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Nutrition and Oxidative Parameters in Pregnancy, Size at Birth and Metabolic Status of the Offspring at 4.5 Years

The MINIMat Trial in Rural Bangladesh

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Abstract
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Undernutrition and oxidative stress in fetal life and infancy may lead to adverse health outcomes in the offspring. We studied nutrition and oxidative parameters in pregnancy and their associations with birth anthropometry and metabolic status in the children.

In Matlab in rural Bangladesh, women were randomized to either early (Early) invitation to food supplementation or to start at their own liking (Usual). Women were also allocated to either; 1) 60 mg iron and 400 µg folic acid (Fe60F), 2) multiple micronutrients including 30 mg iron and folic acid (MMS), or 3) 30 mg iron and folic acid (Fe30F). Micronutrients (hemoglobin, iron, zinc, folic acid, vitamin B-12) were assessed in pregnancy week 14, lipid peroxidation in week 14 and 30, and DNA oxidation in week 19. The offspring were assessed for anthropometric measurements at birth and metabolic status at 4.5 years.

Micronutrient deficiencies were common with zinc and vitamin B-12 deficiency being most prevalent. Anemia was present in approximately one third of women, however, iron deficiency was uncommon seen in only 2%.

Maternal Early food supplementation group resulted in an improved lipid status in the children at 4.5 years compared to Usual food group. Prenatal use of MMS lowered the children's glucose, insulin, HOMA-IR, and growth factors compared to Fe60F.

Lipid peroxidation in early pregnancy was associated with size at birth and insulin and HOMA-IR levels in the children. Lipid peroxidation in late pregnancy, however, was associated with the children's lipid status. Both increasing lipid peroxidation and increasing DNA oxidation was associated with decreasing IGF-1 levels.

The beneficial effects of an Early start of food supplementation show that an improved prenatal nutrition may have lasting effects in the offspring and highlights the importance of early timing food supplementation. Use of MMS, however, resulted in lower insulin levels, which, considering the already low level of insulin in these children, may be a cause of concern. MMS also resulted in growth factors indicative of slower growth and further research appears to be needed before scaling up the use of MMS. Oxidative parameters in pregnancy were associated with longer-term outcomes in the offspring, suggesting that oxidative stress may be involved in the development of later metabolic disease.

Keywords: Pregnancy, micronutrient deficiency, anemia, birth size, food supplementation, micronutrient supplementation, metabolic status, childhood

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Lindström, E., Hossain MB., Lönnerdal, B., Raqib, R., El Arifeen, S., Ekström, EC. (2011) Prevalence of anemia and micronutrient deficiencies in early pregnancy in rural Bangladesh, the MINIMat trial. *Acta Obstet Gynecol Scand,* 90(1):47-56.

II Ekström, EC., Lindström, E., Raqib, R., El Arifeen, S., Brismar, K., Persson, LA. Prenatal food and micronutrient supplementation have effects on metabolic status at 4.5 years of age. The randomised MINIMat trial in rural Bangladesh. *Manuscript.*

III Lindström, E., Persson, LA., Raqib, R., El Arifeen, S., Basu, S., Ekström, EC. (2012) Associations between oxidative parameters in pregnancy and birth anthropometry in a cohort of women and children in rural Bangladesh: The MINIMat-cohort. *Free radical research*, 46(3):253-64.

IV Lindström, E., Persson, LA., Raqib, R., El Arifeen, S., El Arifeen, S., Basu, S., Brismar, K., Ekström, EC. Oxidative parameters in pregnancy and metabolic, oxidative and inflammatory markers in preschool aged offspring: The MINIMat-cohort in rural Bangladesh. *Submitted.*

Contents

Abbreviations

8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin-$F_{2\alpha}$
8-OHdG	8-hydroxydeoxyguanosine
ANOVA	Analyses of variance
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
BMI	Body Mass Index
CI	Confidence interval
CRP	C-reactive protein
DOHaD	Developmental Origins of Health and Disease
Fe30F	30 mg iron and 400 µg folic acid supplement
Fe60F	60 mg iron and 400 µg folic acid supplement
HAZ	Height-for-age z-score
Hb	Hemoglobin
HDL	High-density lipoprotein
HDSS	Health and Demographic Surveillance System
HOMA-IR	The Homeostatic Model of Assessment-Insulin Resistance
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
IGF-1	Insulin-like growth factor 1
IGFBP-1	Insulin-like growth factor-binding protein 1
IQR	Interquartile range
LBW	Low birth weight
LDL	Low-density lipoprotein
MINIMat	Maternal and Infant Nutrition Interventions, Matlab
MMS	Multiple micronutrients supplement
OR	Odds ratio
SD	Standard deviation
SES	Socioeconomic status
SGA	Small for gestational age
TG	Triglycerides
WAZ	Weight-for-age z-score
WHO	World Health Organization

Introduction

Pregnancy is a period of extensive tissue growth and thus a period of increased nutritional requirements. The fetus depends on the interactions between mother and placenta and poor nutrition during this time may have both short and long-term health consequences for the offspring. Many low-income settings are facing a double burden of disease where maternal undernutrition is common and a high proportion of newborns has a low birth weight (LBW) (1) at the same time as chronic metabolic diseases such as diabetes type 2 and cardiovascular disease, often associated with overnutrition, are increasing (2). Fetal undernutrition, as indicated by LBW, has been shown to be associated with an increased risk of metabolic diseases later in life (3) providing a link between under- and over-nutrition, which is of particular relevance to settings facing this double burden of disease.

Nutrition in pregnancy

Nutritional deficiencies in pregnancy and short-term health consequences

Maternal nutrition is critically important for the health of mother and child and nutritional deficiencies during pregnancy may lead to adverse pregnancy outcomes. There is an increased requirement of both macro- and micronutrients (4) increasing the vulnerability of malnutrition in settings with limited resources. Maternal anemia and micronutrient deficiencies are common in many low-income settings (5). As common causes of deficiency such as inadequate diet and frequent infections are shared by several micronutrients, micronutrient deficiencies often coexist. Anemia is considered a major public health problem, where in South-East Asia, almost half of all pregnant women are estimated to be anemic (6). While this has been questioned (7), iron deficiency has commonly been considered to be the main cause of anemia (8), however, deficiency of B-vitamins (9) and infectious diseases are also potential contributors. Maternal anemia may lead to pre-term delivery and LBW (10), however, other micronutrient deficiencies in pregnancy could also be detrimental to the health of both mother and child. The importance of folate during pregnancy is well established and folate deficiency is a risk factor for neural tube defects (11). Further, inadequate vitamin B-12

concentrations may be associated with adverse pregnancy outcomes such as preterm birth (12), and developmental problems have been observed in infants born with low stores of vitamin B-12 (13). Moreover, maternal zinc deficiency has been suggested to be associated with poor maternal and perinatal health such as poor fetal growth and development and LBW (14, 15). In conclusion, there are several lines of evidence to suggest that an adequate micronutrient status in pregnancy is important for fetal development and positive pregnancy outcome. The macronutrient status of the women during pregnancy is also important and a low maternal BMI may as well be associated with adverse pregnancy outcomes (16).

Nutritional deficiencies in pregnancy and longer-term health consequences (the Developmental Origins of Health and Disease)

The importance of prenatal nutrition for longer-term health outcomes for the offspring is highlighted in the Developmental origins of health and disease hypothesis (DOHaD). It proposes that influences early in life may lead to physiological modifications predisposing an individual to an increased risk of developing chronic adult disease in adult life (3, 17, 18). This hypothesis is supported by a large number of epidemiological studies showing associations between LBW used as a crude proxy of poor nutrition in pregnancy to metabolic dysfunction in later life such as hypertension (19), diabetes type 2 (20), and cardiovascular diseases (21). In addition, ecological studies of catastrophes and natural disasters have observed associations between gestational undernutrition and coronary heart disease in Holland (22) and increased hypertension and glucose intolerance in adults exposed to famine in fetal life and infancy in Nigera (23). A rapid weight gain in infancy, so called catch-up growth, has been discussed to aggravate the negative effects caused by LBW (24, 25).

There is also evidence supporting the DOHaD hypothesis from a large number of experimental studies in animal models where mechanisms underlying these associations have been studied in more detail (26, 27). Rodent models have been extensively used, to demonstrate a reduced β-cell mass observed in offspring born to dams following exposure to caloric restriction during pregnancy (28), and insulin resistance as the result of maternal protein restriction (29). Larger animal models have also been used showing a relationship between reduced nutrition in pregnancy and increased blood pressure in the offspring in sheep (30).

Furthermore, the potential role of maternal micronutrient deficiencies, in particular vitamin B-12 and folate, on long-term health outcomes have recently been highlighted. In a study of 6 year old children conducted in India,

an interplay between maternal folate and vitamin B-12 during pregnancy was demonstrated in the children of mothers whom were vitamin B-12 deficient in combination with high folate status as the most insulin resistant as measured by HOMA-IR (31). As components of one-carbon metabolism, vitamin B-12 and folate are key players in DNA methylation and may be involved in epigenetic modifications (32). Both deficiency of zinc, essential in many enzyme systems, and iron have also been suggested to be associated with offspring insulin resistance but studies are inconclusive (33).

Although the DOHaD hypothesis began with fetal undernutrition, the role of maternal overnutrition is now recognized and a U-shaped relationship between birth weight and later health outcomes for the adult has been suggested (34). There are observations of induced obesity in pregnant rats being associated with elevated fasting insulin in the offspring (35) and in humans maternal obesity in pregnancy has been associated with both fetal insulin resistance (36) and insulin resistance in 11-year old children (37).

While the focus of this discussion has been on undernutrition and conditions related to metabolic dysfunction (38, 39), other exposures in early life potentially influencing later health are also being discussed. For example, toxins such as tobacco smoke (40) and endocrine-disrupting chemicals (41) in pregnancy are found to be associated with increased obesity in the offspring. Moreover, there is a growing body of literature supporting associations between early life exposures to various factors and adult health such as impaired immune function (42), mental health (43), and asthma and lung function (reviewed in (44)).

The importance of timing in the Developmental Origins of Health and Disease

Evidence is emerging that the timing of poor nutrition in pregnancy is important. There appears to be critical windows during development where the outcome for the offspring will depend on the stage of fetal development and the vulnerability of the fetus at that specific time (26). This has been seen in animal studies where maternal nutrient restriction in sheep elicited different responses depending on timing. Nutrient restriction from the first week of pregnancy throughout gestation resulted in increased adipose tissue in the offspring despite a decrease in birth weight, while nutrient restriction in late pregnancy only was associated with reduced adipose tissue without a change in birth weight (45). The importance of timing has also been described in human studies. In a longitudinal study from Guatemala, maternal weight gain in mid-pregnancy was found to be associated with infant birth weight, length and head circumference while weight gain in late pregnancy was associated with birth weight only, suggesting the timing of nutritional influ-

ences in fetal life is important for growth (46). Additionally, data from the Dutch hunger winter study demonstrates that the fetal response is dependent on whether the fetus is exposed to famine in early, mid or late gestation. Famine during early gestation was associated with a more atherogenic lipid profile in adult life while famine exposure during late gestation was associated with an increase in plasma glucose compared to unexposed individuals (47). Furthermore, these studies also demonstrated that the adult response to fetal famine does not necessarily have to be mediated by a reduced birth weight.

Sex differences in the Developmental Origins of Health and Disease

It is also known that there are differences in the incidence and manifestation of a wide range of diseases between men and women starting already in fetal life. In a longitudinal study of uncomplicated pregnancies, a difference between fetal growth in male and female fetuses was observed dependent on maternal height and pre-pregnancy weight (48). In relation to DOHaD, there are several reports of sex-specific associations between prenatal nutritional exposures and offspring outcomes in both animal and human studies. Male rats born to dams fed a protein-restricted diet during pregnancy had higher concentration of triglycerides and cholesterol compared to controls, while these variations in metabolic markers were not seen in female offspring (49). In humans, LBW was associated with adult hypertension in both male and females in the Helsinki birth cohort. However, while a large placental surface was also associated with hypertension in men, in women an association was instead observed between a small placental surface and hypertension in later life (50). In data gained from periods of famine exposure during gestation from Holland, men who had been exposed to undernutrition in fetal life had an association between hypertension and placental area, however, no such association was found in the women (51).

Oxidative stress in pregnancy

Oxidative stress is the result of an overproduction of reactive species, a decreased antioxidant status, or both. While reactive species have normal physiological functions in cell signaling, apoptosis and microorganism defense, the imbalance in the body's pro-oxidant-antioxidant status may have potentially harmful effects (52). Oxidative stress has therefore mainly been associated with outcomes such as aging, cardiovascular disease, cancer and impaired immune function. Whereas some disease may be a direct consequence of the reactive species attack on polyunsaturated fatty acids in lipids, amino

acids in proteins or the oxidation of DNA, an increase in oxidative stress may also be a result of the disease itself (53).

Oxidative stress has also been suggested as a potential mediator in the development of the metabolic syndrome (54-56). In adults, increased oxidative stress is often a feature of metabolic diseases and has been observed in individuals with disorders such as obesity (57), hypertension (58), and cardiovascular disease (59). Oxidative stress is also closely linked to inflammation, which may predispose subjects to insulin resistance (60, 61). A causal mechanism in linking oxidative stress to metabolic dysfunction was suggested in studies of hypertensive rats where an increased oxidative stress caused alterations in the contraction of the endothelium thus causing a negative effect on the circulation (62).

There are several human studies that support the relationship between oxidative stress and DOHaD. An increase in oxidative stress has been observed both in neonates born small for gestational age (SGA) by malnourished mothers (63) and in pre-pubertal children born SGA (64). Furthermore, undernutrition in childhood has been associated with a reduced antioxidant status (65, 66). However, intergenerational studies of maternal oxidative stress and offspring outcomes, in particular longer-term, are scarcer. Studies have suggested that an impairment of fetal growth was a consequence of higher maternal oxidative stress as increased oxidative DNA damage was found in women who gave birth to LBW babies when compared to women with babies with normal birth weights (67-69). In rodent models, increased oxidative stress in pregnancy as a consequence of undernutrition *in utero* has been associated with endothelial dysfunction in male offspring (70). In addition, maternal oxygen exposure as a way to induce oxidative stress was associated with vascular dysfunction in the offspring of rats (71).

In further support of an potential link between maternal oxidative stress and later metabolic disease in the offspring is the observation that, in addition to undernutrition, overnutrition may also produce oxidative stress (72). Rapid weight gain in childhood has been put forward to worsen the potential consequences of being born with a LBW (73) and this catch-up growth as a result of nutrition has been suggested to lead to an increased oxidative stress. This was observed in pre-pubertal children born SGA followed by catch-up growth who had an increased oxidative stress compared to both children born with a normal birth weight as well as to SGA children who had not experienced a rapid increase in growth in early childhood (74).

Scientific justification for the studies of the thesis

The majority of epidemiological studies investigating associations between prenatal influences and adult chronic disease are retrospective in design and performed in high-income settings drawing upon historical data. However, these associations are of particular relevance for low-income settings where poor nutrition in early life is common at the same time as chronic metabolic diseases in adult life are increasing.

Furthermore, there is a lack of human studies evaluating longer-term outcomes in the offspring when efforts to improve maternal nutrition have been made and thus whether it is possible to modify risk for adult metabolic diseases. As women in disadvantaged settings may suffer from both macro and micronutrient deficiencies it is important to investigate whether combinations of nutritional interventions in pregnancy may be needed for favorable long-term health of the offspring. In addition, an understanding of the underlying mechanism linking early life nutrition with long-term health may assist in designing appropriate nutrition interventions. Oxidative stress has been associated with metabolic syndrome but whether this has its origin in fetal life has not been evaluated. A conceptual framework that illustrates the focus of this thesis is shown in **Figure 1**.

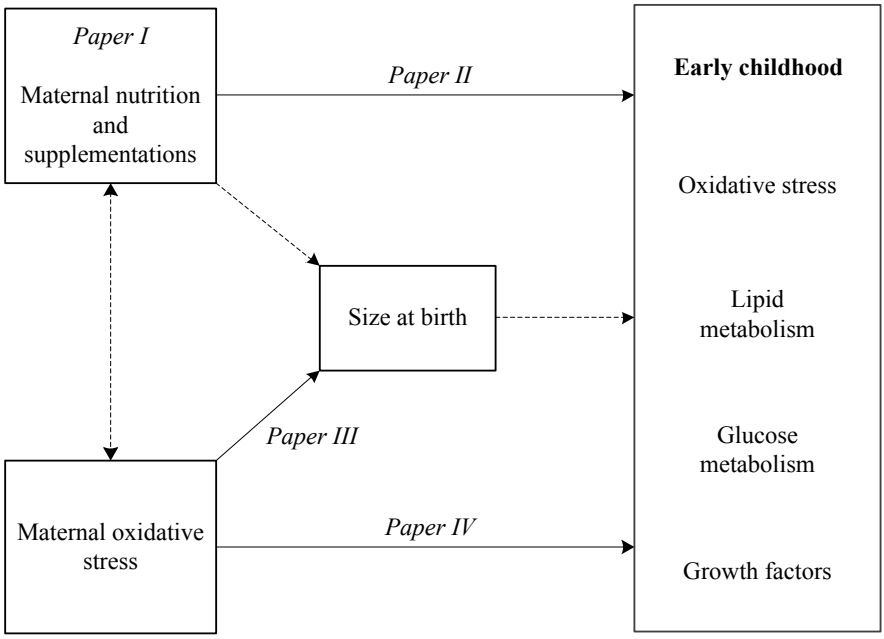


Figure 1. Conceptual framework of exposures of nutrition and oxidative stress in pregnancy and potential associations with offspring birth anthropometry and metabolic status.

Aim and objectives

The overall aim of this thesis is to study maternal nutrition and oxidative parameters in pregnancy and to examine their associations with birth anthropometry and metabolic status in the offspring in early childhood in a cohort of women and children in rural Bangladesh.

Specific objectives:

1. To describe the prevalence of anemia and micronutrient deficiencies as well as examine their determinants in early pregnancy (Paper I)

2. To analyze the effects of prenatal food and micronutrient supplementation on the metabolic status, growth factors and inflammatory status in the offspring in early childhood (Paper II)

3. To analyze associations between oxidative parameters in pregnancy and birth anthropometry (Paper III)

4. To analyze associations between oxidative parameters in pregnancy and metabolic status, growth factors and markers of oxidative and inflammatory status in the offspring in early childhood (Paper IV)

Methods

Study setting

A randomized nutrition intervention trial was performed, in pregnant Bangladeshi women, called the Maternal and Infant Nutrition Interventions, Matlab (the MINIMat-trial, ISRCTN16581394). The study area is located in Matlab, a sub-district approximately 53 km southeast of Dhaka, the capital of Bangladesh. Matlab is a rural area situated in a river delta prone to frequent flooding that is utilized predominantly for agriculture of rice, which together with fishing and day labor are the main sources of income in the area. The general diet consists largely of rice and vegetables with the addition of lentils and beans. Fish is also consumed if afforded while meat is eaten more seldom.

The Matlab area is a field site of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Since 1966, ICDDR, B has been undertaking a Health and Demographic Surveillance System (HDSS) in the area that currently covers a population of about 225,000 people. ICDDR, B provides health services, primarily through local sub-center health clinics, to half of the surveillance area while the other half is provided with governmental health care.

Study design and population

As part of the HDSS, community health workers perform monthly visits to all households to collect demographic and health data. During these visits women were questioned about their last menstrual period and offered a urine pregnancy test if believed to be pregnant. All women identified as pregnant between November 2001 and October 2003 were invited to participate in the MINIMat-trial. Women who gave their consent were enrolled if not more than 14 weeks pregnant as determined by their last menstrual cycle. The pregnancy was later confirmed by ultrasound. A total of 4436 women were enrolled in the MINIMat trial around pregnancy week 8.

The primary outcome of the MINIMat-trial was to study the effects of prenatal nutritional supplementation on infant health with the aim of reducing

LBW and improving infant survival. It is an on-going cohort study in design and women and their children have been examined several times since enrolment; during pregnancy, at birth, and during infancy. All children who were assessed and had their birth weight measured were eligible for an extensive follow-up at 4.5 years of age. The papers included in this thesis are based on data from several data collection time points and use different subsets illustrated in **Table 1**.

To ensure a seasonal representative sub-sample all women who were enrolled throughout January 1 to December 31, 2002, (n=2119) was assigned for biomarker analyses. As the intention was not to perform all biomarker analyses on biological specimen from all women in this cohort a random order for selection of subjects for biomarker analyses was developed. Using statistical software (SPSS) women were given a random number, which then was arranged in order providing a list for selection of samples. The same selection order was used for all biomarkers.

Table 1. Sample size, exposure and study outcomes of the four papers included in this thesis.

Paper	Sample size	Exposure	Study outcomes
I	740 women in pregnancy week 14	Baseline study	Prevalence and determinants of anemia and micronutrient status in pregnancy
II	1351 pregnant women and their offspring at 4.5 years	Food and micronutrient supplementation in pregnancy	Metabolic and inflammatory status, and growth factors in children
III	413 pregnant women and their offspring at birth	Oxidative parameters in pregnancy	Birth anthropometry
IV	337 pregnant women and their offspring at 4.5 years	Oxidative parameters in pregnancy	Metabolic and inflammatory status, growth factors and oxidative stress in children

Randomization to food and micronutrient supplementation

All women enrolled in the MINIMat-trial were randomly allocated to both food and micronutrient supplementation in a 2 by 3 factorial design (**Figure 2**). Randomizations were done in permuted blocks of 12 women.

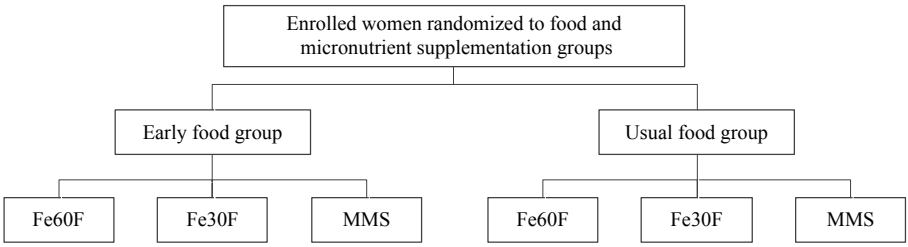


Figure 2. Food and micronutrient supplementation interventions.

Food supplementation

In Bangladesh, at the time of data collection, a government nutrition program provided food supplements to pregnant women with a BMI < 18.5 kg/m^2. In the MINIMat-trial, this food supplement was provided to all women regardless of BMI status and women were randomly assigned to two food supplementation groups.

1. Early food group: Women were encouraged to begin food supplementation as soon as possible after being diagnosed as pregnant, usually around pregnancy week 9.

2. Usual food group: Women were starting food supplementation at the time of their own choosing, as is the usual procedure in the national program, commonly in the second trimester of pregnancy.

The food supplements were locally produced and provided by community nutrition centers 6 days/week throughout pregnancy. They consisted of roasted rice powder (80g), roasted pulse powder (40g), molasses (20g) and soybean oil (12 mL) to be mixed with water. They energy content was 600 kcal and it contained no added micronutrients.

Micronutrient supplementation

Within each food supplementation group women were also assigned one of three types of daily micronutrient supplements. Women received identical looking capsules at the sub-center visit targeted at week 14 in pregnancy and supplementation continued up until 3 months post-partum. Capsule bottles were blinded for both study subjects and community health workers with 4 bottle codes for each micronutrient supplementation type.

The three types of micronutrient supplements were:

1. Fe60F: 60 mg iron and 400 µg folic acid.

2. Fe30F: 30 mg iron and 400 µg folic acid.

3. MMS: multiple micronutrients (contents in **Table 2**).

Table 2. Contents of multiple micronutrient supplementation capsules (MMS) (75).

Micronutrient	**Quantity**
Copper	2 mg
Folic acid	400 µg
Iron	30 mg
Iodine	150 µg
Niacin	18 mg
Selenium	65 µg
Vitamin A	800 µg
Vitamin B1	1.4 mg
Vitamin B2	1.4 mg
Vitamin B6	1.9 mg
Vitamin B12	2.6 µg
Vitamin C	70 mg
Vitamin D	200 IU
Vitamin E	10 mg
Zinc	15 mg

Compliance to supplementations

Food supplementation use was assessed by 4 weeks recall by the women at the households repeated throughout the study period. Each bottle with micronutrients contained 35 capsules and was refilled every month. The adher-

ence to micronutrient supplementation was monitored by an electronic device eDEM® recording each opening of the capsule bottle. The number of bottle openings from week 14 to week 30 has been used as an indicator of adherence to the micronutrient supplements.

Data collection during pregnancy and delivery

Data during pregnancy was collected at scheduled home visits and sub-center clinic visits. **Figure 3** provides an overview of the different data collection points for both women and children and food and micronutrient supplementations during pregnancy. Upon enrolment, maternal weight and height was measured. Extensive demographic and background information was also collected at an introductory home visit including data on morbidity, education, and pregnancy history. A number of household assets noted by the data collector was used to construct asset scores based on the principle component method (76). This asset score was used in statistical analyses as an estimation of socioeconomic status (SES).

Women were also scheduled for 4 follow-up visits to sub-center health clinics at approximately weeks 8, 14, 19, and week 30 in pregnancy. Maternal anthropometric measurements were taken by trained personnel at week 8 and 14. Electronic scales (UNISCALE®, Copenhagen), accurate to ± 100 g, were used for weight measurements. Height was measured with ± 1 mm accuracy with a locally manufactured height board. Body Mass Index (BMI) was calculated by dividing the weight (kg) by the height squared (m^2).

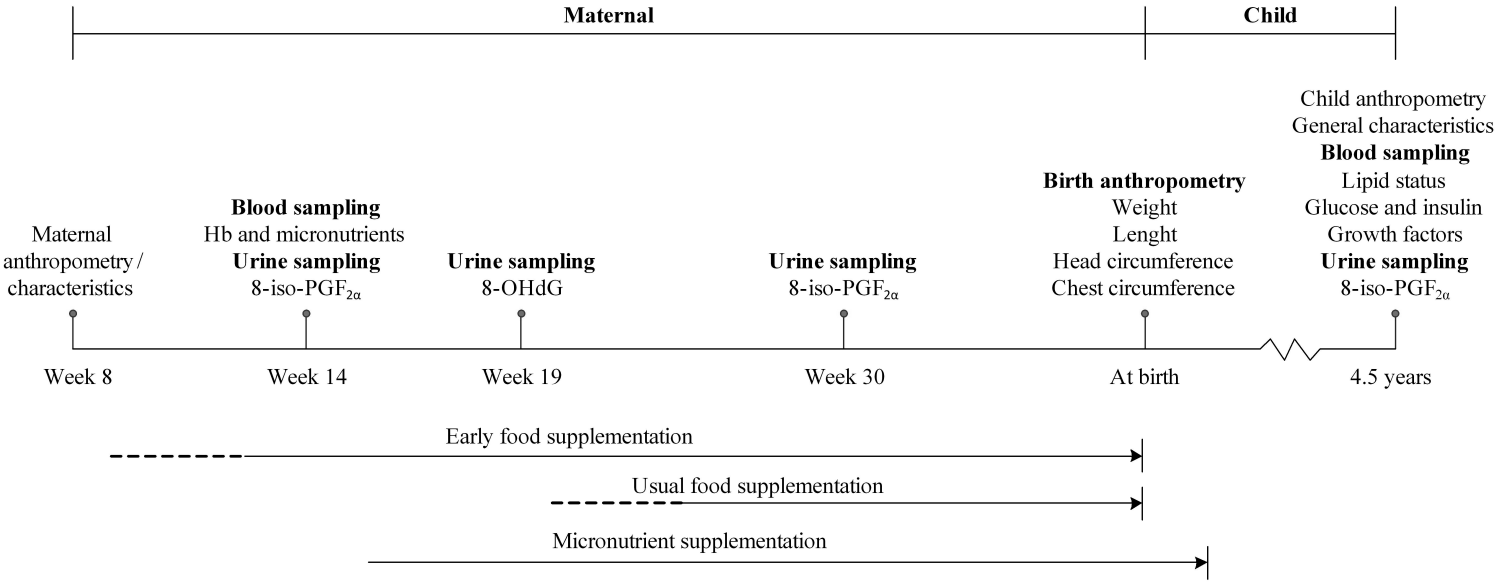
Stool samples were collected at the sub-centers in pregnancy week 8 and analyzed for parasitic infestations at Matlab hospital. Blood samples were collected at the sub-centers in pregnancy week 14. Trained paramedics collected 5 mL of venous blood in Li-heparin treated trace-element-free tubes (Sarstedt Monevette®, Sweden). Hemoglobin (Hb) assessment was done on venous blood by HemoCue photometer (HemoCue® AB, Ängelholm, Sweden). Women who had Hb < 80 mg/L were excluded from the study and referred to Matlab hospital for further health assessment. The plasma was then processed within 4 hours and stored at -70^{o}C in Matlab until shipped on dry ice to the University of California, Davis, USA for biomarker analysis.

To examine potential seasonal variations in micronutrient status we categorized blood collection at pregnancy week 14 into two months periods according to the traditional Bangladeshi calendar (77). These periods begin with the cool and dry season 15 December–14 February (in Bengali: Shhit) followed by the mild temperature season 15 February–14 April (Basanta), the hot and dry season 15 April–14 June (Grisma), the monsoon rains season 15

June–14 August (Barsa), the hot and humid season 15 August–14 October (Sharat) and the main harvest season 15 October–14 December (Hemanta).

Urine samples were collected at sub-centers at pregnancy weeks 14, 19 and 30. The urine was collected in cups, transferred to plastic vials and transported cold to the laboratory at Matlab hospital and stored at -70°C. Urine samples from week 14 and week 30 were shipped on dry ice to Uppsala, Sweden, and samples from week 19 were transported to Dhaka, Bangladesh, for further analyses.

Figure 3. Data collection points for participating women and children.



A birth notification system enabled a home visit to a woman who had been given birth within 72 hours and anthropometric measurements were taken. The infant's birth weight was measured by SECA electronic scales (SECA Gmbh & Co, Hamburg, Germany) with an accuracy of ± 10 grams. Infant's birth length was measured by locally manufactured length boards with an accuracy of ± 1 mm. Head and chest circumference of the infant was measured to the nearest 1 mm with a non-elastic measuring tape. We used the established cutoff of birth weight < 2,500 g to define LBW. For the other anthropometric measurements at birth where no such cutoffs exist we instead used the lowest tertile to define small birth size; low birth length, small birth head, and small birth chest.

Data collection during the 4.5 year follow-up of children

Children were seen at approximately 4.5 years of age during 1 home visit and 1 clinic visit. Data on socioeconomic status and schooling were collected at the home visit. Anthropometric measurements were taken at the sub-center clinic visit by trained nurses. Weight was measured with digital scales (TANITA HD -318, Tanita Corporation, Japan), accurate to ± 10 g. Height was measured with a Leicester Height Measure stadiometer (SECA 214, UK) with an accuracy of ± 1 mm. Undernutrition was classified as weight-for-age < 2 SD and stunting as height-for-age < 2 SD according to the WHO Multicentre Growth Reference Study child growth standards (78).

Fasting venous blood, 5 mL, was collected by trained paramedics using Li-heparin treated trace-element-free tubes (Sarstedt Monevette®, Sweden). Hb assessment was done on venous blood at the sub-centers by HemoCue® photometer (HemoCue AB, Ängelholm, Sweden). Glucose was also measured on venous blood by HemoCue Glucose 201 RT (HemoCue AB, Ängelholm, Sweden). Blood samples were transported on ice to Matlab laboratory, processed within 4 hours and stored at -70^{o}C freezers in Matlab until shipped on dry ice to Uppsala University, Sweden, for biomarker analysis.

Laboratory analyses

All samples from women were analyzed for micronutrients from plasma samples at UC David, California, USA. Ferritin was assessed with radioimmunoassay (Diagnostic Products, San Diego, CA, USA). CRP was measured semi-quantitatively by radial immunodiffusion (The Binding Site, San Diego, CA, USA). Folate and vitamin B12 were determined in the same radio-

immunoassay (SimulTRAC-SNB, MP Biomedicals; Solon, OH, USA). Zinc was assessed in plasma by atomic absorption spectrometry.

8-OHdG in urinary samples of women was assayed at ICDDR, B by ELISA (Japan Institute for the Control of Aging, Shizuoka, Japan) with an 8-OHdG monoclonal antibody (clone N45.1).

8-iso-$PGF_{2\alpha}$ in urinary samples from both women and children's were analyzed in Uppsala, Sweden by a validated radioimmunoassay developed by Basu (79).

Metabolic markers in plasma samples from children were analysed at Akademiska sjukhuset laboratory, Uppsala, Sweden. ApoA1, apoB, cholesterol, HDL, LDL, triglycerides, and CRP was measured by immunoturbidimetry on the Architect ci8200® analyzer (Abbott Diagnostics). Insulin was analysed by immunological sandwich technique using a Modular® Analytics E 170 Module (Roche Diagnostics). Insulin resistance was estimated by the homeostasis model assessment HOMA-IR = (FPI × FPG)/22.5, where FPI is fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/L) (80). Growth factors were analysed at the Karolinska Institute, Stockholm, Sweden. Total IGF-1-I in plasma was measured using radioimmunoassay after acid–ethanol extraction. IGF-1/IGFBP-1-1 was analyzed using radioimmunoassay as described by Póvoa et al. (81).

CRP concentrations were below the detection limit of 0.20 mg/L in 24% of samples from children. These samples were assigned half the detection limit value, 0.10 mg/L, to use in statistical analyses. Insulin concentrations were similarly below the detection limit of 0.20 mU/L in 7% of the samples and were thus assigned half the detection limit value, 0.10 mU/L.

Statistical analyses

All papers evaluated potential confounders and sex-stratified analyses were performed in paper II and III. In Paper I analyses of variance (ANOVA) and chi-square tests was used to analyze seasonal variations in micronutrient status and prevalence of deficiency. Multivariate logistic regression was used to examine potential determinants of anemia and micronutrient deficiencies. In Paper II, the associations between food and micronutrient supplementation groups and biomarkers in the children were analyzed with the general linear model. In Paper III and IV oxidative stress markers were divided into tertiles with the first tertile used as the reference category. Comparisons of differences in means of the children's birth anthropometry (paper III) or metabolic markers (paper IV) between oxidative stress tertiles were

performed using the general linear model with test for linear or quadratic trends. Multiple logistic regression was used to evaluate risk indicators of small size at birth or metabolic conditions associated with oxidative stress tertiles.

All statistical analyses were done in PASW/SPSS; version 17.0 (paper I and II) and version 18.0 (paper III and IV), SPSS Inc. Chicago, IL, USA. Statistical differences were considered significant at $p < 0.05$.

Ethical considerations

Ethical approval for the first phase of the trial was received from the Ethical Review Committee at ICDDR,B. Informed consent was obtained according to the Helsinki declaration in a two-step process. A verbal consent was obtained from all women before performing a pregnancy test at their house. At the first sub-center health clinic visit a written informed consent was then obtained for participation in the trial including consent to receive micronutrient supplementation. Ethical approval for the follow-up of the children at 4.5 years was received from the Ethical Review Committee at ICDDR,B. In addition, an ethics application was submitted to the Regional Ethical Review Board in Uppsala. As the Review Board is limited to research conducted in Sweden, only the part of the research protocol conducted in Sweden (biomarker analyses) was reviewed. A written informed consent was obtained from the child's caregiver for study participation including collection of blood and urine samples.

Previous results from the MINIMat trial

Previous results for the MINIMat trial of key importance for this thesis are the effects of maternal food and micronutrient supplementation on birth weight, infant mortality and child growth. There was no difference in mean birth weights between any of the food or micronutrient supplementation groups (82). However, Early invitation to prenatal food supplementation in combination with multiple micronutrients resulted in a lower infant mortality rate (16.8/1000 live births) compared to the standard care of Usual invitation to food supplementation combined with Fe60F (44.1/1000 live births). In addition, Early food together with MMS had a lower under-five mortality rate (18/1000 live births) than Usual food with Fe60F (54/1000) (82). There was no interaction between prenatal food and micronutrient groups on growth. The Early food supplementation group reduced stunting from infancy up to 4.5 years in boys, but not girls (83). Allocation of prenatal MMS resulted in a higher prevalence of stunting, also observed in boys only (83).

Results

General characteristics of women and children

All four papers included in this thesis use demographic data from the same cohort of women and children. The sample size in each paper varies as not all biomarkers were assessed for all individuals, however, all women were randomly selected for use in different subsets. General demographic characteristics were similar in each study, and the largest subset including 1351 women-children pairs, used in paper II, are presented here and representative of all subjects enrolled.

The median age of the enrolled women was 26 years (interquartile range (IQR) 22-31 years). The majority of women had no previous children (32%), one previous child (27%) or two previous children (21%). Women were generally short and lean and almost one third were underweight with a BMI < 18.5 kg/m^2. The median years of schooling was 5 (IQR 0-9) with 34% of the women not having any formal schooling. Less than 10% of the women report of doing other work outside the household.

At birth, 52% of the infants were male and 48% female. The mean birth weight was 2674 g ± 412 g. A large proportion of infant's, about one third, were born with a LBW. The mean birth length was 47.7 cm ± 2.3 cm.

Children were targeted at 4.5 years (54 months) and the median age of the children was 54 months (IQR 53-55 months). The children were short and light, mean height-for-age z-score (HAZ) was -1.49 ± 0.87 and 31% of the children were stunted (83). The observed mean height of boys was 100.2 cm ± 4.3 cm and mean weight 13.9 kg ± 1.6 kg. The observed mean height for girls was 98.9 cm ± 4.3 cm and the observed mean weight 13.4 kg ± 1.6 kg.

Anemia and micronutrient status in pregnancy week 14 (Paper I)

Anemia and micronutrient deficiencies were common in assessed women around week 14 of pregnancy. The most common deficiencies were zinc (55% of women) and vitamin B-12 (46%). Folate deficiency was less prevalent seen in 18% of women. Even though 28% of the studied women were anemic, iron deficiency was uncommon at this stage in pregnancy with only 8% observed to be iron deficient. Consequently, iron deficiency anemia was observed in only 2% of the population studied (excluding women with elevated CRP).

Determinants of anemia and micronutrient deficiencies

As described in the methods, subjects were stratified according to the traditional Bangladeshi calendar to account for seasonal variations. We observed significant seasonal variations in the mean values of all studied micronutrients and differences in the prevalence of anemia and deficiency of zinc, folate and vitamin B-12. Vitamin B-12 deficiency varied the most significantly with two thirds (66%) observed to be deficient in the cool and dry season (Shhit) and one third (33%) observed to be deficient in the mild temperature season (Basanta). Furthermore, although overall deficiency of folate was less than 20%, in the main harvest season as many as 32% of mothers were observed to be deficient.

To evaluate the potential independent effects of more immediate contributors to anemia a multivariate model was used. When including covariates associated with anemia $p < 0.2$ in bivariate analyses (folate deficiency, food supplementation, *Ascaris* infestation, SES, vitamin B-12 deficiency and BMI <18.5 kg/m^2) in a logistic regression model, only vitamin B-12 deficiency and BMI were found to be predictors of anemia. Women with vitamin B-12 deficiency were more likely to be anemic than women with normal vitamin B-12 status (OR 1.50, 95% CI 1.05, 2.14). Underweight women were also more likely to be anemic than women with a BMI ≥ 18.5 kg/m^2. When adding season to the model, vitamin B12 was no longer significantly associated with anemia ($p=0.07$) while the association with BMI remained (OR 1.68, 95% CI 1.14, 2.47).

A similar approach was performed for establishing determinants of micronutrient deficiencies. Potential contributing factors examined were BMI <18.5 kg/m^2, food supplementation, elevated CRP and parasitic infestation with *Ascaris*, *Trichuris*, *Giardia* and hookworm. Only folate and vitamin B-12 deficiency was found to be associated with any of these factors. Women with *Ascaris* infestation were more likely to have folate deficiency than

women not infested (OR 1.96, 95% CI 1.22-3.16) in a multivariate model adjusted for background factors associated with folate $p < 0.2$ (SES, gestational age and season). Similarly, women with *Ascaris* infestation were more likely to have vitamin B-12 deficiency than non-infested women (OR 1.49, 95% CI 1.06–2.09) in a season-adjusted model.

A summary of the results from paper II-IV are also shown in **Table 3**.

Effects of prenatal food and micronutrient supplementation on the metabolic status of the offspring at 4.5 years of age (paper II)

Metabolic, inflammatory and growth factor status of the children at 4.5 years

At 4.5 years of age, a substantial proportion of the children had lipids outside the normal range using clinical cutoffs as crude risk indicators of metabolic disease. Approximately 20% of the children had high triglycerides (≥ 1.7 mmol/L) and high cholesterol (≥ 4.4 mmol/L). In addition, 95% of the children had high apoA1 (< 1.2 g/L), which was reflected in one third of the children with a high apoB/apoA1 ratio. Low LDL was observed in approximately 80% but only 13% had a high LDL/HDL-ratio. Less than 10% of children had fasting glucose values ≥ 5.6 mmol/L. Almost one fifth had CRP levels above 2 mg/L. Insulin concentrations were generally low (median 1.45 mU/L IQR 0.76-2.50) with correspondingly low HOMA-IR values (median 0.30 IQR 0.16-0.54). Similar to insulin, IGF-1 concentrations were also low with a median of 81 (IQR 77-84).

Effects of prenatal food supplementation on lipid metabolism, glucose metabolism and growth factors

The introduction of Early food supplementation in pregnancy was observed to have beneficial effects on the children's lipid status. Children born to women in the early food group had lower concentrations of apoB, cholesterol and LDL compared to children with mothers in the Usual food group. The differences were numerically small (ranging from 0.11 to 0.13 SD), however, statistical differences were observed and consistent towards a more favorable lipid profile. The positive results persisted in analyses adjusted for maternal BMI and height, SES, child sex and age (apoB and LDL, $p < 0.05$; cholesterol, $p=0.053$). As there was interaction between food supplementation group and child sex for several of the lipid biomarkers we performed

sex stratified analyses. Interestingly, our overall effects of Early food supplementation on child lipids were found exclusively in boys.

While we found no overall effects of food supplementation on the children's glucose metabolism, we did find a tendency of lower fasting glucose in boys born to mothers receiving Early food supplements (p=0.056) compared to the Usual start. We did not find any effects of food supplementation on the children's growth factors.

Effects of prenatal micronutrient supplementation on lipid metabolism, glucose metabolism and growth factors

Children whose mothers had received MMS in pregnancy had lower HDL compared to children born to women in the Fe60F group ($p < 0.05$). We found no other differential effects of the micronutrient supplements on the lipid status of the children.

Prenatal MMS had a differential effect on the children's glucose metabolism in early childhood. Maternal use of MMS resulted in children with lower fasting glucose concentrations (mean difference MMS compared to Fe60F; -0.097 (95% CI -0.177, -0.018). In a sex-stratified analysis this effect on glucose concentrations was observed only in the boys. Furthermore, MMS had a lowering effect on insulin and HOMA as compared to Fe60F, but only when combined with Early food supplements. Few differences were found between Fe60F and Fe30F but interestingly, we found an interaction between type of micronutrient supplementation and child sex and higher insulin concentrations in Fe30F compared to Fe60F, but only in boys.

Micronutrient supplementation was also observed to be associated with the children's growth factors. Children in the MMS group had significantly lower IGF-1, higher IGFBP-1, and consequently lower IGF-1/IGFBP-1 ratio than the Fe60G group. Crude geometric mean for MMS 73 µg/L (95% CI 67, 79) while it was 84 µg/L (78-91) for the Fe60F group. For the IGF-1/IGFBP-1 ratio, the lowering effect of MMS was most pronounced in children whose mothers had received MMS combined with Usual food supplements. In a sex-stratified analysis, the effect on IGFBP-1 and IGF-1/IGFBP-1 appeared only in boys.

Associations between oxidative parameters in pregnancy and offspring outcomes (Paper III and IV)

As a potential mechanism in the development of adult metabolic disease, we have investigated associations between oxidative parameters in pregnancy and outcomes in the offspring, both in the short-term (as assessed by birth anthropometry (paper III)) and the longer-term (assessed by metabolic status in early childhood (paper IV)). Lipid peroxidation was assessed by 8-iso-$PGF_{2\alpha}$ and damage to DNA by oxidation by 8-OHdG. At week 14 of pregnancy, the geometric mean of 8-iso-$PGF_{2\alpha}$ was observed to be 0.99 (95% CI 0.90, 1.09) nmol/mmol creatinine. Approximately 16 weeks later, at week 30, concentrations of 8-iso-$PGF_{2\alpha}$ were observed to be similar with the geometric mean being 1.01 (95% CI 0.89, 1.09). 8-OHdG measured at week 19 had a geometric mean of 5.30 (95% CI 4.86, 5.77) ng/mL.

Associations between early maternal lipid peroxidation (week 14) and offspring outcomes

Lipid peroxidation as assessed by 8-iso-$PGF_{2\alpha}$ at week 14 of pregnancy was associated with a larger infant size at birth as determined by measures of length and chest. Children whose mothers were in the middle tertile of lipid peroxidation had lower odds of being born with a shortest birth length (OR 0.55, 95% CI 0.32, 0.95) compared to the lowest tertile which was used as the reference. In addition, we found increasing chest size with increasing lipid peroxidation. In a sex-stratified analyses, this linear trend persisted in boys, but not in girls. In further analyses of boys only, we also observed the lowest prevalence of smallest birth chest in the highest lipid peroxidation tertile (18% compared to 38% in the lowest tertile ($p < 0.05$)). In girls, the relationship with lipid peroxidation and birth chest circumference appeared to follow a U-shaped distribution. Girls in the middle tertile had the lowest observed odds of smallest birth chest circumference (OR 0.40, 95% CI 0.18, 0.90). In further analyses of girls we also found the lowest prevalence of shortest birth length in the middle tertile (22%) as compared to the lowest tertile (47%; $p < 0.05$).

U-shaped trends were also observed between lipid peroxidation in early pregnancy and insulin and HOMA-IR values, an observation that was also reflected in lower geometric means in the middle tertile of lipid peroxidation compared to lowest tertile and the highest. Lipid peroxidation in early pregnancy was not associated with the children's lipid status or growth factors at 4.5 years.

Associations between late maternal lipid peroxidation (week 30) and offspring outcomes

Fewer associations between lipid peroxidation in late pregnancy (week 30) and birth anthropometry were found. While we found no overall associations, women whose lipid peroxidation was in the middle tertile were observed to have the lowest odds of boys with smallest chest size (OR 0.37, 95% CI 0.14, 0.95). In girls, we found a linear trend with higher lipid peroxidation being associated with smaller head circumference.

U-shaped associations with late maternal lipid peroxidation and several of the assessed lipids; apoB, apoB/apoA1, cholesterol, LDL, and TG were also observed. With the exception of TG, these associations were also apparent in adjusted logistic regression models where women in the middle tertile had the lowest odds of children with these lipids above cutoffs. In addition, we found that increasing late pregnancy lipid peroxidation was associated with decreasing IGF-1 concentrations in the children (linear trend $p < 0.05$).

Associations between DNA oxidation in mid-pregnancy (week 19) and offspring outcomes

No overall associations between DNA oxidation and birth anthropometry was observed. In an analyses stratified by sex, a negative linear trend was seen where higher maternal DNA oxidation was associated with shorter birth length ($p < 0.05$) in girls. Increasing DNA oxidation was associated with decreasing IGF-1 concentrations in the offspring ($p < 0.05$). DNA oxidation was not demonstrated to be associated with any of the other biomarkers (lipids, glucose, insulin, or HOMA-IR).

Table 3. Summary of results: maternal nutrition and oxidative parameters in pregnancy and offspring size at birth and metabolic biomarkers at 4.5 years.

Pregnancy	At birth	Children at 4.5 years
Supplementation		
Early food (vs. Usual)		*All children* ↓ ApoB, LDL *Boys* ↓ ApoB, cholesterol, LDL
MMN (vs. Fe60F)		*All children* ↓ HDL ↓ Glucose ↓ Insulin, HOMA-IR (with Early food) ↓ IGF-1 ↑ IGFBP-1 ↓ IGF-1/IGFBP-1 (all and with Usual food) *Boys* ↓ Glucose ↑ IGFBP-1 ↓ IGF-1/IGFBP-1
Fe30F (vs. Fe60F)		*Boys* ↑ Insulin
Lipid peroxidation		
Week 14 ↑ lipid peroxidation	*All infants* ↑ Length (U-shaped) ↑ Chest *Boys* ↑ Weight, chest *Girls* ↑ Length, chest (U-shaped)	*All children* ↓ Insulin, HOMA-IR (U-shaped)
Week 30 ↑ lipid peroxidation	*Boys* ↑ Chest (U-shaped) *Girls* ↓ Head	*All children* ↓ ApoB, ApoB/ApoA1, cholesterol, TG (U-shaped) ↓ LDL ↓ IGF-1
DNA oxidation Week 19 ↑ DNA oxidation	*Girls* ↓ Length	*All children* ↓ IGF-1

[1]Including results with $p < 0.05$ in adjusted analyses.

Discussion

In general, the women analyzed in our cohort began their pregnancies with a number of micronutrient deficiencies of which zinc and vitamin B-12 were the most prevalent. We also found large seasonal variations in anemia and micronutrient deficiencies. Anemia was observed to be present in around one third of the women but somewhat surprisingly iron deficiency anemia was uncommon, being seen in only 2% of all women (Paper I). An Early initiation of food supplementation in pregnancy resulted in improved lipid status in the children at 4.5 years. Maternal allocation to MMS was observed to lower the offspring's glucose, insulin and HOMA-IR values, especially if combined with Early food supplements. MMS also resulted in lower growth factors, particularly if combined with the Usual invitation to food supplementation (paper II). We found that early maternal lipid peroxidation was associated with both measures of size at birth and lower insulin and HOMA-IR levels in the offspring in a U-shaped manner. Increasing lipid peroxidation in early pregnancy was associated with increasing chest size at birth. Lipid peroxidation in late pregnancy, on the other hand, was associated with children's lipid status in a U-shaped manner. However, increasing lipid peroxidation was also associated with decreasing IGF-1 levels. Further, increasing DNA oxidation in mid-pregnancy was associated with decreasing IGF-1 (paper III and IV). We found sex specific responses in effects of nutritional supplements as well as in associations between maternal lipid peroxidation and size at birth.

Methodological considerations

Validity

The studies included in this thesis are based on data from a community-based randomized intervention trial aiming to be representative of the study population. In paper II, there were small differences (in SES, parity and education status) between women with incomplete and incomplete data, indicating only minor deviation from representativeness. No baseline differences were observed between any of these factors between supplementation groups.

To ensure good quality data and internal validity, all data collection followed standard operating procedures and appropriate refresher training was conducted throughout the study periods. Instruments for anthropometric measurements and blood collection were calibrated daily.

Sample size

While the sample size for the MINIMat trial was based on birth weight as the primary outcome, we regard the sample size of our used subsets to be sufficient for most analyses as interpreted by numerous statistically significant results. However, sample size was limited in paper IV as we could not perform sex-stratified analyses as we would have preferred based on previous findings.

Confounding and causality

Paper II is a randomized trial and it is important to confirm that there are no baseline differences between supplementation groups that could affect the interpretation of the outcome. We have adjusted the intent-to-treat analyses for some important factors to ensure the robustness of the results. Paper I, III and IV were observational studies where the need to control for potential confounding factors is essential. The same strategy to control for potential confounders was used in all the 3 papers. Potential confounders were identified based on previous research and biological knowledge. The identified potential confounders were then tested if associated with both exposure and outcome. If found to be associated, the factor was included in the statistical model where it was retained if found to change the effect estimates substantially (> 10%). Some potential confounders, such as SES, were considered key factors and were kept in the analyses regardless. Although our observed associations between maternal oxidative stress and offspring outcomes are intergenerational and we have adjusted for known confounders causality is not possible to establish. There may be residual confounding of factors which we have not been able to control for, for example within the shared environment of both mother and child. In addition, it is difficult to know if oxidative stress markers are actively involved in or simply a reflection of biological processes. Causality between maternal oxidative stress and offspring outcomes can therefore not be established.

Exposure and outcome assessment

We used well-established and recognized biomarkers for assessment of micronutrients and metabolic status. The oxidative stress marker 8-iso-$PGF_{2\alpha}$ is a major F2-isoprostane synthesized through non-enzymatic peroxidation of arachidonic acid and a reliable marker of *in vivo* lipid peroxidation (79),

considered the current gold-standard. The assessment of 8-OHdG by ELISA has been overestimating concentrations compared to high-performance liquid chromatography as the gold-standard, however, a high correlation between the methods has been observed (84). While absolute values are needed for comparison with other populations our primary need was comparison within the study sample.

As CRP was analyzed semi-quantitatively in women with a cutoff of 10 mg/L there is the possibility that we may have underestimated the role of infections and sub-clinical inflammation. In the children a high-sensitivity assessment of CRP was therefore used.

For most of the metabolic markers we assessed in children there are no established age-specific cutoffs indicating an increased risk of metabolic disease. Where existing cutoffs for older children or adults have been used the interpretation may not be expanded to become risk indicators of disease, but rather be regarded just as lower or higher part of the distribution.

Anemia and micronutrient deficiencies in early pregnancy

Micronutrient deficiencies were common in our population in early pregnancy. The most commonly observed deficiencies were of zinc and vitamin B-12, with approximately half of all women being deficient. It is established that a deficiency of vitamin B-12 at the beginning of pregnancy is of particular concern, as it confers short-term health risks to the mother as well as potentially increasing the risk of a pre-term birth and birth defects (12). Maternal vitamin B-12 status is closely related to the infant status (85) and deficiency in infancy may cause neurological damage (86). Data on the the importance of maternal zinc deficiency are inconclusive. A deficiency in zinc has been suggested to adversely affect pregnancy outcomes, however, a recent Cochrane review of zinc supplementation throughout pregnancy found no positive effect on LBW although a small reduction in preterm births was observed (87). Further, long-term health consequences of zinc and vitamin B-12 deficiencies are discussed in the development of metabolic disease in adult life (33). Major potential mechanisms of action suggested include changes in hormonal secretions dependent on zinc (33). Further, both zinc and vitamin B-12 may also be involved in epigenetic modifications; where vitamin B-12 acts as a methyl donor in DNA methylation and zinc as a structural component in essential enzymes (32, 33). Interestingly, a combination of maternal vitamin B-12 deficiency and high erythrocyte folate has been associated with increased insulin resistance in preschool Indian children

(31). Similarly, in Nepal, women with vitamin B-12 deficiency in early pregnancy gave birth to children with increased HOMA-IR compared to women with adequate vitamin B-12 status (88), however, this association was observed regardless of the women's folate status. As these findings are recent, the underlying mechanisms can only be speculated on. Regardless, as almost half of all women in our cohort were vitamin B-12 deficient and about one-third vitamin B-12 deficient in combination with normal folate in early pregnancy (data not shown), these findings, when taken together with other published data would suggest that the children in our study are a potentially vulnerable group with regards to later cardiovascular disease. Further investigation in our cohort is needed about associations between these potential risk indicators of cardiovascular disease in pregnancy and later offspring health, and if they may be modifiable in early childhood.

Our study revealed that all the micronutrients (iron, zinc, folate, vitamin B-12) analyzed were sensitive to seasonal variations in a significant manner. This finding highlights the importance of considering seasonal variations when designing appropriate interventions to alleviate deficiencies and also when interpreting data relating to prevalence of micronutrient deficiencies to avoid over and under-interpretation of results as studies that are not covering a full year may not be representative. Several factors may contribute to these observed seasonal variations, including dietary intake due to seasonal availability of different foods, the variable cost of food items throughout the year and seasonal disease patterns that may influence nutritional status.

The prevalence of anemia in our population in early pregnancy was almost 30%, which WHO constitutes a moderate public health problem (89). This prevalence is lower than previously published national surveys of rural areas in Bangladesh, where anemia prevalences were reported to be as high as 47% (90). Possible reasons for the disparity may be the larger variations in assessment time in pregnancy in the national survey, and that we are covering one specific area where the anemia prevalence may be lower. Our results are in agreement with other reported anemia prevalence from non-pregnant women of reproductive years, that reported a rate of 33% (91). Interestingly, we also observed variations in anemia prevalence as a result of seasonal variation (20%-36%), which is not addressed in most studies. Iron deficiency, however, was uncommon in early pregnancy and as a result only a low proportion of women were observed to have iron deficiency anemia. Instead, anemia was associated with vitamin B-12 deficiency and women being underweight. In this population many are adhering to a vegetarian diet due to economical reasons and animal sources rich in vitamin B-12 are limited. In addition, parasitic infestations were also found to be associated with vitamin B-12 deficiency. Nevertheless, as iron requirements increase throughout

pregnancy, it is possible that iron deficiency anemia will develop in later pregnancy or after childbirth.

Metabolic status in the children at 4.5 years

While the children observed in our cohort had fasting glucose levels within the normal range the fasting insulin levels were unusually low. This was reflected also in lower HOMA-IR than those observed in similar settings in Nepal (40% lower) (92) and India (60% lower) (93). As a measurement of insulin resistance, a low HOMA-IR is generally considered beneficial. However, in animal models maternal protein restriction resulted in offspring with impaired insulin secretion (94). In our population, where one third of women were underweight, indicative of poor nutritional status and low protein intake, a similar mechanism might be present thus explaining our observed low insulin levels as a consequence of undernutrition. To gain further understanding of the significance of low insulin, we did preliminary analyses whether it was associated with child height. Height has been put forward as an indicator of positive child development and is not affected by obesity like weight, where both low and high values may be unfavorable (95). Our preliminary analyses show that both increasing insulin and HOMA-IR values were significantly correlated with increasing height (data not shown), suggesting that lower insulin levels are indicating shorter body stature. Thus we question whether the lower insulin levels in these children is favorable.

The children had several lipids that deviated in comparison with well-nourished children in the same age group, in particular apoA1 and HDL. Our observed apoA1 and HDL concentrations were approximately 20% lower than in Finnish children (96). However, HDL concentrations were similar to preschool children from similar settings in rural India (93) and Nepal (97). In our cohort, approximately 80% of children had low HDL while as many as 95% had low apoA1 levels suggesting unfavorably low levels in the children.

The observed IGF-1 concentrations in our cohort of children were approximately one standard deviation below that of healthy Danish children of the same age (98). Although IGF-1 concentrations have been observed to vary between study populations (99), a large proportion of the children from our cohort were stunted at this age (83) we thus interpret that lower IGF-1 levels in this population is a reflection of growth restriction.

Prenatal food and micronutrient supplementation

Effects of prenatal food and micronutrient supplementation on glucose metabolism

While the randomized food or micronutrient supplementation groups had no differential overall effects on the children's glucose and insulin metabolism, the combination of Early food and MMS resulted in lower insulin and HOMA-IR. In India prenatal food supplements has been associated with decreased insulin resistance in 15-year olds (100) and children in the Gambia born to mother receiving protein-energy supplements during pregnancy had slightly lower glucose than controls (101). However, in Nepal prenatal supplementation with MMS had no effect of either insulin or glucose in preschool children (92). Observational studies suggest, as previously discussed, that a high prevalence of maternal vitamin B-12 deficiency, alone (88) or in combination with high folate (31), are associated with increased insulin resistance in preschool offspring and as the MMS provided the deficient micronutrients the lowering effect of MMS may be beneficial. However, as previously discussed we are uncertain whether further lowering of already low insulin and HOMA values is a sign of a beneficial effect or not. Possibly, the low values may instead indicate an unfavorable consequence of undernutrition.

Effects of prenatal food and micronutrient supplementation on lipid metabolism

We found that prenatal Early food supplementation had favorable effects on the children's lipid profile. Additionally, there was a sex difference in the effects of prenatal food as the effects were only seen in boys. Few previous studies evaluating longer-term effects of prenatal supplementation have been done. While a prenatal protein-energy drink in Guatemala resulted in adult offspring with higher HDL and lower triglycerides compared to a control group receiving a low energy-protein drink (102), protein-energy supplements during pregnancy had no effects on the lipid profile of 11-17 year olds in the Gambia (101) or in adolescents in India (100). The MINIMat trial is unique in that all women received food supplements and the differential effects we observed are due to timing. It highlights the importance of timing the introduction of these supplements appropriately, as an Early introduction of these supplements had lasting effects on the children's lipid status that was not manifested in a change in birth weight (82).

We hypothesized that the allocation of MMS would have beneficial effects on the offspring, especially as it contained both zinc and vitamin B-12, deficiencies affecting a large proportion of women in our cohort. However, our only finding was of lower HDL in children born to mother in the MMS

group, suggesting an unfavorable effect of MMS. Trials investigating the effects of prenatal multiple micronutrients on lipid status in the offspring are scarce, but no effects on HDL or the other assessed lipids (cholesterol, LDL or triglycerides) of prenatal MMS was found on preschool children in Nepal compared to a control group receiving vitamin A only. However, they found a positive effect of supplements containing a combination of iron, zinc and folic acid in reduced triglycerides in children (92).

Effects of prenatal food and micronutrient supplementation on growth factors

Of potential concern are our observations that prenatal MMS was associated with growth factors that indicate a slower growth potential. Our findings are in agreement with previous results from the MINIMat trial where allocation to MMS increased stunting in the offspring from birth up to 4.5 years in boys (83). The negative effects on growth factors in our study were predominantly seen in combination with Usual food group and so, it appears that initiation of Early food supplementation in pregnancy has a protective effect against the potentially unfavorable effects of prenatal MMS. As the effects on growth factors was seen exclusively in boys this would suggest sex differences in the programming of growth.

Oxidative stress during pregnancy

We found associations between our assessed markers of maternal oxidative stress on both short-term and longer-term health outcomes for the offspring born in our cohort. The nature of these associations, however, differed dependent on the type of oxidative stress marker as well as their timing

Early lipid peroxidation and offspring outcomes

Since oxidative stress commonly is associated with pathological conditions, it was unexpected that higher lipid peroxidation in early pregnancy was associated with a larger infant size at birth. Increasing lipid peroxidation was associated with increasing chest size and a U-shaped relationship was seen for birth length, where infants in the middle range of lipid peroxidation appeared to be protected of shortest birth length. In this population, where LBW is common, we consider a larger infant size to be a favorable outcome. Concerns regarding size and infant body composition have been raised previously by Yajnik et al., who described a group of Indian babies who were short and thin at birth but were observed to have a relatively large proportion of adipose tissue; a group described as the “thin-fat” baby (103). In our co-

hort, however, we observed an increase in chest size as well as an increase in length, an observation that is generally regarded to be positive, so we believe it less likely that our observation is due to an increase in fat tissue only. Previous research on lipid peroxidation in pregnancy and birth outcomes have mostly focused on more immediate adverse pregnancy outcomes such as pre-eclampsia (67, 104). The majority of studies investigating associations between maternal lipid peroxidation and indicators of fetal growth have been conducted later in pregnancy, where an increased lipid peroxidation might be a reflection of different physiological influences. However, Stein et al. do examine lipid peroxidation in early pregnancy (week 12) but in contrast to our results do not find any associations between 8-iso-$PGF_{2\alpha}$ and birth weight (105). However, this study included women with a considerably higher pre-pregnancy BMI and whose infants were heavier at birth than in our population making comparisons difficult. Towards a potential explanation to our findings may be that an increased lipid peroxidation could be a reflection of a normal pregnancy development. There is evidence of a mild increase in oxidative stress during the course of a normal pregnancy (106, 107) with the concept of "oxidative strain" being put forward by Basu. In this concept, they describe that a mild increase in oxidative products may stimulate normal physiological responses as opposed to inducing a pathological condition described by "oxidative stress" (108). Pregnancy is a period of extensive tissue growth where the role of oxidative stress in cell signaling, defense against microorganisms and alterations in vascular tone (109) might be especially important.

Early lipid peroxidation in the middle range was associated with the lowest insulin and HOMA-IR values in the offspring at 4.5 years. As previously discussed, we regard lower insulin levels as potentially unfavorable in these children. In well-nourished adults, higher lipid peroxidation has been associated with higher insulin resistance (110). A proposed mechanism is a disrupted insulin signaling as the result of free radical attack that could lead to insulin resistance (110, 111). However, our study addresses an intergenerational association in a malnourished population making comparisons difficult. In our study, it appears as if early lipid peroxidation in the middle range had both favorable (larger birth size) and potentially unfavorable (lower insulin) associations with offspring outcomes, and further investigation is needed to interpret this.

Late lipid peroxidation and offspring outcomes

Our findings from lipid peroxidation in late pregnancy and associations with birth size are few and somewhat contradictory. Infants in the middle range of lipid peroxidation was protected of smallest chest size, while increasing lipid peroxidation was associated with decreasing head size in girls. Previous

research has found associations between higher maternal lipid peroxidation and smaller birth size (67, 112, 113) or no association (114). The proposed mechanism for associations between increased lipid peroxidation and smaller size at birth have been that oxidative products may alter the blood flow to the fetus resulting in a reduction in fetal oxygen delivery and tissue damage (105, 113). In addition, isoprostanes themselves are vasoconstrictors (115) and an increase in 8-iso-$PGF_{2\alpha}$ may cause a restriction in blood flow negatively influencing fetal oxygen and nutrient supply. Differences in maternal health and nutrition as well as lipid peroxidation indicators used may contribute to the differences in results between studies.

In our cohort, lipid peroxidation in late pregnancy was associated with a potentially favorable, less atherogenic lipid profile in children in a U-shaped manner. Higher lipid peroxidation has been seen observed together with cardiovascular risk factors in cross-sectional studies and is suggested to be involved in atherosclerosis (108), however, there is a paucity of human data that analyses maternal lipid peroxidation and longer-term associations in the offspring. The underlying mechanism to our findings of somewhat higher lipid peroxidation being associated with a more favorable lipid profile, as opposed to a less favorable one, is not clear. Further, increasing lipid peroxidation was associated with unfavorable decreasing IGF-1 concentrations.

DNA and offspring outcomes

Increasing DNA oxidation in mid-pregnancy was associated with decreasing birth length, however, this observation was seen in girls only. Our findings is in accordance to previous studies where women with higher 8-OHdG concentrations in both mid- (67-69) and early gestation (105) gave birth to infants with lower birth weights. It has been suggested that these increases in DNA oxidation may result in an altered gene expression profile as a result of excessive damage to DNA that is unable to be repaired by the body's normal repair systems (105). This seems plausible as pregnancy is a period of rapidly growing tissues. Further, increasing DNA oxidation was associated with decreasing IGF-1 in early childhood, implying longer-term associations with body stature.

Timing in pregnancy

We observed differential effects of timing of both prenatal nutrition and oxidative stress markers. Poor nutrition during pregnancy has been demonstrated to exert different responses in the offspring depending on the timing of critical periods in pregnancy (26). This observation has occurred both in animal experimental studies (45) as well as in human studies. In the Dutch

famine, children exposed to poor nutrition in early gestation were observed to have a less favorable lipid profile in adult life, while poor nutrition exposure in late gestation was associated with decreased glucose tolerance (47). While the timing of exposure to oxidative stress during pregnancy may be important for fetal growth (69), previous research investigating maternal lipid peroxidation at different times during pregnancy and offspring outcomes are difficult to find.

Although it seems plausible that exposures during different stages of fetal development would result in different fetal response, the underlying mechanisms of our observed time differences remain unclear. The Early initiation of food supplements around week 9 of pregnancy resulted in an improved lipid profile in the offspring, perhaps due to beneficial effects on the organ development occurring at this time (116). Fetal β-cells have been suggested to have full function as early the end of the first trimester in humans (117), thus our finding of an association between early maternal lipid peroxidation and insulin status in the offspring may be related to early β-cell development. Our findings of exposures in early pregnancy and associations with insulin metabolism may be in accordance with findings from the Dutch famine where lower glucose tolerance was seen in participants exposed to famine during early- or mid-gestation (118). However, while participants in the Dutch famine were affected by more acute nutrition deprivation our women were experiencing a more chronic undernutrition that had lasted for generations, which make comparisons difficult. The placenta seems to play an important role in the effects of timing with observed differences in placental size and efficiency depending on the gestational time of undernutrition (119). An increase in placental weight in response to undernutrition in mid-pregnancy has been previously observed in sheep (120). This has been suggested as a compensatory mechanism that could potentially improve the transfer of nutrients and oxygen to the fetus. A similar compensatory growth of the placenta has been suggested in humans, but only in the tallest women, presumed to be most well-nourished before pregnancy (121). Our results may relate to the function of the placenta and its ability to provide both nutrients and oxygen to the fetus.

Sex differences

We report here of several sex differences in our study, seen in both effects of prenatal nutrient supplements and in associations between maternal oxidative stress markers and offspring outcomes. Supporting our observations, there are several lines of evidence from animal studies where the response to prenatal nutrition is different in male and female offspring, such as in the hypothalamic-pituitary-adrenal axis in sheep (122), and adult adiposity (123)

and hypertension (124) in rats. Furthermore, research is also emerging from human studies reporting sex differences. Data from the Dutch famine demonstrated an association between undernutrition in fetal life and increased serum cholesterol and triglycerides levels in adult women, but not men (125). In addition, sex-specific differences were also found in adult adiposity after maternal famine exposure (126). When taken together in the context of our study, it seems plausible to hypothesize that other exposures in fetal life, such as oxidative stress, may exert different responses depending on the sex of the fetus.

It is well established that male fetuses demonstrate different and more rapid growth patterns to female fetuses, making them more vulnerable *in utero* (127, 128). Further, it has been suggested that male fetuses are more sensitive to a woman's state of nutrition during pregnancy and therefore more susceptible to nutritional constraints, whereas girls appear to be more affected by nutritional status over a lifetime (50). This susceptibility to maternal nutritional status has also been observed in placental growth where boys in the Helsinki cohort had smaller placentas (relative to birth weight) than girls, suggesting that boys invest less in placental growth thus increasing their vulnerability to undernutrition (50). This observation was confirmed by data derived from Holland where gestational famine altered the relationship between the size of the placenta and risk of adult hypertension in males, but not females (51). Our results further support the notion that males are more vulnerable and reliant on maternal nutrition *in utero* as we demonstrated that boys in our cohort benefited more from the early start of food supplements than girls. The more pronounced association of maternal lipid peroxidation and birth anthropometry observed in boys may also be a reflection of increased sensitivity in male fetuses to maternal nutritional status. Interestingly, women carrying a male fetus had higher DNA oxidation than woman with a female fetus. This may possibly be a result of the increased fetal growth in male fetuses requiring extra maternal resources and potentially leading to an increased oxidative stress, similar to what has been observed in higher maternal lipid peroxidation in pregnancies carrying male children (105).

Public health implications

An Early initiation of prenatal food supplementation, compared to the Usual start, had favorable effects on the lipid profile in the offspring in early childhood. In addition, previous research from this cohort has observed beneficial effects of Early food supplementation in reduced stunting from birth up to 4.5 years in boys (83). Early food supplements in combination with MMS also resulted in decreased infant mortality and under-five mortality com-

pared to Usual food and the standard pregnancy care of Fe60F (82). Put together, the results clearly show several beneficial differential effects of an Early initiation to food supplementation. This highlights the importance of an early timing, and the benefits of targeting women as early as possible in food interventions during pregnancy.

Our observations from prenatal supplementation with MMS, however, are a potential cause of concern as the use of MMS resulted in levels of both insulin and growth factors that may be indicative of slower growth. Although MMS in combination with Early food had beneficial effects on offspring mortality, the combination of MMS with the Usual initiation of food supplementation resulted in the highest infant mortality rate (82). MMS also resulted in a higher prevalence of stunting in the children (83). Our findings taken together with the previous results raise concern for scaling up supplementation with MMS, especially in populations where maternal malnutrition is common.

Conclusions

Maternal malnutrition was prevalent in this population, reflected by a large proportion of underweight women as well as a high prevalence of micronutrient deficiencies in the beginning of pregnancy. An Early invitation to food supplementation in pregnancy, in comparison to the Usual invitation, resulted in a more favorable lipid profile in the offspring at 4.5 years. This suggests longer-term beneficial effects of improved nutrition in pregnancy and also stresses the importance of timing in the initiation of food supplementation.

Allocation to use of prenatal multiple micronutrients resulted in lower insulin and HOMA-IR in the children compared to the standard pregnancy care of iron and folic acid supplements. As the children already had remarkably low levels of insulin we interpret this lowering effect as being potentially unfavorable. Additional concern is that multiple micronutrients resulted in growth factors indicative of slower growth in the children. Further investigation of the effects of multiple micronutrients is needed before scale up of its use in malnourished populations.

Oxidative parameters in pregnancy were associated with longer-term outcomes in the offspring, seen in both birth anthropometry and metabolic status in preschool children. Our findings may be interpreted as providing further evidence to the theory that oxidative stress is involved in the development of metabolic disease in later life. However, if the observed associations are causal or a reflection of other processes needs to be further elucidated.

Future research

There is plenty of future research that could be done to increase our knowledge in this field. Below is a just a few of these presented.

Maternal nutrition is important for fetal growth and previous research have shown differences in the effects of prenatal supplements on size at birth and child growth depending on the nutritional status of the mother. In order to further delineate the role of maternal nutrition, it would be of interest to stratify the analyses of effects of prenatal food and micronutrient supplementation for BMI and height as indicators of current and lifelong nutrition, and whether the differential effects observed are lasting and remains in prepuberty.

We have observed that several metabolic markers appear to be associated with height in the children at 4.5 years. For a more comprehensive picture of the children's nutritional status and to gain further understanding how to interpret the effects of nutritional supplements it would be useful to carefully investigate associations between children's height, and their metabolic markers and body composition. In addition, it would be valuable to study food patterns in the children and how they may influence the effects of prenatal nutrition interventions and current nutritional status, so as to understand if and how potentially unfavorable effects of poor maternal nutrition is modifiable in later life.

Summary in Swedish/svensk sammanfattning

Svag fostertillväxt har genom epidemiologiska studier förts fram som en riskfaktor för hjärtkärlsjukdom. Som stöd finns ett stort antal studier som visar att barn som föds med låg födelsevikt löper en större risk att i vuxen ålder utveckla kroniska sjukdomar som högt blodtryck, diabetes typ 2 och hjärt-kärlsjukdom. Den bakomliggande orsaken tros huvudsakligen vara näringsbrist under graviditeten som avspeglas i en låg födelsevikt. Det är ännu oklart vilka biologiska mekanismer som ligger bakom associationen mellan tillväxt tidigt i livet och sjukdom senare i livet. Den metabola effekten har bland annat föreslagits vara medierad av oxidativ stress vilket innebär ett överskott av reaktiva syreföreningar som oxiderar lipider, DNA och protein i kroppen. Det kan påverka kroppens vävnader genom att orsaka skada på blodkärlen vilket kan vara ett förstadium till hjärt-kärlsjukdom. Högre nivåer av markörer för oxidativ stress har visats just hos personer drabbade av metabola sjukdomar.

I Bangladesh är det vanligt att kvinnor, inte minst under graviditeten, har ett begränsat näringsintag och många barn föds med en låg födelsevikt. Samtidigt sker en ökning i landet av förstadier till hjärt-kärlsjukdom som högt blodtryck och diabetes typ 2 hos vuxna. MINIMat-studien startade år 2001 i Matlab, Bangladesh, med målet att motverka låg födelsevikt genom kosttillskott och tillskott av mikronäringsämnen till kvinnor under graviditeten. Syftet med de studier som ingår i denna avhandling var att studera hur nutrition och oxidativ stress under graviditeten påverkar barnens storlek vid födseln samt hur de påverkar barnens metabola status vid 4,5 års ålder.

Kvinnorna randomiserades till en av två grupper där alla fick 600 kcal kosttillskott, hälften av kvinnorna uppmuntrades aktivt att börja intag av kosttillskott så tidigt som möjligt i graviditeten medan den andra hälften startade när de själva önskade. Kvinnorna randomiserades även till dagligt tillskott av mikronäringsämnen; a) 60 mg järn och 400 µg folsyra, b) multipla mikronäringsämnen (30 mg järn och 13 andra mikronäringsämnen inklusive antioxidanter), c) 30 mg järn och 400 µg folsyra. Kvinnorna lämnade blodprov vid graviditetsvecka 14 som analyserades för hemoglobin, järn, zink, folsyra och vitamin B-12. Kvinnorna lämnade även urinprov som analyseras för markörer för oxidativ stress vid vecka 14 och 30 (8-iso-$PGF_{2\alpha}$ som ett mått på

lipidperoxidation) och vecka 19 (8-OHdG som ett mått på DNA-oxidation). Kvinnornas barn följdes upp och vid födseln mättes vikt, längd samt bröst- och huvudomfång. Vid en uppföljning när barnen var 4,5 år analyserades blodprov för lipidprofil, glukos och insulinnivåer samt tillväxtfaktorer.

Studie 1 visar att många av kvinnorna i studien var drabbade av brist på mikronäringsämnen tidigt i graviditeten. Brist på zink och vitamin B-12 var vanligast och förekom hos ungefär hälften av alla kvinnor. Koncentrationer av alla mikronäringsämnen hos kvinnorna varierade även stort beroende på tidpunkt på året. Runt en tredjedel av kvinnorna hade anemi, medan anemi orsakad av järnbrist, vilket ofta anses vara huvudorsaken till anemi, endast hittades hos 2 % av alla kvinnor.

I studie 2 undersökte vi hur barnens metabola status påverkades av kvinnornas intag av kosttillskott och mikronäringsämnen under graviditeten. Vi fann att barn vars mödrar blivit randomiserade till tidigt kosttillskott hade en mer gynnsam lipidprofil vid 4,5 års ålder. De barn vars mödrar fått multipla mikronäringsämnen under sin graviditet hade lägre nivåer av glukos, insulin och tillväxtfaktorer jämfört med gruppen som fått 60 mg järn i kombination med folsyra, vilket vi preliminärt tolkar som negativt för barnen.

I studie 3 och 4 undersökte vi associationer mellan markörer för oxidativ stress under graviditeten och barnens storlek vid födseln och deras metabola status i tidig barndom. Vi fann att lipidperoxidation i tidig graviditet (vecka 14) var associerat både med barnens storlek vid födseln och med insulinnivåer vid 4,5 år. Lipidperoxidation i sen graviditet (vecka 30) var istället associerat med barnens lipidstatus. Vi fann även att ökade nivåer av lipidperoxidation i vecka 30 och DNA oxidation i vecka 19 var associerade med minskade nivåer av tillväxtfaktorer hos barnen.

Vi har visat att en tidig start av kosttillskott i graviditeten har positiva och bestående effekter på barnens lipidstatus. Tillskott av multipla mikronäringsämnen däremot påverkade både barnens insulin och tillväxtfaktorer på ett sätt som tyder på en sämre tillväxt, vilket vi tolkar som något negativt. Ytterligare studier om effekterna av multipla mikronäringsämnen under graviditeten är därför nödvändiga. Vi fann även att markörer för oxidativ stress under graviditeten var associerade med både barnens storlek vid födseln och deras metabola status i tidig barndom. Detta skulle kunna tyda på att oxidativ stress är involverad i utvecklingen av metabola sjukdomar.

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