included, resulting in a nutrient density model reflecting isocaloric substitution of the macronutrient in question at the expense of the others not included in the model (19). Missing values for covariates (mother's smoking status (4%) and educational level (8%) were assumed to be "missing at random" (20) and were imputed using multiple imputation (*m* = 10) as implemented in SPSS (MCMC algorithm).

In secondary analyses the associations between the highest recorded GWG with maternal inflammation were also explored to facilitate comparison with official recommendations (12). In these analyses, the reference category was optimal and suboptimal GWG combined.

When examining the association between maternal diet and the risk of excessive GWG (Supporting Information), our primary focus was on macronutrient intake and dietary factors identified in earlier reports, i.e., total energy intake, milk, sweets, vegetarian diet, glycemic index, and glycemic load (21-23).

Results

Anthropometric and demographic characteristics of participants, as well as dietary intake, are presented in Table 1. For pre-pregnancy BMI, mean (standard deviation, SD) was 21.1 (1.6) kg/m^2 with 11% of subjects being underweight (BMI <18.5 kg/m^2), 84% normal weight (BMI 18.5–24.9 kg/m^2), and 5% with overweight and obesity (BMI ≥25 kg/m^2). The mean (SD) GWG at week 30 was 10.3 (3.5) kg and 31% had excessive GWG. The mean (SD) total energy intake was 8.5 (2.2) MJ/day. The mean carbohydrate, fat, and protein intake was 51 E%, 31 E%, and 16 E%, respectively. The concentrations of the inflammatory markers were comparable to values observed among other pregnant populations (24-26) (Supporting

Information Table S1); for example, the median concentration of hsCRP was 2.6 μg/mL (interquartile range, IQR = 2.9), for SAA 761 ng/mL (693), and for IL8 3.3 pg/mL (2.3).

Maternal GWG and inflammatory markers

The association between GWG and the inflammatory markers are shown in Table 2. After adjusting for covariates, each 1 kg increase in maternal GWG during the first 30 weeks of gestation was associated with 3% (95% CI: 1–5) higher hsCRP, 3% (95% CI: 1–4) higher SAA, and 2% (95% CI: 1–4) lower IL-8. This corresponded to ~18% to 25% increase in hsCRP and SAA among those with excessive GWG compared with women with non-excessive GWG. Absolute median concentrations of the inflammatory markers hsCRP, SAA, and IL-8 in relation to GWG can be found in Supporting Information Table S2.

Maternal diet and inflammatory markers

Table 3 shows the association between maternal macronutrient intake and markers of inflammation. In the adjusted models, maternal protein intake was positively associated with hsCRP (β = 6%; 95% CI: 1–11) and SAA (β = 6%; 95% CI: 1–10); with β reflecting change per 10 g change in intake per day. To further evaluate the relationship between protein consumption and inflammation markers, the importance of the protein source as either of animal or plant origin was also examined (Table 4). Animal protein intake was positively associated with hsCRP (*P* for trend 0.004) and SAA concentrations (*P* for trend 0.003) in adjusted models. Women in the highest quintile of animal protein intake had 25% higher hsCRP levels (95% CI: 2–53) and 23% higher SAA level (95% CI: 4–47) compared with women in the lowest quintile. Intake of plant protein intake was, however, inversely associated with hsCRP levels (*P* for trend <0.001) and SAA levels (*P* for trend 0.013). The correlation coefficient between animal and plant protein intake was −0.49 (*P* <0.001). Additional adjustment for GWG in week 30 of gestation did not substantially attenuate the observed associations. For example, the mean difference in hsCRP values between women in the

TABLE 3 Multivariable associations of maternal diet with maternal inflammatory biomarkers in week 30 of gestation (n = 671)

	hsCRP (%), β (95% CI)[a]	SAA (%), β (95% CI)[a]	IL-8 (%), β (95% CI)[a]
Total energy intake (MJ/d)[b]	2.4 (−0.6 to 5.4)	2.5 (−0.0 to 5.2)	0.3 (−2.5 to 3.1)
Protein (g/d)[c,d]	6 (1 to 11)	6 (1 to 10)	3 (−2 to 8)
Glycemic index[c,d]	−9 (−18 to 1)	−1 (−9 to 8)	1 (−8 to 9)
Glycemic load[c,d]	0 (−2 to 2)	0 (−2 to 1)	0 (−2 to 2)
Fiber (g/d)[c,d]	−10 (−23 to 3)	−6 (−17 to 5)	−13 (−24 to −1)
SFA (g/d)[c,d]	−2 (−11 to 7)	1 (−6 to 9)	3 (−5 to 11)
MUFA (g/d)[c,d]	6 (−12 to 25)	11 (−5 to 27)	8 (−8 to 25)
PUFA (g/d)[c,d]	−10 (−39 to 19)	6 (−19 to 32)	−19 (−45 to 7)

[a]The percent increase in the outcome variable per 1 MJ increase in total energy intake, otherwise per 10 unit increase for nutrients and glycemic index/glycemic load (95% confidence interval (CI)).
[b]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, and educational level.
[c]Same covariates as in b but also adjusted for total energy.
[d]Energy-adjusted by the residual model.
hsCRP, high-sensitivity C-reactive protein; IL-8, interleukin 8; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SAA, serum amyloid A; SFA, saturated fatty acids.

highest compared with the lowest quintile of animal protein intake was 25% (95% CI: 2–53) in adjusted models (Table 4), compared with 24% (95% CI: 1–52) when further adjusted for GWG.

Concerning other nutrients, only fibers were found to be associated with levels of inflammation, i.e., fiber intake was inversely associated with IL-8. Mean intake in each quintile for the dietary variables in Table 4 are shown in Supporting Information Table S3.

Additional analyses

In stability analyses, total protein intake was subdivided into protein from milk, cheese, ice cream, meat, fish, eggs, cereals, vegetables, fruits, and related products. Higher levels, but mostly nonsignificant

associations, of hsCRP and SAA, were observed with increased intake of proteins from all the animal sources. Significant inverse associations with hsCRP and SAA were observed for consumption of plant proteins from cereals (data not shown).

Additional analyses where the relationship between diet and GWG (Supporting Information Table S4) showed that substituting 1 E% of animal protein intake with plant protein intake was associated with 32% lower risk of excessive GWG (adjusted multivariate nutrient density model). Total energy intake and intake of milk/milk products, meat/meat products, and sweets were also associated with risk of excessive GWG.

Median concentrations of markers of inflammation tended to be higher in women exposed to different obstetric complications

TABLE 4 Multivariable associations of maternal protein and fiber intake with maternal inflammatory biomarkers in week 30 of gestation (n = 671)

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P for trend[a]
		% Change (95% CI)				
hsCRP[b]						
Protein[c]	Ref	9 (−11 to 33)	11 (−10 to 35)	22 (−0 to 50)	26 (3 to 54)	0.026
Animal protein[c,d]	Ref	−3 (−21 to 19)	5 (−14 to 29)	19 (−3 to 46)	25 (2 to 53)	0.004
Plant protein[c,e]	Ref	−5 (−23 to 16)	−22 (−36 to −4)	−19 (−34 to 0.5)	−24 (−38 to −6)	<0.001
SAA[b]						
Protein[c]	Ref	11 (−6 to 32)	16 (−3 to 37)	16 (−3 to 37)	31 (10 to 55)	0.011
Animal protein[c,d]	Ref	3 (−13 to 23)	13 (−5 to 34)	21 (2 to 43)	23 (4 to 47)	0.003
Plant protein[c,e]	Ref	3 (−13 to 22)	−2 (−18 to 17)	−11 (−26 to 6)	−10 (−25 to 8)	0.013
IL-8[b]						
Fiber[c]	Ref	−14 (−28 to 3)	−18 (−32 to −1)	−15 (−30 to 2)	−24 (−37 to −9)	0.028

[a]T-test with maternal protein intake entered as continuous variable.
[b]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking status, educational level, and total energy.
[c]Energy-adjusted by the residual model.
[d]Animal protein included protein from milk/milk products, cheese, ice cream, meat, fish, eggs, and related products.
[e]Plant protein included protein from cereals, vegetables, fruits, and related products.
hsCRP, high-sensitivity C-reactive protein; IL-8, interleukin 8; SAA, serum amyloid A.

compared with women with uncomplicated pregnancy (Supporting Information Table S5). For gestational diabetes (hsCRP: 4.4 vs. 3.0 µg/mL) and hypertension (hsCRP: 4.9 vs. 3.0 µg/mL) these differences were statistically significant. In addition, women giving birth to low-birth-weight neonates (<2,500 g) were found to have higher CRP levels (6.5 vs. 2.9 µg/mL) and SAA levels (1,213 vs. 842 ng/mL) in week 30 compared with women giving births to neonates with normal birth weight (term births). Exclusion of participants that had been hospitalized during pregnancy (15%) or had pregnancy complications (23%) did not appreciably alter effect estimates for the observed associations in Tables 2 to 4 (data not shown).

Discussion

In this study, we examined the association between maternal GWG and diet with low-grade inflammation in lean pregnant women. We found GWG to be positively associated with hsCRP and SAA and inversely associated with IL-8. Examining the influence of maternal diet on these same inflammatory markers, we found some suggestions that high consumption of animal protein during pregnancy may be proinflammatory whereas plant-based protein may be anti-inflammatory.

HsCRP, as well as SAA, are acute-phase reactants (nonspecific inflammatory markers). Although largely produced by hepatocytes, hsCRP and SAA are also produced by adipocytes. IL-8 is, however, a chemokine produced by a variety of tissue and blood cells. IL-8 induces chemotaxis in target cells and phagocytosis at the site of inflammation (7). There is extensive evidence of a relation between weight gain and low-grade inflammation in the nongravid population (27). In line with our results, weight gain during pregnancy has been related to maternal hsCRP levels in some (26,28), but not all, previous reports (29-31). It is generally accepted that inflammation associated with weight gain is related to secretions of proinflammatory biomarkers from adipose tissue (6). However, there are some suggestions that low-grade inflammation may also precede weight gain, possibly by promoting adipose accumulation or indirectly through disturbances of the gut microbiota, which may influence metabolic pathways by modulating inflammation, satiety control, and extraction of calories (26,32). It has also been suggested that the placenta may promote an increase in systemic inflammation and a decrease in insulin sensitivity, thereby influencing GWG (12). However, the reverse could also be true as maternal weight status is associated with placental size and function (33,34). Conversely, we observed an inverse association between weight gain and IL-8, indicating that low weight gain during pregnancy may also induce some aspects of inflammation.

In additional analyses in relation to pregnancy health and birth outcomes, we noted that median levels of hsCRP tended to be higher among women with gestational diabetes and hypertension, compared with uncomplicated pregnancies, which is in agreement with previous studies (3,4). Giving birth to low-birth-weight neonates, but not preterm births, was also associated with higher median concentrations of inflammatory factors in week 30 of gestation. It is however relevant to know that during this time, ultrasound to determine gestational age was in its infancy and estimated gestational age may, therefore, be imprecise. Adjustment or exclusion of participants with

obstetric complications did not substantially attenuate the observed associations between GWG and levels of inflammatory markers.

Given the evidence of a link between diet and GWG (21,22,35), the inflammatory markers found to be associated with GWG, i.e., hsCRP, SAA, and IL8, were further explored in relation to maternal diet. In these analyses, we found that maternal protein intake was associated with both hsCRP and SAA concentrations, and these associations were of similar magnitude as for GWG. Results based on an urban U.S. population of lean gravidae ($n = 520$) found protein intake to be positively associated, and carbohydrate intake to be inversely associated, with maternal CRP concentrations at entry to prenatal care (3). Two 8-week intervention studies among nonpregnant individuals with overweight and obesity have also reported results in agreement with our observations (9,10). The DIOGenes intervention ($n = 932$) found that a low-glycemic-index diet and, to a lesser extent, low-protein diet (10–15 E%) may specifically reduce hsCRP levels (9). In line with our results, the RESEMNA trial ($n = 96$) found that high protein intake (~30% E%), mostly of animal origin, resulted in higher inflammation (10).

Adverse consequences of high-protein diets have not been examined in detail, but there are rare reports suggesting that high protein intake in pregnancy may have consequences on pregnancy outcomes and offspring health (36,37). In the Harlem trial (38), high-protein supplementation, leading to total protein intake of ~20 E%, led to an excess of premature births and associated neonatal deaths. Since then, concerns regarding maternal diets with high protein density have been raised regularly. In comparison, the mean total protein intake in the highest quintile in this study was 19.7% of total energy. Although a link between our findings and the Harlem trial cannot be made, there is a need, with increased popularity of high-protein diets, to examine their safety in more detail.

In addition to our study results indicating that both GWG and different protein sources may be associated with the inflammatory status, our secondary analyses suggest that eating more plant-based protein sources at the expense of animal protein could be beneficial with regard to decreasing the risk of excessive GWG (Supporting Information). As a result, one might suspect that these associations for GWG and protein intake, with respect to hsCRP and SAA values, would be intercorrelated. However, adjusting for GWG in week 30 did not attenuate the observed associations between protein intake and levels of inflammation, suggesting that the associations observed for GWG and protein intake were independent. However, given the relatively low precision of dietary estimates in general (19), we cannot exclude measurement error as the explanation for these independent associations.

In terms of interpreting our findings, it is important to note that separating the potential role of individual nutrients or foods in observational settings is difficult, as many nutrients are highly correlated and have synergistic or interactive effects. Our interpretation of these results is therefore that a high intake of animal versus plant foods may have different relations with levels of inflammatory markers during pregnancy. Concerning other limitations, the cross-sectional design of our study inhibits us from inferring causality. Additionally, the dietary data were collected using a dietary questionnaire, which ranks the participants according to their food consumption; the absolute amount of nutrient intake must, therefore, be interpreted cautiously. The measure of pre-pregnancy weight was

based on self-report in week 30, possibly leading to bias because of under-reporting. However, as women in this cohort were predominantly in normal weight (84%), we suspect that such bias would be minor (39). Although we adjusted for a number of potential confounders, we recognize that we lacked proper assessment of physical activity, which may have an independent impact on inflammatory markers. Moreover, higher whole grain/fiber intake and a vegetarian-based lifestyle correlate with other factors of healthy lifestyle, which may leave room for residual confounding. With regard to the blood measurements, we only had a single measurement of the inflammatory factors in week 30 of gestation, which may have led to misclassification due to random variability. Additionally, the plasma samples were stored for 20 years at $-20°C$ which might have introduced molecular degradation. However, the stability of these inflammatory biomarkers after long-term storage has been documented (13). We also acknowledge that our study population consisted of mainly healthy normal-weight pregnant women, and the results may therefore not be representative of today's pregnant population in general. As a result, the clinical relevance of a $\sim25\%$ increase in concentrations of markers of inflammation as a result of either excessive GWG or high protein intake remains uncertain.

In summary, our results indicate that both GWG and diet are related to inflammatory status of pregnant women. We found evidence that excess GWG and high intake of animal protein during pregnancy may be proinflammatory whereas plant protein intake may be anti-inflammatory. With high-protein diets becoming more popular, further studies are needed to be able to evaluate optimal protein intake and the best balance between animal and plant-based foods during pregnancy.O

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Paper IV

Development of a dietary screening questionnaire to predict excessive weight gain in pregnancy

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Conflict of Interest Statement: IG is the owner of a nonprofit company behind the website www.nmb.is that includes the questionnaire used in the present study. The website is run as a non for profit NGO. LH, TIH, BEB, IH, and HH have no conflicts of interest to declare.

Contributor statement: IG, LH, and TIH designed the research. IG designed the questionnaire and together with colleagues, initiated the data gathering. LH was responsible for the collection of the web-based data. LH performed the statistical analyses with guidance and feedback from TIH. LH wrote the first draft of the paper and had primary responsibility for final content. All authors contributed to and critically reviewed the manuscript. They have all approved the final manuscript.

Supporting information: Additional supporting information can be found online.

ABSTRACT

Excessive gestational weight gain (GWG) is a risk factor for several adverse pregnancy outcomes, including macrosomia. Diet is one of few modifiable risk factors identified. However, most dietary assessment methods are impractical for use in maternal care. This study evaluated whether a short dietary screening questionnaire could be used as a predictor of excessive GWG. The dietary data was collected in gestational weeks 11-14 using a 40-item food frequency screening questionnaire. The dietary data was transformed into 13 predefined dietary risk factors for inadequate diet. Stepwise backward elimination was used to identify a reduced set of factors that best predicted excessive GWG. This set of variables was then used to calculate a combined dietary risk score (range 0-5). Information regarding outcomes, GWG (n=1326) and birth weight (n=1651) was extracted from maternal hospital records. In total, 36% had excessive GWG, and 5% of infants were macrosomic. A dietary risk score (characterized by: a non-varied diet, non-adequate frequency of consumption of fruits/vegetables, dairy and whole grain intake, and excessive intake of sugar/artificially sweetened beverages and dairy) was associated with higher risk of excessive GWG. Women with high (≥4) versus low (≤2) risk score had higher risk of excessive GWG (RR=1.24, 95%CI=1.01; 1.52) and higher odds of delivering a macrosomic offspring (OR=2.28, 95%CI=1.18; 4.38). The results indicate that asking simple questions about women's dietary intake early in pregnancy could identify women who should be prioritized for further dietary counseling and support.

Keywords: Dietary habits, dietary screening, maternal nutrition, gestational weight gain, macrosomia, dietary assessment tools.

INTRODUCTION

Weight gain is an essential aspect of pregnancy (Institute of Medicine and National Research Council, 2009). However, excessive gestational weight gain (GWG) is associated with pregnancy and birth complications as well as macrosomia (Goldstein et al., 2017; Tian et al., 2016). Excessive GWG is also the primary factor contributing to increased postpartum weight retention (PPWR) (Rong et al., 2015), and high weight gain from first to second pregnancy is related to increased risk of stillbirth and infant mortality (Cnattingius & Villamor, 2016). Excessive GWG has also been associated with long-term implications for both maternal and offspring health, i.e., later adiposity and cardio-metabolic risk (Fraser et al., 2011; Mamun, Mannan, & Doi, 2014; Perez-Morales, Bacardi-Gascon, & Jimenez-Cruz, 2015).

Results from observational studies have repeatedly shown that dietary habits characterized by high consumption of fruits and vegetables, wholegrain, fish, and healthy fat and low consumption of food with little nutritional value, is associated with lower risk of excessive GWG and pregnancy complications (Brantsaeter et al., 2009; Chen et al., 2016; Englund-Ogge et al., 2014; Hillesund, Bere, Haugen, & Overby, 2014; Knudsen, Orozova-Bekkevold, Mikkelsen, Wolff, & Olsen, 2008; Renault et al., 2015; Shin, Lee, & Song, 2016; Tielemans et al., 2015; Tryggvadottir, Medek, Birgisdottir, Geirsson, & Gunnarsdottir, 2016; Uusitalo et al., 2009). Results from intervention studies, aiming at improving diet, physical activity or both, have shown that reduction in GWG can be achieved, however the effect size reported in these studies have been modest, or around 0.70 kg (95% CI: 0.48, 0.92kg) on average according to a recent meta-analysis (Rogozinska et al., 2017). The clinical relevance of such a modest reduction on other maternal and birth outcomes is unclear (Rogozinska et al., 2017). The observed effect size in observational studies in terms of reduction in GWG has been considerably larger than in intervention studies (Knudsen, Heitmann, Halldorsson, Sorensen, & Olsen, 2013; Maslova, Halldorsson, Astrup, & Olsen, 2015; Renault et al., 2015). Apart from

problems with compliance, one reason for this difference and the modest effect size seen in intervention studies might be that many interventions recruit their subjects based on weight (Dodd et al., 2014; Guelinckx, Devlieger, Mullie, & Vansant, 2010; Poston et al., 2015). This is done independent of the actual dietary habits of those recruited, which has its limitations, as far from all overweight or obese women have suboptimal diets (Tryggvadottir et al., 2016).

The combined evidence from observational, experimental and intervention studies strongly suggest that a healthy diet is important for short and long-term health of the mother and child (World Health Organization, 2016). Still, transfer of existing knowledge into clinical practice has been relatively slow. One reason for this is the lack of methods to assess dietary intake as the use of detailed questionnaires or dietary interviews designed to cover the whole diet are time consuming and impractical for use in clinical practice (Naska, Lagiou, & Lagiou, 2017; Shim et al., 2014). There is a need for a simple dietary screening tool in the clinical setting. It might be more purposeful and cost-effective to target dietary counseling towards more vulnerable groups, based on the background diet. This study aimed to examine whether a short dietary screening questionnaire, answered by pregnant women in their first trimester of pregnancy can give reliable indications of the risk of excessive GWG and macrosomia.

KEY MESSAGES:
- There is a lack of practical method to assess dietary intake in the clinical setting.
- This study used a short dietary screening questionnaire, answered by pregnant women early in pregnancy. Women with poor dietary habits (with a high dietary risk score) had higher risk of excessive GWG and higher odds of delivering a macrosomic offspring compared with women with the lowest scores.
- By asking simple questions about dietary habits early in pregnancy, we might be able to identify women who should be prioritized for further dietary counseling. This procedure could translate to more cost-effective strategies.

PARTICIPANTS AND METHODS

PREgnant Women of ICEland (The PREWICE cohort)

Between October 1st, 2015 to September 31st, 2016, pregnant women in gestational weeks 11-14 who came for an ultrasound at the prenatal diagnostic unit at Landspitali University Hospital were offered to take part in the study. At that timepoint, a dietary screening questionnaire was administered. About 75% of all pregnant women living in the metropolitan area use the clinic's services. Women who did not understand Icelandic and could therefore not answer the questionnaire were not invited to take part in the study. A total of 2113 (77%) out of 2734 eligible women participated in the study. The ethics committee of Landspitali University Hospital approved the study protocol (21/2015), and written consent was obtained from all participants.

Subjects included in the analyses

Out of the 2113 women answering the dietary screening questionnaire, maternal care records were missing for 417 (~20%), the majority probably due to births outside Landspitali University Hospital. Our ethical approval was gathered from the local ethical committee at Landspitali National University Hospital, only allowing us to record information from the maternal care records for women giving birth at Landspitali. Additional 26 women who had multiple pregnancies and another 19 who had missing dietary data were excluded resulting in 1651 women being eligible for analyses. Of these, 313 (19%) had missing data on total GWG, and 12 had missing data on pre-pregnancy BMI status. The final dataset, therefore, consisted of 1326 women with data on GWG and 1651 with data on offspring birth weight.

The dietary assessment

The dietary screening questionnaire was designed to give a snapshot of the participant 's general diet in comparison to food-based dietary guidelines, but at the same time to predict low consumption of key nutrients for fetal development (such as Omega-3 fatty acid, vitamin D,

and iodine) based on the Icelandic diet. Women could answer the dietary screening questionnaire in 5-10 minutes. It consisted of a 40-item list of common foods for which frequency of consumption was recorded (i.e., times per day, daily, times per week, weekly, times per month, monthly or less than monthly). Women were asked about their diet in the previous four weeks, corresponding to the first trimester of pregnancy (enrolled in 11-14 week of pregnancy).

Prior to use in this study, the questionnaire was pilot tested in a group of 25 pregnant women and compared with a four-day weighed food record, with acceptable correlation (>0.3) for most food groups/items (unpublished data).

Covariates and outcomes

Information on maternal lifestyle and socioeconomic factors, pre-pregnancy weight and height were recorded at recruitment. Self-reported pre-pregnancy weight and height were also available from maternal records and those measures were used if those information at recruitment were missing. Information about maternal age, gestational length and GWG were retrieved from the maternal hospital records as women were weighed in antenatal visits. Total GWG was calculated as the difference between the highest recorded weight ($\geq$ week 36 in pregnancy) and pre-pregnancy weight; this information was used to define GWG. Icelandic recommendations on weight gain in pregnancy determined the definition of excessive GWG in this study. Optimal weight gain was 12.1–18.0 kg for pre-pregnant normal-weight women and 7.1–12.0 kg for overweight women (Thorsdottir & Birgisdottir, 1998; Thorsdottir, Torfadottir, Birgisdottir, & Geirsson, 2002). Offspring birth weight was measured at birth by medical staff and was collected from the medical records. Macrosomia was defined as a birth weight of 4500g or higher (Chatfield, 2001).

The dietary risk score

The frequency questions from the 40 items were transformed into predefined 13 dietary risk factors for inadequate diet (see Figure 1). These factors are based on the Nordic (Nordic Nutrition Recommendations, 2014) and Icelandic dietary recommendations (Embætti landlæknis, 2015) and evidence on the association between diet, nutrient intake and the health of the mother and child (Englund-Ogge et al., 2012; Gunnarsdottir et al., 2016; Hrolfsdottir et al., 2016; Olafsdottir et al., 2006; Olsen et al., 2007; Renault et al., 2015). Stepwise backward elimination was used to identify the best combination of these factors for predicting excessive GWG. Model performance was assessed by Nagelkerke's R2. The following six dietary risk factors (predictors) were included in the final model: not eating a varied diet, fruits/vegetables <5 times per day, dairy <2 times per day, whole grain products <2 times per day, sugar/artificially sweetened beverages ≥5 times per week, dairy ≥5 times per day. To construct a total dietary risk score, each participant got 1 for fulfilling the risk criteria, and 0 for not fulfilling the risk criteria. The scores of the six dietary risk factors were then summed up, ranging from 0 to 5 scores as it is not possible to be in both milk risk groups (too low/too high).

Statistical analyses

Assumptions of normality of model residuals were checked, using histograms and QQ plots. Normally distributed variables were described by their mean and standard deviation (SD), non-normally distributed continuous variables by their median and 75-25th percentile, and categorical variables using frequencies (percentages). Students t-tests were used to compare normally distributed continuous variables, whereas, for skewed and categorical variables, Mann-Whitney U test and Chi-square tests were used, respectively.

As excessive GWG is a relatively prevalent outcome (~36% in our sample) associations with excessive GWG in terms of relative risk (RR) were assessed using multivariable Poisson

log-linear regression. However, for macrosomia which is relatively rare event (~5% in our samples) associations were quantified in terms of odds ratios (OR) using logistic regression as OR and RR would be comparable for this outcome and logistic regression was more robust in terms of convergence when adjusting for covariates. Associations were stratified by pre-pregnancy BMI status and early GWG (GWG in weeks 8-15 of gestation), as both high pre-pregnancy weight status and excessive GWG early in pregnancy are well known risk factor of excessive GWG (Knabl et al., 2014; Olafsdottir, Skuladottir, Thorsdottir, Hauksson, & Steingrimsdottir, 2006).

We selected covariates in our adjusted multivariable models *a priori* and on the basis of their potential influence on dietary habits and GWG (Gaillard et al., 2012; Olafsdottir et al., 2006; Restall et al., 2014; Rogozinska et al., 2017; Stuebe, Oken, & Gillman, 2009). When examining the association between the dietary risk score and GWG we included: maternal pre-pregnancy BMI, maternal age, parity, maternal smoking during pregnancy, gestational length when highest weight was recorded and experience of nausea during this pregnancy. Whereas when examining the association between the dietary risk score and macrosomia the following covariates were included: offspring sex, maternal pre-pregnancy BMI, maternal age, parity, maternal smoking during pregnancy and total gestational length.

Missing values for covariates (maternal pre-pregnancy BMI (0.5%), parity (1.2%), educational level (0.9%), maternal smoking during pregnancy (1.5%) total gestational length (0.8%)) were assumed to be "missing at random" and were imputed using multiple imputation (m=10) as implemented in SPSS (MCMC algorithm) Statistical significance was accepted at $p<0.05$. All analyses were done in SPSS 24.0.

RESULTS

Anthropometric and demographic characteristics of the study population are presented in Table 1. For pre-pregnancy BMI, mean (standard deviation (SD)) was 24.1 (6.5) kg/m^2 with 4% of subjects being underweight (BMI<18.5 kg/m^2), 55% normal weight (BMI 18.5-24.9 kg/m^2), 24% overweight (BMI≥25.0-30.0 kg/m^2) and 18% obese (BMI≥30.0 kg/m^2). The mean (SD) age was 30 years (5), and vast minority were nonsmokers (16% smoked prior to and 6% during pregnancy), and had a university education or higher academic degree (59%).

The mean (SD) total GWG was 14.0 (6.3) kg, and 36% of the mothers were defined with excessive GWG and 5% gave birth to a macrosomic infant. Mothers who experienced excessive GWG had slightly longer pregnancy duration (39.8 vs. 39.6 weeks) and their offspring had a higher mean birth weight (3844 g vs. 3689 g) compared with women with optimal weight gain. They also were more likely to be single, have a lower educational level, and smoke before and during pregnancy (table 1). The percentage of women gaining optimal, suboptimal and excessive weight gain during pregnancy, by pre-pregnancy BMI status is presented in table 2. As expected, overweight and obese women had a lower mean GWG when compared to those of normal or underweight. In addition, overweight and obese women were more likely to have excessive GWG.

Stepwise backward elimination was used to identify a reduced set of the 13 predefined dietary risk factors for inadequate diet (Figure 1), that best predicted excessive GWG. After elimination the remaining factors were: a non-varied diet, non-adequate intake of fruits/vegetables, dairy and whole grain intake, as well as excessive intake of sugar/artificially sweetened beverages and dairy. Table 3 shows the percent of women who fulfilled the risk criteria for each of these dietary risk factors. In total 20% reported that they avoided or excluded some food groups (a non-varied diet), with dairy being most commonly excluded (9%). Moreover, most women did not meet public recommendations for fruits and vegetables (87%),

whole grain (92%), and dairy intake (77%). In total 28% reported that they drank sugar and/or artificially sweetened beverages more than five times per week and 2% drank milk products five times or more per day. A higher proportion of women gaining excessive GWG fulfilled the risk criteria for sugar and artificially sweetened beverages (≥5 times per week), compared to women with optimal GWG (p=0.02). The other dietary risk factors did not differ significantly between women gaining excessive vs. optimal GWG (Table 3). The frequency of intake of main food groups by adherence to the recommendation of gestational weight can be seen in supplemental material – table S1.

In table 4 the results for the multivariable association between the dietary risk score, GWG and offspring macrosomia are presented. In fully adjusted models, the dietary risk score, modelled as continuous, was associated with higher risk of excessive GWG (RR=1.10, 95%CI=1.01, 1.19), and higher odds of macrosomia (RR=1.43, 95%CI=1.11; 1.86), When dichotomizing the exposure, women with the highest risk score (≥4 scores) had 1.24 higher risk of excessive GWG (95%CI=1.01; 1.52) and 2.28 higher odds of offspring being born macrosomic (≥4500 g) (95%CI=1.18; 4.38) compared with women with the lowest scores (≤2 scores) (table 4).

In stratified analyses (table 4), the association between the dietary risk score and excessive GWG tended to be stronger among obese women. With macrosomia as the outcome, the association was significant only for normal- and underweight women. For obese women a nonsignificant inverse association was however, observed. However, exclusion of women with gestational diabetes (GDM) (n=264) resulted in a similar trend among all the BMI groups (supplemental table S2). The associations between the dietary risk score, GWG and macrosomia were not dependent on weight gain in the first trimester (table 4).

As use of backward elimination for selecting factors that predict GWG involves some arbitrary decisions in terms of where to stop the elimination process, we examined the stability

of our findings by creating standardized risk score based on fewer and more dietary factors being retained in the model (Supplemental Table S3). The combination of the six dietary risk factors resulted in the strongest predictive outcome.

DISCUSSION

This study aimed to evaluate whether a short dietary screening questionnaire, answered by pregnant women in their first trimester of pregnancy could give reliable indications of risk for excessive GWG and macrosomia. We found that a risk score, including: non-varied diet, non-adequate intake of fruits/vegetables, dairy and whole grain, as well as excessive intake of sugar/artificially sweetened beverages and dairy, was associated with higher risk of excessive GWG and macrosomia. Our results suggest that by asking simple questions about women's dietary intake early in pregnancy, we might be able to identify women who should be prioritized for further dietary counseling and support.

Results from numerous observational studies have shown that a healthy dietary pattern during pregnancy is associated with a decrease in the odds of excessive GWG and various pregnancy complications (Brantsaeter et al., 2009; Chen et al., 2016; Englund-Ogge et al., 2014; Hillesund et al., 2014; Knudsen et al., 2008; Renault et al., 2015; Shin et al., 2016; Tielemans et al., 2015; Tryggvadottir et al., 2016; Uusitalo et al., 2009). However, these results have not been mirrored in nutritional intervention studies (Rogozinska et al., 2017). One reason might be the "one size fits all" approach most commonly used, where researchers recruit and test a specific dietary intervention, independent of participant´s background diet. To change this approach, a practical method to assess dietary intake is needed.

Associations between dietary intake in pregnancy and pregnancy outcomes have commonly been evaluated using detailed food frequency questionnaires or face to face interviews which might take up to an hour to answer if it is designed to cover the whole diet

(Naska, Lagiou, & Lagiou, 2017; Shim et al., 2014). The short dietary screening questionnaire used in this study was however, designed to give a snapshot of a participant's general diet in comparison with food-based dietary guidelines. From this, we identified a reduced set of variables that best predicted excessive GWG.

The results of our study are in line with previous studies using more detailed dietary assessment. Soft drinks and intake of foods high in sugar have been linked to higher risk of excessive GWG in several studies (Hrolfsdottir et al., 2016; Olafsdottir et al., 2006; Renault et al., 2015; Stuebe et al., 2009). Previous Icelandic (Olafsdottir et al., 2006) and Danish (Hrolfsdottir et al., 2016) studies have also found high milk intake in pregnancy to be associated with excessive GWG; potentially related to insulin-like growth factor-1 (IGF-1) mediated growth-promoting effects (Olsen et al., 2007; Qin, He, & Xu, 2009; Sferruzzi-Perri, Owens, Pringle, & Roberts, 2011). Dietary pattern analyses have, however, shown that healthy dietary patterns that include dairy may be protective for excessive GWG (Hillesund et al., 2014; Shin et al., 2016). In the Norwegian MoBa cohort (Hillesund et al., 2014), adherence to a diet including dairy, fruits/vegetables, whole grains, potatoes, and fish and regular meals was associated with lower risk of gaining excessive GWG among women with pre-pregnancy BMI<25 kg/m2. In the NHANES data (Shin et al., 2016), similar posteriori-derived dietary pattern was also inversely associated with GWG. These results harmonize with our findings and indicate that specific food patterns may play a role in weight management during pregnancy.

Macrosomia is a known risk factor for offspring obesity and metabolic syndrome later in life (Hermann, Dallas, Haskell, & Roghair, 2010; Rasmussen & Johansson, 1998). Interventions focused on women at higher risk may, therefore, represent a significant strategy to tackle obesity from a population health perspective. Maternal GWG (Tian et al., 2016) and diet seem to be important risk factors for macrosomia, with a recent systematic review and

meta-analysis of nine randomized controlled trials (n=7458) reporting that personalized maternal nutrition guidance can significantly reduce the rate of offspring macrosomia (RR 0.29; 95 % CI:0.18, 0.45) (Ge, Wang, & Fan, 2015). The current study showed that women with poor dietary habits, i.e., with a high dietary risk score (≥4 scores), had ~2 higher odds of giving birth to a macrosomic infant compared with women with the lowest scores (≤2 scores). This association was, however, not observed among obese women. This may be related to the fact that about half of the obese women (49%) received treatment for GDM which may have resulted in improved dietary habits and lower GWG (Brown et al., 2017). Importantly, our results indicate that prevention efforts should not only target heavy women because more than 40% of the macrosomic cases were among women with a pre-pregnancy normal BMI. When stratified by pre-pregnancy BMI status, the association between the dietary risk score and macrosomia was strongest among lean women (pre-pregnancy BMI<25). To our knowledge, limited attention is drawn to the diet quality of normal-weight women in general in the maternal health care provided by the primary health care.

GWG is a complex biological phenomenon, maternal physiological and metabolic changes may influence it, along with placental metabolism (Catalano & deMouzon, 2015; Institute of Medicine and National Research Council, 2009). In addition to dietary behavior, genetic vulnerability (Andersson et al., 2015), gut microbiota composition (Collado, Isolauri, Laitinen, & Salminen, 2008), and the rate of physical activity (Olson & Strawderman, 2003) may all influence women´s weight gain. Further development of the dietary screening questionnaire and the dietary risk score presented in this study might include harmonization with other known modifiable risk factors, such as low level of physical activity. However, it could be used in the present form as a first screening tool for maternal dietary counseling. Standard maternity care could very easily include this procedure as it only takes 5-10 minutes to answer the dietary screening questionnaire. Focusing more on maternal diet, prioritized by

urgency and expected impact might translate into more cost-effective strategies within the primary care.

The high rate of participation, prospective data collection and information from medical records are the main strength of this study. It is a limitation, to our study that the short dietary screening questionnaire has only been validated against a four-day weighed food records in a pilot study among 25 pregnant women. However, the correlation for most food groups was acceptable (r>0.3). Moreover, pre-pregnancy weight and height were based on self-reported data, possibly leading to bias due to under-reporting. Former studies have though demonstrated a relatively strong validity (Phelan et al., 2011; Shin, Chung, Weatherspoon, & Song, 2014). Even though our covariate adjustments had minimal influence on our effect estimates compared with unadjusted models, we cannot exclude residual confounding or confounding by unmeasured covariate(s). The methodology was based on both predefined (dietary recommendations) and data-driven (stepwise backward elimination) methods, tested in an Icelandic population, the results might therefore not apply to other populations. Testing this methodology with independent data, as well as examining other important outcomes, e.g., GDM and risk of deficiency of nutrients, important for fetal growth, will allow for more rigorous conclusions.

In summary, our results stress that dietary counseling to promote healthy GWG and diet during pregnancy should focus not only on targeting overweight and obese women but also women of normal weight. Today, the latter get limited attention regarding this matter in the primary health care setting. By asking simple questions about women's dietary habits early in pregnancy, we might be able to identify women in more need of support and counseling to meet the GWG recommendations and to find women at higher risk of giving birth to a macrosomic infant. This procedure could translate to more cost-effective strategies in the clinical setting.

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Risk factors for inadequate diet	
1. Not eating a varied diet	8. Sugar and artificially sweetened beverages ≥5 times/week
2. Vegetables and fruits<5 times/day	9. Sweets, ice cream, cakes, cookies ≥2.5 times/week
3. Fish intake <2 times/week	10. French fries ≥1 time/week
4. Dairy intake <2 times/day	12. High dairy intake ≥5 times/day
5. Wholegrain products <2 times/day	11. Processed meat products ≥1 time/week
6. Beans, nuts, seeds <3.5 times/week	13. Quality of fat - using butter (or other unsaturated fat sources) rather than oil (≥50%)
7. Vitamin-D <5 times/week	

Figure 1. The predefined dietary risk factors for inadequate diet

The risk factors were mainly based on the Icelandic Food Based Dietary Recommendations (Embætti landlæknis, 2014), which are based on the Nordic Nutrition Recommendations (Nordic Nutrition Recommendations, 2014). The cutoffs for sugar/artificially sweetened beverages and high dairy intake were set in line with Nordic studies. They show that high intake of these products is associated with high GWG (Hrolfsdottir et al., 2016; Olafsdottir et al., 2006; Renault et al., 2015) and adverse birth outcomes (Englund-Ogge et al., 2012; Olsen et al., 2007).

Table 1. Birth outcomes and characteristics of mothers at baseline in relation to maternal gestational weight gain (Icelandic recommendations)

	All[a] (n=1326)	Optimal GWG[b] (n= 517; 39%)	Suboptimal GWG[c] (n= 333; 25%)	P value[e]	Exc. GWG[d] (n=476; 36%)	P value[f]
Maternal age (y)	30.2 ± 5.2	30.0 ± 5.1	30.1 ± 5.4	0.60[g]	30.0 ± 5.3	0.20[g]
Height (cm)	167.4 ± 6.0	167.3 ± 5.9	167.0 ± 6.0	0.55[g]	167.8 ± 6.1	0.20[g]
Birth weight (g)	3721 ± 491	3689 ± 477	3596 ± 471	0.01[g]	3844 ± 494	<0.01[g]
Gestational age (weeks)	39.6 ± 1.2	39.6 ± 1.1	39.4 ± 1.3	0.02[g]	39.8 ± 1.2	<0.01[g]
Pre-pregnancy weight (kg)	68.0 (19.0)	64.0 (16.0)	68.0 (27.0)	<0.01[h]	72.0 (16.0)	<0.01[h]
Pre-pregnancy BMI (kg/m^2)	24.1 (6.5)	22.7 (5.1)	24.2 (9.9)	<0.01[h]	25.6 (5.2)	<0.01[h]
Pre-pregnancy BMI (groups)				<0.01[i]		<0.01[i]
Underweight (%)[j]	4	4	4		3	
Normal weight (%)[k]	55	68	55		40	
Overweight (%)[l]	24	15	11		43	
Obese (%)[m]	18	13	30		14	
Nulliparous (%)	39	37	35	0.52[i]	42	0.10[i]
Single (%)	6	4	6	0.15[i]	7	0.02[i]
Smoked before pregnancy (%)	16	12	11	0.76[i]	23	<0.01[i]
Smoking during pregnancy (%)	7	5	6	0.29[i]	9	<0.01[i]
Education (%)				<0.01[i]		<0.01[i]
Elementary schooling	13	10	14		15	
High sch. and technical sch.	29	28	27		31	
University education	35	31	39		36	
Higher academic	24	30	20		19	
Feeling nauseous (%)				0.12[i]		0.58[i]
No	10	10	11		9	
Yes, not throwing up	48	48	42		51	
Yes, throwing up sometimes	33	33	33		33	
Yes, throwing up daily	10	9	14		7	

Abbreviations: BMI, body mass index; Exc, excessive; GWG, gestational weight gain.

[a]Values are mean ± standard deviation or median (IQR) for continuous variables and percentages for categorical variables.

[b]Optimal GWG was determined in accordance with the Icelandic recommendations, i.e., underweight and normal-weight women 12-18 kg total GWG and overweight and obese women 7-12 kg total GWG.

[c]Suboptimal GWG was determined in accordance with the Icelandic recommendations, i.e., underweight and normal-weight women <12 kg total GWG and overweight and obese women <7 kg total GWG.

[d]Excessive GWG was determined in accordance with the Icelandic recommendations, i.e., underweight and normal-weight women >18 kg total GWG and overweight and obese women >12 kg total GWG.

[e]Differences between optimal and suboptimal GWG groups.

[f]Differences between optimal and excessive GWG groups.

[g]F-test (Type III) of differences among groups.

[h]Mann-Whitney U test of differences among groups. [i]Chi-square test of differences among group.

[j]Underweight, BMI <18.5 kg/m2. [k]Normal weight, BMI 18.5-24.99 kg/m2. [l]Overweight, BMI ≥25 kg/m2 [m]Obesity, BMI ≥30.

Table 2. The percentage of women gaining suboptimal, optimal and excessive weight during pregnancy

	GWG (kg) mean ± std.	Suboptimal	Optimal	Excessive
All (n=1326)	14.0 ± 6.3	25%	39%	36%
		Suboptimal (≤12.0 kg)	Optimal (12.1-18.0 kg)	Excessive (> 18.0 kg)
Pre-prengnancy BMI < 25 (n=772)	15.4 ± 5.1	25%	48%	26%
		Suboptimal (≤7.0 kg)	Optimal (7.1-12.0 kg)	Excessive (> 12.0 kg)
Pre-pregnancy BMI ≥ 25 (n=554)	12.0 ± 7.1	25%	26%	49%

Abbreviations: BMI, body mass index; GWG, gestational weight gain.

Table 3. Percent of women fulfilling the predefined risk criteria by gestational weight gain (GWG) (n=1326)

Risk factors	All (n=1326)	Optimal GWG[a] (n=517; 39%)	Suboptimal GWG[b] (n=333; 25%)	P[d]	Excessive GWG[c] (n=476; 36%)	P[e]
Not eating a varied diet	**20%**	18%	22%	0.16	21%	0.20
Vegetables and fruits <5 times per day	**87%**	87%	85%	0.26	89%	0.55
Dairy intake <2 times per day	**77%**	78%	75%	0.42	78%	0.82
Wholegrain products <2 times per day	**92%**	90%	91%	0.64	93%	0.15
Sugar and artificially sweetened beverages ≥5 times per week	**28%**	24%	29%	0.07	31%	0.02
Dairy intake ≥5 times per day	**1%**	1%	2%	0.09	2%	0.06

[a]Optimal GWG was determined in accordance with the Icelandic recommendations, i.e., for underweight and normal-weight women 12-18 kg total GWG and overweight and obese women 7-12 kg total GWG.
[b]Suboptimal GWG was determined in accordance with the Icelandic recommendations; i.e., underweight and normal-weight women <12 kg total GWG and overweight and obese women <7 kg total GWG.
[c]Excessive GWG was determined in accordance with the Icelandic recommendations; i.e., underweight and normal-weight women >18 kg total GWG and overweight and obese women >12 kg total GWG.
[d]Chi-square test of differences among groups (optimal vs. suboptimal GWG groups).
[e]Chi-square test of differences among groups (optimal vs. excessive GWG groups).

Table 4. The association between the dietary risk score, excessive gestational weight gain, and macrosomia

	Excessive GWG			Macrosomia		
	RR (95% CI)[a]			OR (95% CI)[b]		
	Cases (%)/n	Crude	Adjusted[c]	Cases (%)/n	Crude	Adjusted[d]
Low scores (≤2)	99 (32%)/305	ref	ref	14 (4%)/ 377	ref	ref
Medium scores (3)	217 (34%)/632	1.06 (0.87, 1.28)	1.04 (0.86, 1.27)	40 (5%)/766	1.43 (0.77, 2.66)	1.40 (0.74, 2.64)
High scores (≥4)	160 (41%)/389	1.27 (1.04, 1.55)*	1.24 (1.01, 1.52)*	37 (7%)/ 508	2.04 (1.09, 3.83)*	2.28 (1.18, 4.38)*
Stratified analyses (continuous)[e]						
All women	476 (36%)/1326	1.12 (1.03, 1.22)*	1.10 (1.01, 1.19)*	91 (6%)/1651	1.36 (1.06, 1.73)*	1.43 (1.11, 1.86)*
BMI < 25 kg/m2	202 (26%)/772	1.13 (0.99, 1.29)	1.07 (0.94, 1.22)	38 (4%)/950	1.56 (1.07, 2.28)*	1.62 (1.09, 2.38)*
BMI 25-30 kg/m2	206 (64%)/320	1.05 (0.96, 1.16)	1.05 (0.94, 1.15)	33 (8%)/395	1.45 (0.96, 2.19)	1.53 (0.94, 2.47)
BMI ≥ 30 kg/m2	68 (29%)/234	1.21 (0.93, 1.57)	1.26 (0.96, 1.64)	20 (7%)/306	0.78 (0.45, 1.36)	0.88 (0.46, 1.66)
Early GWG < 2 kg[f]	220 (26%)/840	1.20 (1.05, 1.37)*	1.18 (1.03, 1.35)*	48 (5%)/1001	1.32 (0.94, 1.86)	1.35 (0.93, 1.96)
Early GWG ≥ 2 kg[f]	222 (57%)/389	1.11 (1.01, 1.22)	1.08 (0.98, 1.19)	29 (6%)/490	1.37 (0.89, 2.11)	1.55 (0.96, 2.49)

Abbreviations: BMI, body mass index; GWG, gestational weight gain.

*indicates significant associations.

[a]Poisson log-linear regression model, reflecting the risk of excessive GWG. Excess GWG was determined in accordance with the Icelandic recommendations, i.e., for underweight and normal-weight women >18 kg and overweight and obese women >12 kg total GWG.

[b]Logistic regression model, reflecting the odds of giving birth to a macrosomic infant (birthweight ≥4500g).

[c]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, gestational length when highest weight was recorded and nausea.

[d]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, total gestational length and offspring sex.

[e]When stratifying by pre-pregnancy BMI and early GWG, maternal pre-pregnancy BMI, age, and gestational length were continuous in the adjusted model.

[f]GWG in weeks 8-15 of gestation. Differences in number of cases to overall number of cases are because of missing values. In total, 97 had missing values on early GWG of the 1326 women with data regarding total GWG and 160 of the women with data on birth weight. Adjustments made for gestational length when early GWG were recorded.

Table S1. Food intake of main food groups (per week) by adherence to the recommended gestational weight gain (GWG) (n=1326)

Intake per week	ALL median	ALL IQR	Optimal GWG (39%)[a] median	Optimal GWG (39%)[a] IQR	Suboptimal GWG (25%)[b] median	Suboptimal GWG (25%)[b] IQR	P[d]	Excessive GWG (36%)[c] median	Excessive GWG (36%)[c] IQR	P[e]
Vegetable and fruit	14.8	9.5 - 28.0	16.5	10.0 - 28.0	14.0	9.5 - 28.0	0.54	14.6	10.0 - 28.0	0.88
Vegetable	7.0	5.0 - 14.0	7.0	5.0 - 14.0	7.0	5.0 - 14.0	0.27	7.0	5.0 - 14.0	1.00
Fruit	7.0	5.0 - 14.0	7.0	5.0 - 14.0	7.0	5.0 - 14.0	0.97	7.0	5.0 - 14.0	0.83
Fish	1.3	0.8 - 2.0	1.5	1.0 - 2.0	1.3	0.6 - 2.0	0.15	1.3	0.8 - 2.0	0.10
Lean fish	1.0	0.5 - 1.0	1.0	0.5 - 1.0	1.0	0.5- 1.0	0.29	1.0	0.5 - 1.0	0.33
Fatty fish	0.3	0.1 - 0.5	0.5	0.1 - 1.0	0.3	0.1 - 0.5	0.03	0.3	0.1 - 0.5	0.02
Dairy products	7.9	3.9 - 12.8	7.9	3.9 - 12.6	7.9	3.4 - 13.2	0.80	7.9	4.4 - 12.7	0.52
Sour dairy products	2.5	1.0 - 5.0	2.5	1.0 - 5.0	2.5	0.8 - 5.0	0.85	2.5	1.0 - 5.0	0.48
Wholegrain products	2.8	1.1 - 7.1	3.0	1.3 - 7.1	2.8	1.1 - 5.5	0.01	2.6	1.1 - 5.9	0.02
Bread wholegrain	2.5	0.5 - 5.0	2.5	0.5 - 5.0	2.5	0.5 - 5.0	0.04	2.5	0.5 - 5.0	0.25
Beans, nuts, seeds	0.5	0.3 - 2.5	0.5	0.3 - 2.5	0.5	0.3 - 1.0	0.29	0.5	0.3 - 2.1	0.84
Oil/unsaturated fat used when cooking	2.5	2.5 - 5.0	5.0	2.5 - 7.0	2.5	2.5 - 5.0	0.21	2.5	2.5 - 5.0	0.05
Butter/saturated fat for cooking and spread on bread	6.0	3.0 - 9.5	7.1	3.0 - 10.0	6.0	3.0 - 9.5	0.14	5.5	3.0 - 8.0	0.01
Vitamin D and cod liver oil	7.1	2.6 - 9.5	7.1	2.6 - 10.0	7.1	1.0 - 7.5	0.03	7.1	1.2 - 9.5	0.29
French fries and fried potatoes	0.5	0.5 - 1.0	0.5	0.5-1.0	0.5	0.5 - 1.0	0.85	0.5	0.5 - 1.0	0.36
Sweets, ice cream, cakes, and cookies	3.5	1.5 - 5.0	3.5	1.5 - 5.1	3.0	1.5 - 5.0	0.11	3.0	1.5 - 5.0	0.37
Sugar- and artificially sweetened beverages	2.0	0.6 - 5.0	1.5	0.6 - 3.5	2.0	0.6 - 5.0	0.24	2.0	0.6 - 5.0	0.03
Sugar-sweetened beverages	1.0	0.1 - 2.5	1.0	0.1 - 2.5	1.0	0.3 - 2.5	0.13	1.0	0.1 - 2.5	0.90
Artificially sweetened beverages	0.3	0.1 - 1.0	0.1	0.1 - 1.0	0.1	0.1 - 1.0	0.84	0.5	0.1 - 2.0	<0.01
Red meat	1.0	0.5 - 2.5	1.0	0.5 - 2.5	1.0	0.5 - 2.5	0.32	1.0	0.5 - 2.5	0.16
Processed meat products	0.5	0.1 - 1.0	0.5	0.1 - 1.0	0.5	0.1 - 1.0	0.24	0.5	0.1 - 1.0	0.40

[a]Optimal GWG was determined in accordance with the Icelandic recommendations; i.e., for underweight and normal-weight women 12-18 kg total GWG and overweight and obese women 7-12 kg total GWG.

[b]Suboptimal GWG was determined in accordance with the Icelandic recommendations; i.e., underweight and normal-weight women <12 kg total GWG and overweight and obese women <7 kg total GWG.

[c]Excessive GWG was determined in accordance with the Icelandic recommendations; i.e., underweight and normal-weight women >18 kg total GWG and overweight and obese women >12 kg total GWG.

[d]Mann-Whitney U test of differences among groups (optimal vs. suboptimal GWG groups).

[e]Mann-Whitney U test of differences among groups (optimal vs. excessive GWG groups).

Table S2. The association between the dietary risk score, GWG, and macrosomia (GDM cases excluded).

| | GDM[a] excluded (264 cases excluded) | | | |
| | Excessive GWG | | Macrosomia | |
	cases (%)/n	RR (95% CI)[b,c]	cases (%)/n	OR (95% CI)[d,e]
Low scores (≤2)	89 (34%)/266	ref	11 (3%)/332	ref
Medium scores (3)	184 (35%)/533	1.01 (0.82, 1.24)	31 (5%)/645	1.37 (0.67, 2.83)
High scores (≥4)	136 (42%)/ 326	1.20 (0.97, 1.49)	30 (7%)/410	2.57 (1.22, 5.40)*
Stratified analyses (continuous)[f]				
All women	409 (36%)/1125	1.09 (1.00, 1.19)*	72 (5%)/1387	1.51 (1.12, 2.03)*
BMI < 25 kg/m2	190 (26%)/732	1.07 (0.94, 1.23)	35 (4%)/899	1.72 (1.14, 2.59)*
BMI 25-30 kg/m2	182 (66%)/275	1.03 (0.93, 1.14)	26 (8%)/333	1.38 (0.79, 2.42)
BMI ≥ 30 kg/m2	37 (31%)/118	1.31 (0.87, 1.97)	11 (7%)/155	1.14 (0.44, 2.98)
Early GWG < 2 kg[g]	195 (27%)/712	1.13 (0.99, 1.31)	38 (5%)/843	1.54 (1.00, 2.39)*
Early GWG ≥ 2 kg[g]	186 (57%)/325	1.09 (0.99, 1.22)	23 (6%)/406	1.43 (0.85, 2.42)

Abbreviations: BMI, body mass index; GDM, gestational diabetes; GWG, gestational weight gain.

*indicates significant associations.

[a]Criteria that were used: *Metzger, B. E., Gabbe, S. G., Persson, B., Buchanan, T. A., Catalano, P. A., Damm, P., . . . Schmidt, M. I. (2010). International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care, 33(3), 676-682. doi:10.2337/dc09-1848.*

[b]Poisson log-linear regression model, reflecting the risk of excessive GWG. Excess GWG was determined in accordance with the Icelandic recommendations i.e., for underweight and normal-weight women >18 kg and overweight and obese women >12 kg total GWG.

[c]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, gestational length when highest weight was recorded and nausea.

[d]Logistic regression model, reflecting the odds of giving birth to a macrosomic infant (birthweight ≥4500g).

[e]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, total gestational length and offspring sex.

[f]When stratifying by pre-pregnancy BMI and early GWG, maternal pre-pregnancy BMI, age, and gestational length were continuous in the adjusted model.

[g]GWG in weeks 8-15 of gestation. Differences in number of cases to overall number of cases are because of missing values. In total, 88 had missing values on early GWG of the 1125 women with data regarding exc. GWG and 138 of the 1387 women with data on birth weight (GDM cases excluded).

Table S3. Different combinations of the dietary risk score and the risk of excessive gestational weight gain (GWG)[a]

	Excessive GWG[b] RR[c] (95%CI)	
	Crude	Adjusted[d]
SCORE-1[e]	1.06 (0.99, 1.14)	1.05 (0.97, 1.12)
SCORE-2[f]	1.09 (1.02, 1.17)*	1.08 (1.01, 1.16)*
SCORE-3[g]	1.10 (1.02, 1.19)*	1.09 (1.01, 1.17)*

*indicates significant associations.

[a]Standardized coefficient reflecting the risk of excessive GWG per standard deviation increase in the dietary risk score.

[b]Excess GWG was determined in accordance with the Icelandic recommendations, i.e., for underweight and normal-weight women >18 kg and overweight and obese women >12 kg total GWG.

[c]Poisson log-linear regression model.

[d]Adjusted for maternal pre-pregnancy BMI, age, parity, smoking during pregnancy, educational level, gestational length when highest weight was recorded and nausea.

[e]All 13 dietary risk factors included, i.e., not eating a varied diet, vegetables and fruits <5 times per day, fish intake < 2 times per day, dairy intake < 2 times per day, whole grain products < 2 times per day, beans, nuts, seeds <3.5 times per week, D-vitamin <5 times per week, quality of fat - using butter rather than oil (≥50%) french fries and fried potatoes ≥ 1 times per week, sweets, ice cream, cakes, cookies ≥ 2.5 times per week, sugar- and artificially sweetened beverages ≥ 5 times per week, dairy intake ≥ 5 times per day, processed meat products ≥ 1 times per week.

[f]Three dietary risk factors included, i.e., factors most strongly associated with excessive GWG in the multivariable model: sugar- and artificially sweetened beverages ≥ 5 times per week, whole grain products < 2 times per day and dairy intake ≥ 5 times per day.

[g]The dietary risk score, which included the six dietary risk factors, i.e., a non-varied diet, vegetables and fruits <5 times per day, dairy intake < 2 times per day, whole grain products < 2 times per day, sugar- and artificially sweetened beverages ≥ 5 times per week, dairy intake ≥ 5 times per day.

Appendix 1

	Paper I	Paper II	Paper III	Paper IV
Citation	IJO 2015	JAHA 2017	Obesity 2016	Manuscript
Pregnancy cohort	DaFO88 cohort	DaFO88 cohort	DaFO88 cohort	PREWICE cohort
Design	Prospective observational study	Prospective observational study	Cross-sectional study	Prospective observational study
Study sample in main analysis	N=308 mother-offspring pairs	N=434 mother-offspring pairs	N=671 mothers	N=1326 mothers (weight gain) N=1651 mother-offspring pairs (birth weight)
Exposure	Maternal GWG (antenatal records), i.e.: • GWG at week 30: continuous term • GWG total: used to classify GWG according to the IOM guidelines	Maternal micronutrient intake: • FFQ, face-to-face interview	Maternal GWG (antenatal records) • GWG at week 30: continuous term • GWG total: used to classify GWG according to the IOM guidelines • FFQ, face-to-face interview	Maternal general diet: • Short FFQ (diet screening questionnaire)
Outcomes	• Height, weight and waist circumference • Serum leptin and adiponectin • Plasma insulin • HOMA-IR • Serum triglycerides • Total cholesterol • LDL • HDL • Resting pulse • SBP • DBP	 • SBP • DBP	*Main analyses:* • hsCRP • SAA • IL- 6 • IL-8 • IL-1b • TNF-α *Additional analyses:* Risk of excessive GWG[1]	 Risk of excessive GWG[2] Macrosomia (≥4500 g)
Covariates	Maternal pre-pregnancy BMI, age, parity, education, smoking in pregnancy offspring sex.	Maternal pre-pregnancy BMI, age, parity, education, smoking in pregnancy offspring sex.	Maternal pre-pregnancy BMI, age, parity, education and smoking in pregnancy. When examining maternal diet and inflammation, maternal total energy intake was also included in the model.	Maternal pre-pregnancy BMI, age, parity, education, smoking in pregnancy, gestational length, nausea. When examining macrosomia, offspring sex was also included in the model (nausea excluded).
Statistical methods	Linear regression Logistic regression	Linear regression Substitution model	Linear regression Logistic regression	Stepwise backward elimination Logistic regression Poisson log-linear regression

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; FFQ, Food Frequency Questionnaire; GWG: gestational weight gain; HDL, high-density lipoprotein, Homa-IR, homeostasis model assessment-estimated insulin resistance; hsCRP, a high-sensitivity C-reactive protein; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; LDL, low-density lipoprotein; SAA, Serum amyloid A; SBP, systolic blood pressure; TNF-α, Tumor necrosis factor alpha.

[1] Excessive GWG was determined in accordance with the Institute of Medicine recommendations for each pre-pregnancy BMI category, i.e., for underweight women >18 kg, normal weight >16 kg, overweight >11.5 kg, and obesity >9 kg total GWG.
[2] Excessive GWG was determined in accordance with the Icelandic recommendations, i.e., for underweight and normal weight women >18 kg, overweight and obese women >12 kg GWG.

Number:

HÁSKÓLI ÍSLANDS

FOOD SELECTION

Do you avoid or not eat certain types of food?   Yes   No

If yes, mark the types of food that you avoid or do not eat.

Cereal products	
Vegetables	
Fruits	
Fish	
Meat	
Eggs	
High-fat foods	
Dairy products	

CONSUMPTION OF VARIOUS FOODS
Keeping in mind the last 4 weeks, put one cross in each row.

How many times per month or week or day do you eat the following types of food?

(Questions on fish and meat (including processed meat products) apply to main meals, not sandwich meats or spreads.)

	Per month			Per week			Per day			
	<1	1	2-3	1	2-3	4-6	1	2	3-4	≥5
Vegetables										
Fruits										
Lean fish (e.g., haddock or cod)										
Fatty fish (e.g., salmon, trout or large halibut)										
Red meat (beef, lamb or pork)										
Poultry										
Processed meat products, meat dough products or sausages										
Soured dairy products (sour milk, skyr or yogurt)										
Cheese										
Whole-grain products, other than bread*										
Bean dishes, nuts or seeds (not in breads)										
French fries and/or packaged snacks										
Cakes and/or crackers										
Candy and/or ice cream										

*E.g., brown rice, barley or whole-wheat pasta, as accompaniment or part of main meals.

HIGH-FAT FOODS
Keeping in mind the last 4 weeks, put one cross on each row.

How often do you use the following high-fat foods?

	Per month			Per week			Per day			
	<1	1	2-3	1	2-3	4-6	1	2	3-4	≥5
Oil or other soft fat for food preparation										
Butter or other hard fat for food preparation										
Butter or oil-blended butter on bread										

BEVERAGES
Keeping in mind the last 4 weeks, put one cross on each row.

How many <u>portions</u> per month <u>or</u> per week <u>or</u> per day do you drink of the following beverages?

Assume that one portion is about 250 ml.

Remember to include in the estimate milk on morning cereal or mush and in coffee. Carbonated or noncarbonated beverages include all types of carbonated beverages, sports drink, fruit drink (other than pure fruit juices) and energy drink.

	Per month			Per week			Per day			
	<1	1	2-3	1	2-3	4-6	1	2	3-4	≥5
Pure fruit juice										
Whole milk										
Low-fat milk										
Low-fat milk fortified with vitamin D										
Fortified nonfat milk (vitamin D- and protein-fortified)										
Nonfat milk										
Carbonated and noncarbonated drinks with added sugar										
Carbonated and noncarbonated drinks with sweeteners										
Coffee										
Alcohol										

BREAD
Keeping in mind the last 4 weeks, put one cross on each row.

How many <u>slices</u> per month <u>or</u> per week <u>or</u> per day do you eat of the following kinds of bread?

	Per month			Per week			Per day			
	<1	1	2-3	1	2-3	4-6	1	2	3-4	≥5
Whole-grain bread. This means breads marked with the Keyhole, or specified as fiber rich or whole grained.										
Other breads. This means "usual" wheat breads (Heimilisbrauð, Bónus/Krónu-brauð, ciabatta, soft-cheese bows, etc.)										
Rye bread with added sugar. Here, this pertains only to Icelandic rye bread. Please record other rye bread, e.g., Danish rye bread, as whole-grained bread.										

SUPPLEMENTS
Keeping in mind the last 4 weeks, put one cross on each row.

How often per month <u>or</u> per week <u>or</u> per day do you use the following supplements?

	Per month			Per week			Per day			
	<1	1	2-3	1	2-3	4-6	1	2	3-4	≥5
Cod liver oil										
Vitamin D										
Folate/folacin/folic acid										
Iron										
Multivitamins with vitamin A										
Multivitamins without vitamin A										
Other supplements or food additives*										

*Other supplements, which ones? _____

Thank you for participating!