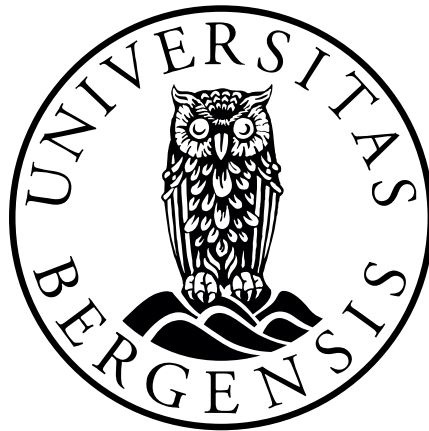# Folate and reproductive health

An epidemiologic study of folic acid supplement use and its relation to birth outcomes in Norwegian pregnant women

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Acknowledgements

Contents

PAPERS

Abstract

Background: Randomized trials and observational studies have consistently shown that maternal intake of folic acid supplements before and early in pregnancy reduces the risk of neural tube defects in infants. These reports constitute the basis for recommending fertile women to use folic acid supplements before and during early pregnancy and for introducing food fortification programs with folic acid in numerous countries. Furthermore, increasing evidence suggests that prenatal use of vitamins including folic acid may also have a protective effect on other adverse pregnancy outcomes and complications as well.

Objectives: In Norway, periconceptional folic acid supplement use is low, despite official recommendations and information campaigns to fertile women. This thesis aimed to examine the patterns of folic acid supplement use in Norwegian pregnant women and to identify important predictors of periconceptional use 1 month before pregnancy and throughout the first 3 months of pregnancy. We also tested whether various folate indicators, including prenatal folic acid supplement use, were associated with placental abruption and infant birth size. These outcomes have been linked to folate in previous studies, but results are inconsistent, possibly due to small sample sizes and methodological limitations.

Materials: For the purpose of this thesis, we used observational data from two well-established data bases: the Medical Birth Registry of Norway (MBRN) and the Norwegian Mother and Child Cohort Study (MoBa). MBRN is a national health registry in which registration of all live births and stillbirths in Norway has been compulsory since 1967 (50,000-60,000 births per year), whereas MoBa is a population-based prospective study of Norwegian pregnant women which includes more than 100,000 pregnancies between 1999 and 2008.

Results: Our first paper (based on MoBa) showed that 71.6 percent of the MoBa participants in 2000-2003 had taken folic acid-containing supplements at some time

before or during pregnancy. Of these, more than 70 percent had started use after becoming pregnant, the majority during the first and second month of pregnancy. Only 10.2 percent of the participating women had used folic acid-containing supplements regularly from 1 month before pregnancy throughout the 3 first months of pregnancy. Women who had used folic acid supplements regularly during the periconceptional period were more likely to be older, to be married or living together, to be non-smokers, to have higher incomes, to have higher education, to have lower parity, to have planned their pregnancy, and to have received fertility treatments. Demographic and socioeconomic factors were the strongest predictors.

Our second paper (based on MoBa) showed that food folate intake, supplemental folic acid use, total dietary folate intake, and maternal plasma folate and homocysteine concentrations were not significantly associated with gestational age, infant birth weight, head circumference, crown-heel length, or small for gestational age (SGA). Consistent with previous studies, infant birth size was strongly predicted by maternal smoking (adjusted odds ratio (OR) for SGA = 2.3; 95 percent confidence interval (CI): 1.6, 3.3).

Our third paper (based on MBRN) showed that intake of folic acid and other vitamin supplements before and during pregnancy statistically significantly reduced the risk for placental abruption by up to 30 percent. Associations between vitamin supplement use and placental abruption were strongest for women using both folic acid and multivitamin supplements (adjusted OR = 0.68; 95 percent CI: 0.56, 0.83), followed by multivitamins alone (adjusted OR = 0.72; 95 percent CI: 0.57, 0.91) and folic acid alone (adjusted OR = 0.81; 95 percent CI: 0.68, 0.98).

Because the response rate of MoBa is low (43 percent), there is concern as to which extent the results of MoBa are valid for the total Norwegian population. To evaluate potential bias due to self-selection in MoBa, our fourth paper aimed to study differences in prevalence estimates and association measures between study participants and all women giving birth in Norway, using data from the MBRN. We

found no bias in 8 studied exposure-outcome associations, even though several exposures and outcomes were overrepresented or underrepresented in MoBa.

Conclusions: Most women started folic acid supplementation too late with respect to the prevention of NTDs. Demographic and socioeconomic factors were the strongest predictors for periconceptional folic acid use 1 month before pregnancy throughout the 3 first months of pregnancy. This thesis further supports the old hypothesis that folate deficiency can be involved in development of placental abruption. Maternal intake and status of folate during second trimester appeared not to be associated with infant birth size, possibly due to the low number of individuals with low folate status in MoBa.

List of publications

The following 4 papers formed the basis of this thesis:

1. Nilsen RM, Vollset SE, Gjessing HK, Magnus P, Meltzer HM, Haugen M, Ueland PM. Patterns and predictors of folic acid supplement use among pregnant women: the Norwegian Mother and Child Cohort Study. *Am J Clin Nutr* 2006; 84:1134-41.

2. Nilsen RM, Vollset SE, Monsen AL, Ulvik A, Haugen M, Meltzer HM, Magnus P, Ueland PM. Infant birth size is not associated with maternal intake and status of folate during second trimester in Norwegian pregnant women. *J Nutr* 2010 Jan; In press.

3. Nilsen RM, Vollset SE, Rasmussen SA, Ueland PM, Daltveit AK. Folic acid and multivitamin supplement use and risk of placental abruption: a population-based registry study. *Am J Epidemiol* 2008; 167:867-74.

4. Nilsen RM, Vollset SE, Gjessing HK, Skjaerven R, Melve KK, Schreuder P, Alsaker ER, Haug K, Daltveit AK, Magnus P. Self-selection and bias in a large prospective pregnancy cohort in Norway. *Paediatr Perinat Epidemiol* 2009; 23:597-608.

Abbreviations

AED	Antiepileptic drugs
BMI	Body mass index
CI	Confidence interval
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
EDTA	Ethylenediaminetetraacetic
FFQ	Food frequency questionnaire
FIGLU	Formiminoglutamic acid
GAM	Generalized additive logistic regression models
ICD-10	International Classification of Diseases, Tenth Revision
MBRN	Medical Birth Registry of Norway
MoBa	Norwegian Mother and Child Cohort Study
MRC	Medical Research Council
MTHFR	Methylenetetrahydrofolate reductase
NTD	Neural tube defect
OR	Odds ratio
RR	Relative risk
SAM	S-adenosylmethionine
SGA	Small for gestational age
THF	Tetrahydrofolate

1. Introduction

1.1 Background

Folate is a water-soluble B-vitamin that occurs naturally in foods (1). It is essential for DNA synthesis and normal cell division in humans (2). Folate is also involved in the metabolism of homocysteine and helps maintain normal levels of this amino acid (3). Overt deficiency of folate may cause a variety of health problems, like megaloblastic anemia (4), neurological disorders (5), and hyperhomocysteinemia (6). A chemically stable synthetic form of folate, folic acid, is used in dietary supplements and fortified foods.

During the last 20 years, folate has attracted much scientific and public health interest due to its role in neural tube defects (NTDs). In the early 1990s, 2 large randomized clinical trials demonstrated that daily use of folic acid supplements before and during early pregnancy can prevent as much as 70 percent of NTDs (7, 8). Although the prevention mechanisms of folate are unknown, these trials constitute the basis for recommending fertile women in numerous countries to use folic acid supplements before and during early pregnancy (9). Several countries have, in addition, introduced mandatory fortification of staple foods (usually flour) with folic acid to increase intake of this B-vitamin.

Despite recommendations and public health campaigns, folic acid supplement use and dietary intake of folate before and during early pregnancy is still low in Norway and other countries (10). Explanations for this may be that a large proportion of women do not plan their pregnancy, and of those who do plan pregnancy, many are still unaware of the benefits and recommended timing of folic acid supplement use (9, 10). To improve folate intake among fertile women, we thus need to increase awareness and knowledge of folate, both in the public and among health care professionals. In order to do this, both existing and future epidemiologic studies are needed.

Several studies have suggested that the use of folic acid supplements before and during pregnancy may also prevent other birth defects and adverse pregnancy outcomes in addition to NTDs (11). However, results are not conclusive. Hence, there is a need for extensive epidemiologic research on maternal folate status and other pregnancy outcomes and complications as well. If folate can reduce the risk of several adverse outcomes, this would provide yet another reason for more promotion of folic acid supplement use and dietary folate intake among fertile women.

1.2 Folate

Before reviewing the literature of folic acid supplement use and its relation to pregnancy outcomes, some historical and biochemical background of folate will be provided in this section. In addition, I will introduce some basic concepts that will be used throughout the thesis.

The discovery of folate

The first observation that led to the identification of folate as an important nutrient was done by Lucy Wills in 1931 (12). Through studies of pregnant women in Bombay, India, she found that a yeast extract could effectively treat macrocytic anemia, which was a common pregnancy complication. Wills and her group further demonstrated that this type of anemia could be produced in monkeys fed by the same diets as the women in Bombay, and that oral administration of yeast or liver extract could cure anemia in both monkeys and human patients (13).

Like Wills and colleagues had demonstrated in monkeys, several other researchers reported that anemia could be produced in monkeys and chickens by feeding them deficient and purified diets (1). They further found that the anemia appeared to respond positively to dietary supplement of the brewer's yeasts, as well as liver extract, alfalfa and wheat bran. These protective factors were named *vitamin M, vitamin B$_c$, factor U* and *factor R*, but the corrective substance was not identified.

In 1940, Snell and Peterson, 2 microbiologists, reported the existence of an unidentified water-soluble factor that was necessary for the growth of *Lactobacillus casei* (14). This factor, which became known as the *L. casei factor*, was present in both yeast and liver. In 1941, Snell's group further reported that they had obtained a nearly pure form of an acid that promoted the growth of *Lactobacillus casei* (15). This acid was obtained from spinach leaves and stimulated growth of *Lactobacillus casei* under the same conditions as the *L. casei factor*. Because this acid was found in most green leaves, they named the factor by virtue of its sources, *folic acid*, from the Latin word *folium* for leaf.

During the early 1940s it was not clear whether *folic acid* and the anti-anemic factors in liver and yeast were related. However, in 1941, Robert Stokstad and his group managed to isolate pteroylglutamic acid from liver (16). They further managed to synthesize the compound in 1945 (17). Shortly thereafter, pteroylmonoglutamic acid was found to be the substance that had been identified in liver as *vitamin M*, *vitamin B_c*, *factor U*, and the liver *L. casei factor*. Furthermore, the yeast derived *L. casei factor* was found to be the diglutamyl derivative of pteroylglutamic acid (1). These and other derivatives became generically known as *folic acid* or *folate*.

Forms of folate

Folate occurs in different chemical forms distinguished by their oxidation state and their type of one-carbon substitution (18). The original term, folic acid, usually refers to the fully oxidized form of folate, which has no biological function of its own. This is the chemically stable synthetic form of folate used in vitamin supplements and fortified foods and consists of an aromatic pteredine ring, attached to a paraminobenzoic acid conjugated to one *L*-glutamic acid residue (18, 19). Biologically active folate usually differs from folic acid in 3 respects: (i) reduction to di- or tetrahydro forms of the pteridine ring, (ii) additional single-carbon units at the N5 and N10 position, and (iii) additional number of glutamate chains. Folate in human tissues is found as polyglutamate derivatives, whereas folate in human blood

and urine is found as monoglutamate derivatives (18). The main circulating form of folate is the monoglutamate 5-methyltetrahydrofolate (THF) (18, 19). About half of the total body folate content is stored in the liver (18).

Folate absorption

Rich sources of natural-occurring folates are liver, yeast, green leafy vegetables, and cereal products, but high levels are also found in fruits and dairy products (1, 20, 21). Most of the natural folates consumed in foods are polyglutamates, predominantly as 5-methylTHF and 10-formylTHF (18, 19). Before absorption across the intestinal mucosa can take place, the polyglutamate form needs to be hydrolyzed to the folate monoglutamate form in the gut lumen. This is done by the folate deconjugase, a brush border enzyme in the jejunum (18, 19). Because synthetic folic acid already exists as a monoglutamate, it may directly be transported through the intestinal brush border without this enzymatic conversion. For this reason the bioavailability in humans is considered higher for synthetic folic acid than naturally occurring folate (20). Before passing into the blood circulation, folic acid and other folate forms that are not already in the 5-methylTHF from need to be reduced and methylated to form 5-methylTHF. This is done by the enzyme dihydrofolatereductase during transit through the intestinal mucosa (18). After passing into the circulating blood, 5-methylTHF enters a cell, during which an enzyme adds a chain of polyglutamate residues. The polyglutamation is necessary in order to retain folate within the cell.

Folate metabolism

Folate participates in 2 different metabolic cycles within the cell, i.e. one involving the synthesis of DNA and the other the supply of methyl groups for methylation reactions (18, 22). In these cycles, folate undergoes a number of metabolic changes, in which the fully reduced form of folate, THF, serves as an acceptor or donor of a one-carbon unit, reactions commonly referred to as the folate-dependent *one-carbon metabolism*.

Nucleotide metabolism

Folate is involved in the synthesis of DNA by having an essential role in the synthesis of the pyrimidine nucleoside, thymidine, and of purines (18, 22). In the synthesis of thymidine, the folate intermediate 5,10-methyleneTHF can directly donate its one-carbon unit in the thymidylate synthase reaction, which converts deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). In the synthesis of purines, folate-linked one-carbon units are incorporated into the C2 and C8 of purines via 10-formylTHF. During periods of rapid cell division and growth such as infancy and pregnancy, sufficient supply of folate to cells is critical to maintain normal cell division.

Methylation reactions

Folate is also needed in the metabolism of homocysteine and methionine (23). Homocysteine is an intermediate product of methionine metabolism and is itself metabolized by 2 pathways: the re-methylation pathway which regenerates methionine, and the transsulfuration pathway, which converts homocysteine into cysteine. The remethylation pathway is comprised of 2 intersecting biochemical pathways, one involving 5-methylTHF and vitamin B12, the other involving betaine (23). Methionine in turn can be utilized to produce S-adenosylmethionine (SAM), which is the primary methyl group donor used in many biological methylation reactions, including the methylation of DNA and RNA (23). Because of the role of folate in the remethylation of homocysteine to methionine, deficiency of folate may contribute to impaired DNA methylation and to accumulation of homocysteine, the latter associated with cardiovascular disease and other chronic diseases (3).

Interrelations of B-vitamins

The utilization of folate as methyl donor depends on several other B-vitamins. Vitamin B2 is the cofactor for methylenetetrahydrofolate reductase (MTHFR), the enzyme that reduces 5,10-methyleneTHF to 5-methylTHF (24). Vitamin B12 is required by the enzyme, methionine synthase, involved in the conversion of

homocysteine to methionine (23). In addition, vitamin B6 is involved in the synthesis of 5,10-methyleneTHF from THF (18). Hence, in order to ensure maximal utilization of folate as a methyl donor, adequate intake of vitamin B12, B6 and B2 is also important. Blood folate and vitamin B12 are strongly associated with homocysteine, and supplemental folic acid and vitamin B12 has been used in homocysteine lowering therapy in individuals with elevated levels of this amino acid (25).

Folate status

The main cause of low folate status in humans is low intake of folate from foods. Low folate status can also occur due to malabsorption, which is often recognized in alcoholics (26) and individuals with intestinal and gastric diseases, such as celiac disease (27). Furthermore, deficiency of other micronutrients, such as zinc (28) and iron (29) are associated with impaired food folate utilization.

Folate requirements are increased in periods during rapid cell division and growth. Several studies have reported that women with normal pregnancies have more circulating folate before and during early pregnancy than folate concentrations after and late during pregnancy (30-32). Furthermore, studies have reported that women who have recently undergone a viable pregnancy may become folate deplete in the next pregnancy, especially if the inter-pregnancy interval is short (33). Folate status is likely to deteriorate in multiple pregnancies.

Folate status is also affected by particular defects or polymorphisms in the genes encoding for folate-dependent enzymes. Most important polymorphisms is C-to-T substitution (677C->T polymorphism) that occurs at locus 677 of the MTHFR gene (24). The TT genotype reduces the enzyme activity and impairs the conversion of 5,10-metyleneTHF to 5-methylTHF, which in turn is used in the conversion of homocysteine to methionine.

Studies have shown that whole blood folate varies according to assay format and the common MTHFR 677C->T polymorphism (34). The radioassay measures higher

folate concentrations in subjects with TT compared to CC genotype, while the opposite is found when folate is measured with the microbiological assay (35). This has been explained by a larger proportion of the total cellular folate as formylated forms of folate in subjects with the TT genotype (36). Formylated folate species may be overestimated by the folate binding protein assay, because they have higher affinity than 5-methylTHF for the binder (37).

Several drugs can interfere with folate status. Methotrexate is an antifolate drug used in the treatment of cancer and rheumatoid arthritis. It inhibits the production of the active form of THF from the inactive form dihydrofolate, resulting in reduced DNA synthesis and cell division (38). Furthermore, individuals using some antiepileptic drugs (AEDs), especially phenytoin and carbamazepine, are at particular high risk of folate deficiency (39). Possible mechanisms by which these drugs interfere with folate status include reduced absorption, increased folate metabolism in the liver and altered enzyme activity (40).

1.3 Epidemiology

In this section, I will briefly review the literature of folate and its relation to NTD, placental abruption and infant birth size. I will point to some limitations that should be considered in future studies of placental abruption and infant birth size.

Early works on folate

The first report that addressed the importance of folate in reproductive health was published by Bryan Hibbard in 1964 (41). He assessed folate status as urinary excretion of formiminoglutamic acid (FIGLU) in 1484 low-income obstetric patients from Liverpool, United Kingdom. Abnormal FIGLU excretion was not only related to megaloblastic anemia, but also to placental abruption and spontaneous abortion. He also showed that abnormal FIGLU excretion was related to adverse outcomes in previous pregnancies, including infant low birth weight, congenital malformations,

and perinatal mortality. He hypothesized that increased adverse pregnancy outcomes in many cases reflected poor folate status due to several factors, like a "demand and supply" problem during pregnancy, a less efficient absorption during pregnancy, and a defective utilization of folate due to certain drugs (42). Hibbard later showed that poor folate status was particularly associated with 3 outcomes: placental abruption, congenital malformations, and small for gestational age (SGA) (43).

Neural tube defects (NTDs)

Shortly after Bryan Hibbard's report in 1964, Elisabeth Hibbard and Richard Smithells suggested that folate deficiency in pregnancy may be related to central nervous system malformations (44). Smithells and his group started a series of observational and intervention studies demonstrating that vitamins, including folate, reduced the risk of NTDs (45-47), which was a common malformation (4.5 per 1000 births) in the United Kingdom in the 1970s and 1980s (48). In a study from 1983 (45), almost 1000 women with previous NTD pregnancies were enrolled in a non-randomized trial and assigned 0.36 mg folic acid plus multivitamins from 2 months before conception and throughout the first trimester. The overall NTD recurrence rates were 0.7 percent for 454 fully supplemented mothers and 4.7 percent for 519 non-supplemented mothers, a difference that was statistically significant (45). At the same time, several randomized trials were conducted by others, but findings yielded insignificant results, possibly due to the limited number of subjects (49). Unfortunately, Smithells and his group were not permitted by their institution to perform a randomized trial, and for that reason their findings did not lead to any public health action (50).

The proposed role of folic acid in the prevention of first occurrence and recurrence NTDs was eventually confirmed in the early 1990s in 2 large randomized trials. In 1991, the Medical Research Council (MRC) in United Kingdom conducted a randomized multicenter study of folic acid supplement use among women with a previously affected NTD infant. This study demonstrated that women who had been given folic acid supplements of 4 mg from 1 month before pregnancy throughout the

first pregnancy trimester were associated with a 72 percent statistically significant risk reduction of recurrent NTDs, relative to those who were assigned placebo (7). Other vitamins showed no significant protective effect. In 1992, Czeizel et al conducted a large randomized trial in Hungary of multivitamin use among women with no previous NTD infants (8). Women were assigned a supplement of 0.8 mg folic acid and other nutrients from 1 month before pregnancy and through the second missed menstrual period. No NTD cases occurred in 2104 supplemented women, compared with 6 cases among 2052 non-supplemented women (P <0.029).

In a case-control study in the Unites States and Canada in 1993, Werler et al (51) showed that a dose of 400 µg of folic acid contained in multivitamins was found to reduce significantly the risk of NTDs by approximately 60 percent. The protective effect appeared to be confined to daily use in the periconceptional period; no appreciable reductions in risk were observed for women who began supplementation after the first missed menstrual period. In a community-based intervention study in China in 1999, Berry et al (52) further demonstrated that a dose of 400 µg folic acid alone during the periconceptional period could prevent up to 80 percent of NTDs in a high prevalence area (5 per 1000 births) in North China and 40 percent in a low prevalence area (1 per 1000 births) in South China.

There has been a considerable interest in discovering the mechanisms by which folate can prevent NTDs. Since folate plays an essential part in the methylation of homocysteine to methionine, both elevated homocysteine (22, 53) and MTHFR TT genotype of both mother and child (24) have been proposed. The mechanisms remain uncertain, however. Regarding the MTHFR TT variant, it may explain only a fraction of NTDs prevented. In Italia, for instance, the TT variant is common in the population, although the prevalence of NTD occurrence is low (50, 54). Recently, it has been suggested that NTDs may be related to auto-antibodies against folate receptors (55). This finding was not verified in a study in Ireland in 2009 (56), demonstrating the difficulty in establishing a specific mechanism of folate in NTD.

Placental abruption

After Hibbard's observation of an association between excessive FIGLU excretion and placental abruption in 1964 (41), many other studies, including a randomized trial, evaluated the association of folate deficiency or folic acid supplementation with this pregnancy complication (43, 57-60). Unfortunately, results from these studies were diverging and few researchers pursued the hypothesis further into the 1980s.

In the 1990s and early 2000s, several observational studies showed a consistent relation between homocysteine and placental abruption (61-64). Furthermore, a meta analysis in 1999 showed that both folate deficiency and elevated homocysteine was associated with placental abruption (65). Consequently, many researchers hypothesized that elevated homocysteine in some cases may be the underlying cause of some abruptions: homocysteine can cause vascular damage, which may result in blood clots behind the placenta (66). However, since plasma homocysteine analysis in these studies was made after the onset of abruptions, a specific function of homocysteine in placental abruption is uncertain (11). More recently, several studies have been undertaken to explore the possible association between placental abruption and several maternal polymorphisms of enzyme genes in the folate metabolism, including MTHFR (67-72). However, also these results are inconsistent, demonstrating the difficulty in establishing a specific role of folate in abruption.

A major limitation of previous epidemiologic studies is the small sample size used. The prevalence of placental abruption occurs in about 0.5 to 1 percent worldwide (73), and clearly large samples are needed to detect a precise effect of various risk factors. Furthermore, surprisingly few epidemiologic studies have addressed whether supplemental folic acid or multivitamin use during pregnancy can reduce occurrence of the complication, even though blood folate, vitamins B12, B6, A and E have been associated with reduced risk of placental abruption (62, 64, 74). In paper 3, we tested this hypothesis by using data from a large population-based registry in Norway (55,000-60,000 births per year), where both folic acid and multivitamin use before and during pregnancy, as well as placental abruption, are recorded.

Infant birth size

Hibbard demonstrated that blood folate concentration early in pregnancy was associated with the size of the infant at birth (43). This association was mainly found for SGA and to a less extent for preterm birth. Although the administration of folic acid in pregnancy had been of short duration, he further found that low folate concentrations were less common for those who had been given folic acid supplements (43).

Over the years, several large randomized trials have examined the effect of prenatal use of vitamins containing folic acid on infant low birth weight (<2500 g), preterm birth (<37 weeks gestation), and SGA. While some trials have observed a relation with low birth weight or SGA (75, 76), others have shown no significant association of folic acid supplementation with these outcomes, neither alone (77, 78) nor in combination with other vitamins (79). Also, numerous observational studies have examined the relation of prenatal use of folic acid-containing supplements, dietary folate intake, and maternal blood folate status, with low birth weight, preterm birth and SGA. While some studies have found a relation (80-87), others have not (88-91). A Dutch study on several B vitamins measured in blood before and during pregnancy in healthy, well-nourished women demonstrated no association between the vitamin concentrations and infant birth weight (92). Diverging results are also reported regarding elevated homocysteine (61, 80, 88-91, 93), and the evidence of an association between maternal and fetal MTHFR polymorphisms and these outcomes is not conclusive (11).

The conflicting results obtained from trials and observational studies may be due to methodological issues, such as small population size, lack of control for confounding factors, and timing of folate exposures. Nevertheless, in order to draw firm conclusions regarding folate and infant birth size, one also should include information on several folate exposures (e.g., circulating folate, food folate intake, folic acid supplement use) and several birth size parameters, which most studies have not. In paper 2, we used a subsample of a large population-based pregnancy cohort in

Norway to evaluate the association of various folate indicators with gestational age, infant, birth weight, head-circumference, crown-heel length, and SGA.

1.4 NTD prevention strategies

In this section, I will focus on the international and national strategies for the prevention of NTDs. I will point to some important issues that I think need further investigation in Norway and other countries.

NTD occurrence

The neural tube is the precursor to the central nervous system. During early pregnancy, and within the first 28-30 days of pregnancy, the neural tube gradually closes to form the spinal cord and brain (49, 50). Failure of closure results in NTDs, of which there are 2 main forms depending on whether the cranial or caudal end of the neural tube is involved (49, 50). The most common types of NTDs are:

- *Spina bifida (caudal):* The spinal cord is not completely closed. The opening in the spine may or may not be covered by a layer of skin. This malformation is associated with disabilities later in life.

- *Anencephaly (cranial):* The skull and brain is not completely developed. The opening is often not covered by bone or skin. This malformation is incompatible with life.

- *Encephalocele (cranial):* The skull is not completely closed. Parts of the infant's brain may come through the opening in the skull. The degree to which they can be corrected depends on the size of the encephalocele.

In the European Union, NTDs affect at least 4500 pregnancies each year (48). These include live births, stillbirths, and pregnancy terminations after prenatal diagnosis. The highest NTD prevalence in Europe is found in United Kingdom and Ireland,

where the frequency was 45 per 10,000 births in 1980 and 10 to 15 per 10,000 in the 1990s (48). In Norway, about 1 in 1000 is affected, corresponding to around 60 cases per year, mostly spina bifida and anencephaly (Table 1; Medical Birth Registry of Norway (MBRN) Online Birth Statistics: www.fhi.no).

Table 1. Number of NTDs in Norway as registered by MBRN, 2007

NTDs	Total (n = 59,632)	Live births (n = 58,969)	Stillbirths (n = 404)	Induced abortions (n = 259)
All	61	16	1	44
Anencephaly	18	0	1	17
Spina bifida	35	13	0	22
Encephalocele	8	3	0	5

Recommendations

The MRC and Hungarian randomized trials in 1991 and 1992 laid the ground for a major public health opportunity to facilitate NTD prevention. Today, these reports constitute the basis for recommending fertile women to use folic acid supplements before and during early pregnancy and for introducing food fortification programs with folic acid in numerous countries (9).

The first recommendation in Norway on folate use and pregnancy was issued by the Norwegian Board of Health in 1993 (94). This recommendation stated that women with a previous affected infant should been given 4 mg of daily folic acid during the periconceptional period to prevent recurrence of a NTD. There was no folic acid recommendation for women without a history of NTD, apart from increasing their intake of folate from foods. In 1998, a second recommendation of periconceptional folic acid use was issued by the Norwegian Nutrition Council (95). This recommendation stated that all women who may become pregnant should take a daily

folic acid supplement of 400 µg from 1 month before pregnancy throughout the first 2-3 months of pregnancy to reduce the risk of NTDs. Women with an increased risk of delivering a child with NTD (for instance, women with previous affected infant and those using AED) were recommended to take higher doses of 4 mg of daily folic acid during the periconceptional period. Mandatory food fortification with folic acid to increase intake, as implemented in the United States and other countries, has not been introduced in Norway (see next section below).

After the folic acid recommendations were issued in Norway in 1998, several public health campaigns have been conducted to disseminate the recommendations to fertile women (96-98). When the recommendations were launched, a public folder, poster and guidelines for health personnel were made (96). Pharmacies were asked to distribute the public folders to all persons who were buying contraceptive pills, other contraceptive prevention, or folic acid tablets. The message on periconceptional folic acid use and NTDs was also advertized in womens' magazines and in various health journals for health personnel. In 2002, an official website regarding pregnancy and folate was established by the Norwegian Nutrition Council (96). This website is now under revision (www.helsedirektoratet.no).

Despite official recommendations and large public health campaigns, the overall folic acid supplement use and dietary intake of folate is still low among Norwegian fertile/pregnant women (97, 99, 100). In a study from Oslo in 2003, only 17 percent of the participating women reported folic acid supplement use prior to pregnancy. A substantially lower percentage (2 percent) was observed by the same authors among immigrants. Nevertheless, recent numbers from the MBRN show a marked increase in the preconceptional use of folic acid in pregnant women from 5 percent in 1999 to 27 percent in 2007 (MBRN Online Birth Statistics: www.fhi.no), indicating a growing folate interest among fertile women. From 1999 through 2007, however, there has not been a reduction in NTDs in Norway (Figure 1; MBRN Online Birth Statistics: www.fhi.no).

The most important predictors for not taking folic acid supplements periconceptionally are unplanned pregnancies, low socioeconomic levels, and young age (9). However, also other important determinants of supplement use exist, and further investigation of such predictors should be carried out to help design more effective intervention programs to improve periconceptional intakes of folic acid in Norway and other countries. Also, more information is needed regarding timing of folic acid supplement use. Apparently, a substantial portion of women starts folic acid supplementation too late with respect to NTD prevention (96), but it is unclear how late in pregnancy they start. In paper 1, we took advantage of a large pregnancy cohort in Norway to examine both patterns and predictors of folic acid supplement use.
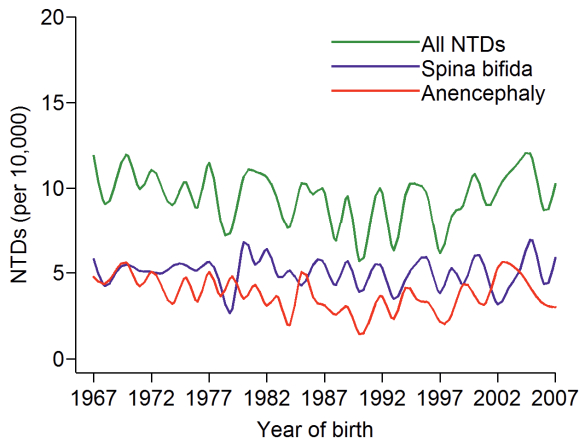
Figure 1. NTD occurrence by year of birth as registered by MBRN, 1967-2007. Numbers from January 1999 through 2007 include information on pregnancy terminations after prenatal diagnosis.

Food fortification

Food fortification refers to the situation in which a country has enriched certain food products (usually staple foods) with vitamins and other micronutrients to ensure that minimum dietary requirements are met. This can be done either voluntary or

mandatory, or both. Mandatory food fortification is usually regulated by law, whereas voluntary food fortification is regulated by specific permission from health authorities.

The rationale of fortifying foods with folic acid in many countries is the low compliance with folic acid recommendations among pregnant women and that most women do not meet the recommended dose of 400 µg folic acid through diets alone. In Norway, only voluntary food fortification with folic acid is allowed. However, by 2009 only few food production companies had taken advantage of this possibility (www.mattilsynet.no). Although voluntary food fortification has proven to increase maternal blood folate status markedly in some countries (101), the effect on the NTD occurrence is still less than expected (9).

In 1996, the Food and Drug Administration in the Unites States issued regulations requiring that grains be fortified by folic acid by January, 1998. The level of folic acid was set at 140 µg per 100 g of cereal grain product (9, 102). The average of folic acid intake was estimated to increase by 100 µg per day. To meet the recommended daily dose of 400 µg/day folic acid, women were recommended to consume additional folic acid supplements (9). After legislation in the United States was passed, other countries, including Canada and Chile, have introduced mandatory food fortification with folic acid (103, 104). In 2007, the number of countries with national regulations for mandatory wheat-flour fortification was 54 (105).

The decision to fortify foods with folic acid by law in Norway has been postponed due to concerns regarding potential side-effects of high dose folic acid in certain subgroups of the population (96). Particularly, there has been a concern as to whether folate may promote the growth of premalignant and malignant lesions in humans (106, 107). If this is true, the benefits of mandatory food fortification to prevent NTDs probably do not outweigh the risk of cancer in certain groups.

A comparison of NTD prevalence before and after fortification in the United States shows a decline in the prevalence of spina bifida and anencephaly (23 and 11 percent,

respectively) (108). However, NTDs were declining before fortification, suggesting that other factors than fortification may play a part. In Ontario, Canada, voluntary and mandatory fortification (flour, pasta, and cornmeal) was introduced in 1995 and 1998, respectively. During 1995-1999, the NTD occurrence decreased by 48 percent (109). In Chile, wheat flour fortification with folic acid has been mandatory since 2000. Compared with pre-fortified periods, the prevalence of spina bifida and anencephaly in the post-fortified period fell by 51 and 42 percent, respectively (110).

2. Objectives

The present thesis aimed to study folic acid supplement use and its relation to birth outcomes in Norwegian pregnant women. All research questions were addressed in 4 papers using data from the Norwegian Mother and Child Cohort Study (MoBa) and the Medical Birth Registry of Norway (MBRN).

The main research questions were:

1. To which extent do Norwegian pregnant women follow the recommendations regarding periconceptional folic acid supplement use? [**Paper 1**]

2. Which maternal factors predict periconceptional folic acid supplement use among Norwegian pregnant women? [**Paper 1**]

3. Is infant birth size associated with second-trimester folate status in Norwegian pregnant women? [**Paper 2**]

4. Is periconceptional folic acid and multivitamin supplement use associated with placental abruption in Norway? [**Paper 3**]

Because the response rate of MoBa is low (43 percent), there is concern as to which extent the results of MoBa are valid for the total Norwegian population. This thesis therefore also aimed to study the following methodological questions:

5. Are prevalence estimates of exposure and outcome variables in MoBa different from those of all women giving birth in Norway? [**Paper 4**]

6. Are exposure-outcome associations in MoBa different from those of all women giving birth in Norway? [**Paper 4**]

3. Materials and methods

3.1 Study populations

In this section, I give a description of the study populations and the sample selection criteria used for each paper in the thesis.

The Medical Birth Registry of Norway (MBRN)

MBRN is a national health registry in which registration of all live births and stillbirths in Norway has been compulsory since 1967 (111). It was initiated by the Directorate of Health with the aims to monitor and identify causes for perinatal morbidity and mortality in both mother and child (111, 112). The registry comprises extensive medical information on the mother's health before and during pregnancy, on delivery, and on the newborn. The information is collected from hospital records at admittance for delivery and from an antenatal form, which the mother brings to the birth clinic. All data are further registered in a standardized notification form at the time of birth (Appendix 1). The midwife and/or the physician attending the birth are responsible for completing the notification form, and sending it to the registry within a few weeks after birth. In December 1998, a revised version of the notification form was introduced to include new variables (Appendix 1), such as maternal dietary supplement intake and smoking. The papers in the present thesis were based on data from the revised form only.

The Norwegian Mother and Child Cohort Study (MoBa)

MoBa is a population-based prospective study of Norwegian pregnant women that includes more than 100,000 pregnancies between 1999 and 2008 (113). The objective of the study is to identify genetic and environmental risk factors for various health problems in mothers and their children both during pregnancy and in childhood. The

cohort was established in Western Norway in 1999 and was gradually expanded to a national level during the study period. Women were recruited to the study through a postal invitation after they had signed up for the routine ultrasound examination at their local hospital (around 18 weeks of gestation). In 2008, 50 hospitals had been included in the study. At the ultrasound examination, participating women and their partners were asked to donate blood samples (Appendix 2). In addition, after delivery, a blood sample was collected from the umbilical cord and a second blood sample was taken from the mother. During pregnancy, the mother received 3 questionnaires and the father received 1. The mothers were further asked to respond to 4 additional questionnaires during early childhood (0-7 years). The present thesis was based on the maternal questionnaires 1-3 during pregnancy (Appendix 2) and on the maternal blood sample collected around gestational week 18.

Tracking and linking data

MoBa uses a database tracking system to register pregnant women and to follow the progress of participants through the various phases of the study. Since a woman can take part in the survey with several pregnancies, the unit of observation is the pregnancy and not the woman. To retrieve registered pregnancy outcomes and other pregnancy-related information, participants of MoBa are linked to MBRN by using the national identification number (113). MoBa and MBRN are both administered by the Norwegian Institute of Public Health in Bergen.

Sample selection criteria

Paper 1: We included 25,935 MoBa participants with births during the period 2000-2003. Of these, 23,201 pregnancies (89 percent) had complete information on selected follow-up criteria (Table 2). If a woman had several pregnancies during this study period, we used only her first pregnancy. We also excluded women who had missing information on dietary supplement intake data in the baseline and the follow-up questionnaire. The final sample for analyses comprised 22,500 women.

Table 2. Inclusion criteria for papers 1 through 4

Inclusion criteria	Description	Paper			
		1	2	3	4
Time period	Year of birth	2000-2003	2002-2003	1999-2004	2000-2006
MBRN notification form	Information collected at birth	Yes	Yes	Yes	Yes
MoBa baseline questionnaire	Prenatal information (median 18 weeks)	Yes	Yes		
MoBa food frequency questionnaire	Prenatal information (median 18 weeks)		Yes		
MoBa maternal blood samples	Prenatal information (median 18 weeks)		Yes		
MoBa follow-up questionnaire	Prenatal information (median 30 weeks)	Yes			
Initial study sample	Number of subjects with all data sources	23,201	14,838	349,043	398,849
Final study sample	Number of subjects included in the analyses[a]	22,500	2934[b]	280,127	398,849

[a] Exclusions are described in the text.

[b] Random sample of subjects available for retrieval from the MoBa Biobank.

Paper 2: We analyzed data in a random sample of 3000 MoBa women during the period July 1, 2002 - December 31, 2003. Initially, there were 17,588 women with registered births during this period. Of these, 14,838 women (84 percent) had complete information on selected follow-up criteria, including blood samples (Table 2). At the time of sampling, only 6723 blood samples had been processed and were ready for retrieval from the MoBa Biobank. We randomly selected 3000 of these samples. We excluded multiple births and women who had no information on both gestational age and infant birth weight, leaving 2934 women for analyses.

Paper 3: We analyzed data from MBRN during the period 1999-2004. Initially, there were 349,043 infants during this period. We excluded 12,944 infants from multiple births and further 55,972 births (16 percent) where information on supplemental vitamin use was missing, leaving 280,127 singleton births (representing 226,724 women) for analyses (Table 2).

Paper 4: We analyzed data from MBRN during the period 2000-2006. Women who agreed to participate in MoBa ($n = 73,579$) were compared with all women giving birth in Norway ($n = 398,849$). Women who had more than 1 pregnancy during the study period and who agreed to MoBa participation in more than 1 of the pregnancies were defined as unique participants with each pregnancy. The study had no exclusions of subjects (Table 2).

3.2 Folate indicators

Information on maternal folate intake and status was obtained from both MBRN and MoBa (Table 3). In this section, I provide a brief description of the various folate indicators used in the various papers.

Folic acid supplement use (Paper 1)

Information on dietary supplement use in MoBa was collected in a baseline and a follow-up questionnaire returned by the women around gestational weeks 18 and 30, respectively (Appendix 3 and 4). Women who used dietary supplements were asked to report in detail which vitamins/minerals they were taking based on the label of their supplement container, and when and how often the supplements were taken. Dosage was not asked for. A woman was defined as a folic acid user if she reported use of supplements containing folic acid more than once a week during a 4-week period (Appendix 3 and 4). Periconceptional intake was defined as starting folic acid supplement use 1 month before pregnancy and continuing use throughout the first 3 months of pregnancy (Table 3).

Table 3. Folate indicators included in papers 1 through 4

Folate indicator	Source	Description	Categorization
Paper 1			
Folic acid supplement use	MoBa baseline and follow-up questionnaire	Use of folic acid supplements more than once a week during a month	No, yes
Periconceptional folic acid use	MoBa baseline and follow-up questionnaire	Folic acid use from 1 month before pregnancy through the first 3 months of pregnancy	No, yes
Paper 2			
Folic acid supplement use	MoBa food frequency questionnaire	Mean intake during the first 18 weeks of pregnancy	0, 1-399, ≥400 µg/day
Food folate intake	MoBa food frequency questionnaire	Mean intake during the first 18 weeks of pregnancy	Quartiles
Plasma folate	MoBa blood sample	Maternal blood samples around 18 weeks of gestation	Quartiles
Papers 3 & 4			
Folic acid and multivitamin supplement use	MBRN notification form	Regular use before and/or any time during pregnancy	No, yes

Dietary folate intake (Paper 2)

Information on dietary food intake and supplement use in MoBa was also collected using a semi-quantitative food frequency questionnaire (FFQ). The FFQ consisted of 263 questions about 255 food items and was designed to capture dietary habits and intake of dietary supplements during the first 4-5 months of pregnancy (114, 115). The FFQ has been thoroughly validated (116).

Participants reported their food intake by selecting mean frequencies of intake of each food item from never to several times monthly, weekly, or daily (Appendix 5). In addition, global questions were asked regarding the weekly/monthly intake of hot

meals, fruits and vegetables. If the total number of detailed items deviated from the number given in the global question, the global questions were used as scaling factors (115). For instance, if a woman reported that she had chicken/turkey for dinner twice a week in the global question, but the total number of hot meals with chicken/turkey was 3 in the detailed questions, intake of each chicken/turkey item was scaled down to a total of 2. In the same way, if the answer to the global question was 3 and the total number of chicken/turkey meals was 2, the amount was scaled up. However, the total number of hot meals was finally scaled down to a maximum of 365 per year. Scaling was also applied to spreads on bread relative to the reported number of bread slices. Energy and nutrient intakes (amount/day) were calculated with the use of FoodCalc (www.ibt.ku.dk/jesper/foodcalc) and the Norwegian food composition table (www.matportalen.no/matvaretabellen).

Dietary supplement use was reported by participants either from a predefined list of the most commonly used supplements or by detailed textual descriptions (Appendix 6). The frequency of supplement use ranged from never to 7 times per week, and the quantity was reported by the number of tablets, capsules, or spoons. The folic acid content in the dietary supplements was obtained from a database that included more than 1000 different dietary supplements (115). A data program connected to the database read all food supplements recorded by the women. The database was continuously updated with new supplements throughout the study period.

In statistical analyses, food folate and total folate intake from both food and supplements were analyzed as continuous variables or as relative cut-offs by dividing the distribution of folate intake into quartile exposures (Table 3); folic acid supplement use was divided into 3 categories: 0, 1-399, and ≥400 µg/day.

Plasma folate status (Paper 2)

Concentrations of plasma folate and total homocysteine were assessed from maternal blood samples collected at the time of the routine ultrasound examination at their local hospital. The median gestational age for blood sampling was 18 weeks.

Blood samples (non-fasting) used for the preparation of plasma were collected into ethylenediaminetetraacetic (EDTA) tubes, centrifuged within 30 minutes after collection, and placed in the hospitals' refrigerator (4 °C). They were shipped by mail overnight to the Biobank of MoBa. On the day of receipt, which was usually 1-2 days after blood donation, EDTA plasma were aliquoted onto polypropylene micro-titre plates (300 µL per well, 96 well format), sealed with heat-sealing foil sheets, and stored in freezer at -80 °C. A description of the blood sample collection and laboratory methods is found elsewhere (117).

Plasma folate concentration was measured by microbiological assay, using a chloramphenicol resistant strain of *Lactobacillus casei* (118). The assay determines biologically active folate species, predominantly 5-methylTHF, and has a coefficient of variation that corresponds to 4 percent within day and 5 percent between days, at population median (www.bevital.no). The sample handling did not involve addition of ascorbic acid. Plasma total homocysteine concentration, which included both free and protein-bound fractions of homocysteine, was determined using a mass spectrometry method (119).

In statistical analyses, plasma folate and plasma homocysteine were analyzed as continuous variables or as relative cut-offs by dividing the distribution of plasma concentrations into quartile exposures (Table 3).

Vitamin supplement use (Papers 3 and 4)

Information on vitamin supplement intake in MBRN was collected during hospitalization at the time of birth and was recorded into the notification form using check boxes. Information on supplement use included questions on regular use of folic acid supplements before or during pregnancy and regular use of multivitamin supplements before or during pregnancy (Appendix 7). Information on dosage, frequency or exact duration of folic acid use was not recorded. Vitamin supplement use was classified as use of folic acid and/or multivitamin supplements before or any time during pregnancy (Table 3). We also categorized the women by time-period of

vitamin use (i.e., both before and during pregnancy, during pregnancy only, and before pregnancy only), and by supplement type (i.e., multivitamin alone, folic acid alone, and both folic acid and multivitamin).

3.3 Pregnancy outcomes and complications

Information on pregnancy outcomes and complications were obtained from the MBRN. I here provide a description of the main outcome variables used in the various papers (Table 4).

Gestational age was calculated in completed weeks or days based on second trimester ultrasound measurements. If ultrasound measurements were missing (about 2.8 percent of births), gestational age was based on the date of last reported menstrual period. Overall, gestational age information was missing for around 0.8 percent of births. Preterm delivery was defined as <37 completed weeks of gestation (Table 4).

Infant birth weight, head circumference, and crown-heel length were measured by the attending health personnel immediately after birth and used as markers of infant birth size. Low birth weight was defined as infant birth weight <2500 g (Table 4).

Infant SGA was used as a crude marker of intrauterine growth, and was constructed from national reference weights from gestational weeks 20 through 44, classified as infant birth weight below the 10th percentile within strata of infant gender and gestational age (120) (Table 4). To be consistent with the reference population, SGA was obtained by using gestational age based on the last menstrual period date.

Placental abruption is defined as the premature separation of a normally situated placenta (121). The condition was recorded in MBRN using a check box or open text coded according to the *International Classification of Diseases,* Tenth Revision (ICD-10). Placental abruption is usually a clinical diagnosis based on prenatal signs and symptoms, like antepartum haemorrhage, uterine pain or tenderness, or fetal distress.

According to current practice in Norwegian hospitals, the diagnostic criteria are extended to include retro-placental impression or blood clot behind the placenta.

Other categorical outcomes were birth defects (any defect and major defects), stillbirth, and neonatal death. All birth defects were recorded using textual descriptions and coded according to the ICD-10. Stillbirth was defined as the death of an infant before or during delivery. Neonatal death was defined as infant death within the first month of life. Preeclampsia and gestational diabetes was recorded using check boxes or textual descriptions coded according to ICD-10. Apgar score was assessed 5 minutes after delivery.

Table 4. Pregnancy outcomes for papers 1 through 4

Pregnancy outcome	Categorization	Paper			
		1	2	3	4
Gestational age[a]	<37, ≥37 weeks	Yes	Yes	Yes	Yes
Infant birth weight[a]	<2500, ≥2500 g		Yes		Yes
Head circumference[a]			Yes		Yes
Crown-heel length[a]			Yes		Yes
Infant SGA	<10th, ≥10th percentile		Yes		
Placental abruption	No, yes			Yes	Yes
Preeclampsia	No, yes			Yes	Yes
Gestational diabetes	No, yes				Yes
Birth defects	No, yes				Yes
Stillbirth	No, yes				Yes
Neonatal death	No, yes				Yes
Apgar score[a]					Yes

[a] Continuous variables.

3.4 Other relevant variables

Covariates were abstracted from both MoBa questionnaires and MBRN. Inclusion of covariates and potential confounders in statistical models is discussed later in Chapter 5.2. All variables are shown in Appendix 8 and described in the papers. Notably, maternal smoking in MoBa was assessed both from self-reported data and from cotinine in blood plasma. Cotinine is the primary metabolite of nicotine and is considered a sensitive marker of tobacco exposure (122). Plasma cotinine was determined by using a mass spectrometry method (119), and a woman was considered as an active smoker if her cotinine concentration was ≥85 nmol/L (122, 123).

3.5 Statistical analyses

All statistical analyses were performed using SAS (Statistical Analysis System) version 9.2 software for Windows (SAS Institute, Inc., Cary, North Carolina) and R version 2.8.1 (www.r-project.org/). All P values were 2-sided, and values <0.05 were considered statistically significant.

Data were described as relative frequencies or means together with a variability measure, such as standard error, standard deviation, or confidence interval (CI). Potential confounding factors and relevant effect modification variables were chosen *a priori* based on information in previous literature. Inclusions of confounders and interaction terms in statistical models are discussed in more detail in Chapter 5.2 and 5.4, respectively.

Associations between dichotomous outcomes and exposures were assessed using simple and multiple log-binomial regression models. In these models, relative risk (RR) and odds ratio (OR) and their corresponding 95 percent CIs were obtained using a log-link or logit-link function, respectively. We estimated OR when the prevalence of the study outcome was low (papers 2, 3, and 4) and RR when the prevalence of the

study outcome was frequent (paper 1). The latter was done because ORs for common conditions are not good approximations of RR (124).

Paper 1: All analyses were adjusted for maternal age, marital status, maternal education, parity, and year of birth. Prevalence of folic acid use was adjusted by using a direct standardization method. This was performed in a 3-stage operation, thoroughly described in the paper. If a woman participated with several pregnancies, only the first was included in the analyses. Predictors were assessed by using log-link binomial regression and RR. A test for linear trend or group difference in prevalence of supplement use over the levels of a predictor was calculated by incorporating the predictor as a linear variable or as a categorized variable in the models (Wald test), respectively. This paper also used a log-link binomial regression model for repeated measures to examine a group-by-time effect on supplement use from 2 months before pregnancy through the eighth month of pregnancy. That is, we tested whether the patterns of folic acid use were different for, for example, epileptics and non-epileptics.

Paper 2: All analyses were adjusted for maternal age, marital status, maternal education, parity, prepregnancy body mass index (BMI), and plasma cotinine. Additional adjustments were done for specific analyses. Food folate and plasma folate values were divided into quartiles, whereas folic acid supplement use was divided into 3 categories: 0, 1-399, ≥400 µg/day. Associations were assessed by using linear regression models for continuous outcomes (i.e., gestational age, infant birth weight, head-circumference, and crown-heel length) and logistic regression models for dichotomous outcomes (i.e., SGA). A test for linear trend or group difference in means or prevalence over the exposure levels was calculated by incorporating the exposure as a linear variable or as a categorized variable in the models (Wald test), respectively. Correlation between pairs of measures was assessed by Spearman correlation coefficient. A dose-response association between continuous exposure data and SGA was examined using generalized additive logistic regression models

(GAMs) (125). GAMs are readily used to explore and visualize dose-response relations, especially when relations are non-linear.

Paper 3: All analyses were adjusted for maternal age, marital status, parity, self-reported smoking, pregestational diabetes, and chronic hypertension. Associations of folic acid supplement use and multivitamin use with placental abruption were assessed using logistic regression models. Because analyses included women with several pregnancies during the study period 1999-2004, the potential intra-individual correlation between 2 or more pregnancies from the same woman was taken into account by using generalized estimating equations for logistic regression models (126). We also used Cox regression analysis with a binary time-varying covariate (gestational age <37weeks or ≥37 weeks) to test whether hazard ratios for vitamin use were significantly stronger for preterm abruption than for term abruption. Effect modification of the vitamin - abruption association by smoking and preeclampsia was explored by stratification and by including the relevant interaction term in regression models, evaluated by likelihood ratio tests.

Paper 4: Bias in prevalence of 23 exposure and outcome variables was measured as the ratio of relative frequencies, whereas bias in exposure-outcome associations of 8 selected relations was measured as the ratio of ORs. Bias in continuous variables was measured by calculating the difference in means. Women who had more than 1 pregnancy during the study period and who agreed to MoBa participation in more than 1 of the pregnancies were defined as unique participants with each pregnancy. Logistic regression analyses were performed with adjustment for maternal age, marital status, parity, and self-reported smoking at end of pregnancy. To quantify the uncertainty of the ratio of adjusted ORs, we computed 95 percent CIs, with correction for the inter-dependency between MoBa participants and the total population. This was achieved using a nonparametric bootstrap method (127), thoroughly described in the paper. The nonparametric bootstrap method was also used to obtain corrected CIs for ratios of relative frequencies and differences in means.

3.6 Ethical approval

Informed consent was obtained from each participant before inclusion in the MoBa study. All studies were approved by the Regional Committee for Medical and Health Research Ethics and by the Norwegian Data Inspectorate.

4. Discussion of specific results

In this section, main results of papers 1 through 4 are summarized, discussed, and compared with results of previous studies.

4.1 Patterns of folic acid supplement use

In Norway, women who may become pregnant are recommended to take 400 µg folic acid daily from 1 month before pregnancy through the first 2-3 months of pregnancy (95). The recommendations for folic acid supplement use are intended to prevent spina bifida and related NTDs during early pregnancy.

In paper 1, we examined the patterns and predictors of maternal supplemental folic acid use from 2 months before pregnancy through the eighth month of pregnancy. A woman was defined as a folic acid user if she reported use of supplements containing folic acid more than once a week during a 4-week period. In Norway, the usual prenatal folic acid tablets contain 400 µg, whereas most multivitamin tablets include 200-400 µg folic acid. The study was based on 22,500 pregnant women in MoBa in 2000-2003, who had returned 2 self-administered questionnaires around gestational weeks 18 and 30, and who were registered with births in MBRN.

Our study showed that 16,116 of the participants (71.6 percent) had taken folic acid-containing supplements at some time before or during pregnancy. Of these, more than 70 percent had started use after becoming pregnant, the majority during the first and second month of pregnancy. Only 2303 women (10.2 percent) had used folic acid-containing supplements regularly from 1 month before pregnancy throughout the 3 first months of pregnancy. Of these, 81 percent had used supplements containing folic acid daily. Overall, 8 of 10 women reported that they had planned their pregnancy.

A low prevalence of periconceptional folic acid supplement use during 2000-2003 was also seen in MBRN (128) and in a study of pregnant women in Oslo (99), and is

consistent with a Norwegian survey of fertile women in 2000, which showed that only 8.5 percent of the women knew that the critical period for folic acid supplement to reduce the risk of neural tube defect is before and early in pregnancy (97). A low prevalence of folic acid supplement also agrees with reports from other countries where recommendations have been issued (10, 129). In the Danish National Birth Cohort, full compliance with recommendations (>80 percent of 400 µg/day from 4 weeks before conception until the 6 first weeks of pregnancy) was reported to be 14 percent during 2000-2002 (130), which is comparable to our data.

Since the folic acid supplement recommendations were issued in 1998, the use of folic acid-containing supplements before and during early pregnancy has increased substantially in Norway (Table 5), indicating that folic acid promotion among fertile women is efficient, at least to some degree. In 2006, the prevalence of periconceptional folic acid use in MoBa was at the same level as that reported in the United States, Netherlands, and Canada (131-134). In the United States, folic acid use in the periconceptional period increased from 15 percent in 1988 to 40 percent in 2002, which is a slower increase than that seen in Norway (Table 5).

Table 5. Folic acid supplement use according to month of pregnancy and year of birth, MoBa, 2000-2006

Year of birth	No. of subjects	2 months before pregnancy	1 month before pregnancy	First month of pregnancy	Second month of pregnancy
	n		%		
2000	1923	7	11	16	31
2001	3698	9	12	19	35
2002	8042	10	13	21	36
2003	11,734	14	19	30	46
2004	12,518	26	31	48	65
2005	14,622	30	35	53	70
2006	13,451	33	39	58	75

We found that about 80 percent of the women planned their pregnancy. Hence, our findings of low use before pregnancy and high use during pregnancy strongly indicate that most women during 2000-2003 were not familiar with the recommended timing of folic acid supplement use. Thus, it may still be feasible through effective health campaigns to raise the use prior to pregnancy even more. In order to do this, future campaigns should focus on the timing, as well as on certain risk groups, which I will discuss in the next section.

We used data from MoBa during 2000-2003, ranging from 2 months before pregnancy to approximately the eighth month of pregnancy. Apart from a smaller study from the United States (135), ours is the first large-scale study to have examined detailed folic acid patterns over the course of pregnancy. Previous studies on folic acid awareness and use among fertile women have mainly focused on the time period around neural tube closure and not beyond the first 3 months of pregnancy. Exploring the use of folic acid supplements beyond the first 3 months is also of relevance, because folic acid use during second and third trimester may have a beneficial effect on other adverse birth outcomes besides NTDs (11).

4.2 Predictors of folic acid supplement use

Periconceptional folic acid supplement use is usually defined as regular use of 400 µg folic acid pills from 1 month before pregnancy throughout the 3 first months of pregnancy. Because we did not have information on the exact folic acid dosage and frequency of use, we extended the definition in paper 1 to include women who used any supplements containing folic acid more than once a week throughout the periconceptional period (Appendices 3 and 4).

We found that maternal age, marital status, smoking, maternal and paternal education, maternal income, parity, pregnancy planning, and fertility treatment, were all independent predictors of periconceptional folic acid use. Consistent with other studies (131, 134-137) lower education, living alone, and unplanned pregnancy were

the strongest determinants for not taking folic acid in our study. However, those with unplanned pregnancies quickly caught up folic acid supplement use during early pregnancy, unlike lower educated and single women (Figure 2).
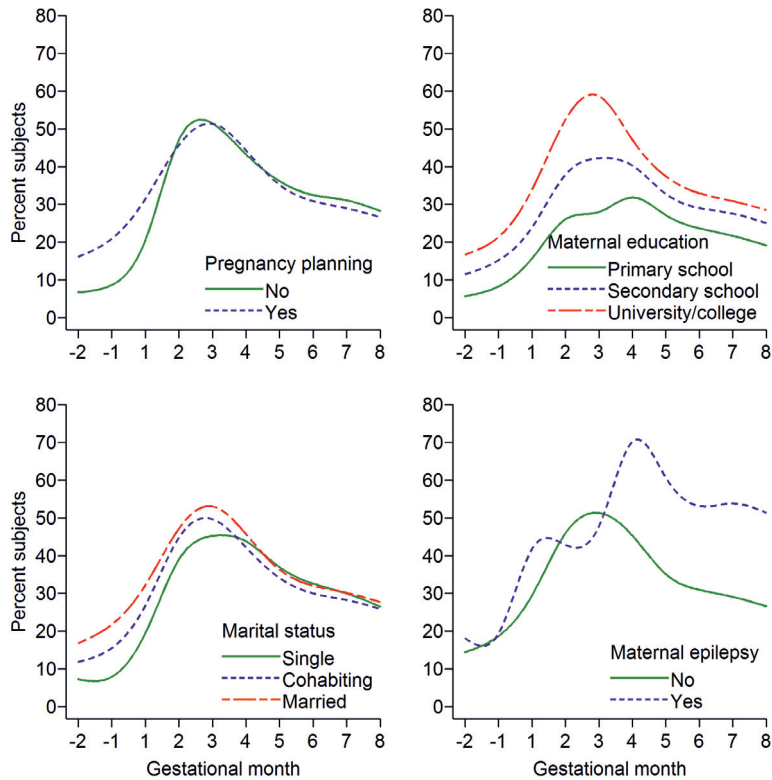
Figure 2. Monthly prevalence of folic acid supplement use according to maternal characteristics, MoBa, 2000-2003

We also found that high parity was a marked predictor for not taking folic acid. This is a particularly important issue, because earlier studies have reported that women who have recently undergone a viable pregnancy may become folate deplete in the next pregnancy, especially if the inter-pregnancy interval is short (33). Similarly, smokers appeared to consume significantly less folic acid supplements than non-smokers. According to recent studies, smokers tend to have lower blood folate levels than non-smokers regardless of dietary folate intake (138-140). Increased

periconceptional folate intake in smokers and women with high parity may thus have relevance for NTDs and perhaps other pregnancy outcomes as well.

Surprisingly, women who suffered from epilepsy did not use folic acid supplements more than others during the periconceptional period (Figure 2), despite the increased need of folate for patients on certain AEDs (95). Although the use of folic acid was low for this group during the periconceptional period, it increased substantially from 3 months of pregnancy and was twice as high as folic acid supplement use for those without the disease throughout the eighth month of pregnancy. One explanation for the increased use late during pregnancy may be that folic acid information was provided by their doctors at their first antenatal visit, which usually occurs within 2-3 months of pregnancy.

Our data suggest that recommendations on periconceptional folic acid use are not efficient for women with low socioeconomic status (i.e., lower education, low income, and single mothers). The use of supplement in lower educated and single mothers tended to be extremely low both before and during pregnancy (Figure 2). Some authors have speculated whether these groups may be less conscious about general health issues (134). Another possibility may be that the health campaigns designed to promote folic acid supplement use do not reach these women, perhaps because they do not use information channels where information on folic acid can be found (e.g., health magazines or health programs on TV).

4.3 Folate indicators and infant birth size

Over the years, numerous studies have examined the relation between infant birth size and folate status (11). Most of these studies, however, are small and often confined to only 1 folate indicator and a few birth size parameters. Furthermore, varying findings have been obtained, possibly due to methodological issues, such as the timing of folate exposures, lack of control for confounding factors, and a variation in population characteristics and nutritional baseline status.

In paper 2, we examined the relation between infant birth size and several maternal folate indicators, measured during the second trimester. The folate indicators included food folate intake, folic acid supplement use, plasma folate, and plasma total homocysteine. The birth size parameters were gestational age, infant birth weight, head circumference, crown-heel length, and SGA. The paper also assessed the association of maternal smoking, which is an established risk factor for small infant birth size. The study was based on 2934 women with singleton births in MoBa during 2002-2003, who had donated a blood sample, returned the baseline questionnaire and FFQ around gestational week 18, and who were registered with births in the MBRN.

About 53 percent of all women had a total dietary folate intake of 400 μg or more per day. Four women had a total folate intake ≤100 μg/day, and 342 women (11.7 percent) had a total folate intake ≤200 μg/day. We found that food folate, supplemental folic acid, total dietary folate intake, plasma folate, and plasma total homocysteine were not statically significantly related to gestational age, infant birth weight, head circumference, or crown-heel length. There was a tendency for increased SGA risk at lower folate levels, but analyses yielded insignificant associations, possibly due to low number of individuals with low folate intake.

Because most studies differ from each other with respect to population characteristics, nutritional baseline status, and the timing and methods used to collect exposures and outcomes data, a direct comparison of our study with previous studies is difficult. Nevertheless, our results agree with those in a longitudinal study of healthy Japanese women that found no association between folate intake during pregnancy and various continuous birth size parameters (91). These authors, however, found an inverse relation between total homocysteine and infant birth weight in the third trimester, but this was not reflected in the analyses of serum or red blood cell folate.

Our results are in contrast with a recent study from the Netherlands which demonstrated that periconceptional folic acid supplementation was associated with higher placental and infant birth weight, and decreased risks of low birth weight and SGA (<5th percentile) (86). The authors, however, did not include information on

several folate indicators, which would have added value to the interpretation of these findings. Recently, a study from the United Kingdom reported a higher incidence of SGA (<10th percentile) at low folate intake during late pregnancy in an adolescent population (140). These authors also showed a greater risk of SGA with lower concentrations of serum and red blood cell folate during the third trimester.

In our study, mean supplemental folic acid intake was higher among women who were married or cohabiting, had higher education and lower parity, had lower prepregnancy BMI, and did not smoke. However, we found no significant difference in mean food folate intake according to these variables, not even according to smoking. This is an interesting finding, suggesting that supplement users are not more motivated than nonusers to consume more folate from foods. Mean food folate intake by supplement groups is shown in Table 6.

Table 6. Mean intake of food folate among supplement users and nonusers, MoBa, 2002-2003

Food folate	Nonusers (0 µg/day)	Users (1-399 µg/day)	Users (≥400 µg/day)
		µg/day	
Mean	264	271	270
Median	249	258	259
5th percentile	140	146	152
95th percentile	429	437	424

The correlation between plasma folate and food folate intake was relatively low in our study ($r = 0.07$, $P <0.001$). Nevertheless, this finding is consistent with an intervention study from 2000 (141), which showed that increase in foods naturally rich in folates (400 µg/day) was a relatively ineffective means of increasing red blood cell folate status in women compared with equivalent intakes of folic acid-fortified food. There may be several reasons for this. First, the folates from foods (polyglutamates) are more unstable for cooking and storage and are less bioavailable

than synthetic folic acid (monoglutamates) (1). Second, we used non-fasting blood samples from MoBa, which may have added pre-analytical variation in plasma folate values and thus attenuated correlation with food folate. Third, the MoBa FFQ measured the mean food folate intake during the first 4-5 months of pregnancy, which may not entirely represent the intake at the time of blood sampling.

Consistent with previous literature (142, 143), we found that second trimester smoking strongly influenced infant birth weight and SGA, suggesting that smoking remains a strong risk factor for infant birth size in our population. We also found that plasma cotinine was significantly inversely correlated with plasma folate ($r = -0.15$). This correlation was almost unchanged after adjustment for total dietary intake ($r = -0.12$), suggesting that smoking alone affects blood folate status. This is also consistent with other studies that have examined blood folate status and smoking (138, 139).

4.4 Vitamin use and placental abruption

Placental abruption is a complication of pregnancy that causes a premature separation of the placenta from the uterus wall (121). This usually occurs in the third trimester, often leading to blood loss that can be life-threatening for both mother and child. In Norway it affects 4 in 1000 pregnancies, resulting in about 200-240 cases each year.

Placental abruption is associated with a number of factors, including placental abruptions in previous pregnancies (144), maternal smoking (145), and vitamin deficiency (65). Although folate and other vitamins measured in blood have been associated with placental abruption (62, 64, 65, 74), few studies have addressed whether supplemental folic acid or multivitamin use during pregnancy can reduce occurrence of this complication.

In paper 3, we examined whether supplemental folic acid or multivitamin use during pregnancy can reduce occurrence of placental abruption. The study was based on 280,127 singleton deliveries recorded in MBRN in 1999-2004. We found that intake

of folic acid and other vitamin supplements before and during pregnancy statistically significantly reduced the risk for placental abruption by up to 30 percent. Associations between vitamin supplement use and placental abruption were strongest for women using both folic acid and multivitamin supplements (OR = 0.68; 95 percent CI: 0.56, 0.83), followed by multivitamins alone (OR = 0.72; 95 percent CI: 0.57, 0.91) and folic acid alone (OR = 0.81; 95 percent CI: 0.68, 0.98), suggesting that other vitamins beside folate may have a role in preventing placental abruption. Our data further showed that the vitamin - abruption associations were stronger when the placental abruption was preterm (<37 weeks gestation), suggesting a different pathogenesis of placental abruptions before term in comparison with those at term.

Our findings are supported by a recent study from Canada in 2009 that found mild to severe placental abnormalities due to low folate intake in pregnant mice (146). In that study, half of the folate-deficient placenta examined had severe disorders, including some degree of separation of the placenta from the maternal decidua, which is similar to placental abruption in humans. Our findings also agree with previous results from a meta-analysis (65) and other reports (61-64) showing that maternal folate deficiency and elevated plasma total homocysteine are associated with increased risk of placental abruption. Our results, however, are in contrast with those from a recent report from Canada, which showed that food fortification with folic acid had no impact on the prevalence of placental abruption (147).

The present paper is the first large study that examines an association of supplemental folic acid and multivitamin use with placental abruption, comprising 1070 abruptions among 280,127 singleton deliveries. Although the associations are moderate, this study suggests that current recommendations to assure adequate intake of folate before and during early pregnancy to prevent NTDs may protect against placental abruption as well. The absolute risk of placental abruption among users and nonusers of folic acid supplements alone were 32 and 44 per 10,000 births (see Table 3 in the paper), respectively. Thus, if all pregnant women took folic acid supplements on a

regular basis before and/or during pregnancy, 12 in 10,000 abruptions could be avoided in Norway each year (corresponds to 60-70 placental abruptions each year).

4.5 Self-selection and bias

To evaluate potential bias due to self-selection in MoBa, we studied differences in prevalence estimates and association measures between MoBa participants and all women giving birth in Norway. Women who agreed to participate in MoBa (43.5 percent of invited; $n = 73,579$) were compared with all women giving birth in Norway ($n = 398,849$) using data from the population-based MBRN in 2000-2006.

Compared to all women giving birth in Norway, participating women were more often older, primiparous, non-smokers, and vitamin supplement users. MoBa participants also tended to experience less stillbirths and neonatal deaths than those who were not included in the study. Despite differences in various exposure and outcome variables, we found no statistically significant relative deviation in 8 studied exposure-outcome associations if they were based on the MoBa participants instead of the total population. For instance, the risk (measured in OR) for infant low birth weight (<2500 g) for smokers was 1.77 (95 percent CI: 1.53, 2.05) among MoBa participants and 1.85 (95 percent CI: 1.76, 1.94) among all women giving birth in Norway, relative to non-smokers.

Many authors have discussed the consequences of self-selection and low participation rate in prospective studies (148-150). Consistent with our findings, bias is often found in the prevalence of exposures and outcomes, but to a less extent in effect estimates of exposure-outcome associations. In general, this is not surprising as data on the exposures in prospective studies are usually collected before the outcome under study. However, both the risk factor and the outcome may sometimes be strongly associated with some underlying factor that is also associated with participation. For that reason, researchers should always consider the possibility of selection bias related to their exposures and outcomes.

A selective sample which is not representative of the target population may have advantages, because it may provide better control for confounding factors that vary in the general population (151). For instance, in studies of causality, one usually wants to select study groups that are homogenous and highly comparable with respect to certain characteristics, rather than to make the study sample representative (151). A weakness of a selective sample, however, is that the number of exposed may be lower/higher than that found in the target population, resulting in a lower statistical power of effect estimates. In a case-cohort study based on MoBa, one therefore always should calculate the sample size needed based on the study's exposure and outcome prevalence in the full cohort.

5. Methodological considerations

We performed observational research based on existing data from MoBa and MBRN. When interpreting results from observational data one should recognize both the methodological strengths and limitations of the data.

5.1 Study design

All papers in this thesis comprised sufficient statistical power to detect moderate to strong estimates of associations between exposures and outcomes. Although inferences of some sub-group analyses were problematic due to small numbers, precision of the estimates was generally not a problem when the inferences were made from the overall results.

The major strength of MoBa is the prospective design in which folate indicators are measured before the outcome under study. Well-designed prospective designs are generally robust for systematic errors and are considered to have high internal validity. However, as described in the previous chapter, the possibility of systematic errors, such as selection bias, should still always be discussed in light of the exposure and outcome analyzed, especially if the response rate is low. Furthermore, the study participants of MoBa are not representative of the total Norwegian pregnant population. This could influence the external validity of study results.

The major strength of MBRN is the compulsory registration of all live births and stillbirths in Norway. The full coverage of births makes it possible to produce association measures with high precision and high external validity. Unless data are not extensively missing in the registry, selection bias is not considered a problem. Nevertheless, some exposure data in MBRN, including prenatal vitamin supplement use, are collected during hospitalization around the time of birth. This retrospective design could be problematic by introducing information bias in studies.

Timing of exposures

Timing of folate exposures may be important when investigating pregnancy outcomes (152). If information on exposures is collected at an irrelevant time point in pregnancy, no association may be found. For instance, there will be no causal association between third-trimester folic acid supplement use and NTDs, as the neural tube closes already within the first month of pregnancy.

During pregnancy, there is an increase in the red cell mass, enlargement of uterus, and a growth of the placenta and infant (153). Thus, adequate folate intake with respect to normal cell growth and fetal development is probably equally relevant at any stage of pregnancy. In paper 2, plasma folate was measured around 18 weeks of gestation and dietary folate intake during the first 4-5 months of pregnancy. However, there were no significant associations of these variables with gestational age, infant birth weight, head-circumference, crown-heel length or SGA, which are applied, but simple, indicators of fetal growth and development (154).

A low folate intake prior to pregnancy has been associated with a number of placental abnormalities in mice, including placental abruption (146). Thus, maternal folate status may be particularly important at conception and during the trophoblast invasion of the human decidua. In paper 3, women were classified according to folic acid and/or multivitamin use before and during pregnancy, during pregnancy only, and before pregnancy only. The magnitude of the associations was similar for all time-windows. However, time-windows largely overlapped and, therefore, no firm conclusions could be drawn regarding timing and supplement use in this study.

Patterns of exposures

Also patterns of folate exposures during pregnancy may be important when investigating pregnancy outcomes (152). Although intake of food folate appears relatively stable over the course of pregnancy (90), some studies, including ours (paper 1) have shown significant changes in supplement use during pregnancy, with

more use earlier, and less use during the third trimester (135). Likewise, several studies have shown that blood folate concentrations decline significantly during gestation, partly due to hemodilution and increased folate excretion (30-32). Thus, studying different time-windows of supplement use or blood folate status in relation to birth outcomes may produce different results, which further confirm the need for a longitudinal design of folate exposures and pregnancy outcomes.

5.2 Internal validity

Internal validity refers to the extent to which scientific inference can be drawn for the population under study (151). In order to obtain high internal validity in observational research, case and comparison groups should be selected and compared in a manner that reduces systematic errors. The 3 main sources of systematic errors that may influence internal validity are selection bias, information bias, and confounding (151).

Selection bias

Selection bias is a systematic error that results from the methods used to include study participants and from factors that influence study participation (151). Selection bias usually refers to a situation when risk estimates of exposure-outcome associations of the responding population differ from those eligible for the study, including the non-participants.

We have shown in paper 4 that self-selection in MoBa at recruitment does not necessarily lead to biased association measures, even though exposures are underrepresented or overrepresented in the cohort study. Furthermore, in papers 1 and 2, subjects were selected according to short-term follow-up criteria (Table 2). The total loss of subjects at follow-up was only 11 and 16 percent, respectively, and may therefore not have influenced exposure-outcome associations in these papers. In paper 1, we also provided prevalence estimates of folic acid supplement use from 2 months before pregnancy through the eighth month of pregnancy. Because women who used

folic acid and multivitamin supplement use were markedly overrepresented in MoBa (paper 4; relative deviation 31-43 percent), these prevalence estimates cannot directly be used to describe the total pregnant population in Norway.

In paper 3, we used data from MBRN to examine associations of folic acid and multivitamin use with the risk of placental abruption. In this study, we excluded subjects that had no information on vitamin supplement use (n = 55,972). Notably, the information on vitamin use was missing for 17 percent of non-abruptions (55,569/ 334,626) and 27 percent of placental abruptions (403/1473). To examine potential selection bias due to missing data, we compared women with and without data on vitamin use for women with or without placental abruption, according to maternal characteristics (see Table 4 in paper 3). This comparison showed essentially no differences in characteristics between the groups, suggesting no selection bias. Because placental abruption is a serious condition that needs immediate medical attention, the registration of vitamin supplement use may not have been completed in many placental abruption cases. This may explain the larger percent of missing vitamin data among women with placental abruption in MBRN.

Information bias

Information bias is a systematic error that results from incorrect measurement or classification of the exposure or outcome variable under study (151). Information bias is usually grouped into 2 types: differential and non-differential misclassification.

Differential misclassification
Misclassification of exposure or outcome variables that depend on the value of the other is referred to as differential misclassification and can either overestimate or underestimate the risk measure. An example of this is recall bias, a situation in which for instance dietary intake of certain food items is better recalled by women who had a baby with birth defect than those who did not. In the following, I discuss differential misclassification with regard to papers 2 and 3.

In paper 2, all folate exposures were collected from self-administered questionnaires and from blood samples during second trimester (from MoBa), whereas information on birth outcomes, such as infant birth weight and SGA, were based on hospital records at the time of birth (from MBRN). By using independent data sources for exposures and outcomes in a prospective design like this, we have no reason to suspect that any misclassification of the various folate indicators and birth outcomes could have been differential.

In paper 3, information on prenatal vitamin supplement use for women with and without placental abruption was collected retrospectively during hospitalization at the time of birth and not during the earlier stages of pregnancy. Although such study design may be susceptible for recall bias in general, we do not suspect that the use of vitamin supplements has been recalled differently among mothers with and without placental abruptions. Vitamin supplements are taken voluntarily, often on a regular basis, to obtain increased health benefits, like prevention of NTDs, and are thus not easily forgotten.

Non-differential misclassification

Misclassification of exposure or outcome variables that do not depend on the value of the other is referred to as non-differential misclassification. If the exposure and outcome variables are dichotomous, non-differential misclassification leads to attenuated risk estimates. With more than 2 levels, exaggerated risk estimates may occur (151). In the following, I discuss possibilities of non-differential misclassification for the papers 1 through 3.

Supplement use: In the MoBa baseline and follow-up questionnaires (Appendix 3 and 4), women were asked to report in detail which vitamins/minerals that were taken according to the label on their supplement containers. This is a rather time-consuming and complex procedure, which may have caused underreporting of the use of supplements. In MBRN (Appendix 7), information was collected during hospitalization by the attending health personnel. Information on supplement use may

have been underreported or registered incorrectly by the hospitals. Also women themselves may have underreported supplement use or provided incorrect information.

Dietary folate intake: In the MoBa FFQ (Appendix 5), which assessed dietary habits during the first 4-5 months of pregnancy, food intake were reported by stating how often women consumed numerous food items from never to several times monthly, weekly, or daily. Similarly, the frequency of vitamin supplement use ranged from never to 7 times per week, and the quantity was reported by the number of tablets, capsules, or spoons. The validity of estimated dietary folate intake, thus, relies on the participants' ability to recall exactly what they have consumed during the first part of pregnancy.

Folate degradation: EDTA plasma samples were transported from the hospitals to the BioBank of MoBa by ordinary mail, usually 1-2 days after blood sampling. Under such conditions, folate concentration in EDTA plasma is likely to decrease, especially in the absence of a stabilizer like ascorbic acid (155, 156). However, with the strong inverse correlation between plasma folate and total homocysteine (Spearman $r = -0.48$), which is stable at room temperature, such degradation likely occurs at a constant rate over the whole distribution of plasma folate. This suggests that remaining biologically active folate still reflects overall folate status and that the relative difference in folate concentration between women may have been upheld.

Infant birth size: Infant birth weight, head circumference, and crown-heel length were measured by the attending health personnel immediately after birth, whereas gestational age was based on second trimester ultrasound measurements or the date of the last menstrual period. Any biases in these measures are likely to be random and not systematic. Infant SGA was calculated based on weights from a national reference population (120). Since MoBa participants delivered infants with higher mean birth weight compared with all women giving birth in Norway, this resulted in a lower percentage of SGA in MoBa.

Placental abruption: Placental abruption in MBRN has not been validated against hospital charts. Thus, whether the report of placental abruption was subject to some underreporting or overreporting in MBRN is not known. Nevertheless, the prevalence of placental abruption among singleton pregnancies in MBRN was 0.38 percent, which is similar to what have been reported in other North-European countries. Placental abruption was reported in 0.41-0.51 percent among singleton births in Sweden (145, 157), and in 0.42 percent among all births in Finland (158).

Confounding

Confounding is a situation in which a third factor explains all or part of the observed association between the exposure and the outcome under study (151). In order to be a confounding factor (confounder), the factor must be associated with the exposure in the source population, as well as being a risk factor for the outcome, and it must not be an intermediate step in the causal pathway between the exposure and outcome (151). To reduce the effect of a possible confounder, the observed association should be corrected for its effect.

A problem in epidemiologic research is over-adjustment bias and unnecessary adjustment (159). Over-adjustment bias can be defined as control for an intermediate variable on a causal path from exposure to outcome. Unnecessary adjustment can be defined as control for a variable that does not affect the causal relation between exposure and outcome but may affect its precision. Opposed to unnecessary adjustment, lack of important data can lead to unknown or unmeasured confounding. Several approaches may be used when evaluating confounding in observational research. In papers 1 through 4, potential confounding factors were chosen *a priori* and examined according to their influence on the risk estimates.

Paper 1: Potential confounding factors were evaluated in multiple regression analyses. Variables that had little or no influence on study results were not included in the final statistical models. The remaining factors for adjustment were maternal age,

marital status, maternal education, parity, and year of delivery. The RRs of predictors for supplement use were attenuated after adjustment.

Paper 2: Potential confounding factors were evaluated in unadjusted logistic regression models. Variables that were significantly associated with both folic acid supplement use and SGA were included in the final statistical models: maternal age, marital status, maternal education, parity, prepregnancy BMI, and smoking. For dietary analyses, additional adjustment was made for folic acid supplement use and total energy intake (160). Furthermore, because dietary habits and plasma vitamins change during pregnancy, the analyses were adjusted for gestational age at blood sampling and at the time of returning the FFQ. Overall, adjustment had little impact on the observed results.

Paper 3: Potential confounding factors were maternal age, marital status, parity, smoking, pregestational diabetes, and chronic hypertension. All variables were associated with vitamin supplement use and placental abruption in both unadjusted and adjusted logistic regression analyses. We had no information on prepregnancy BMI, educational level, and total energy intake, which could have resulted in unmeasured confounding. However, adjustment for existing variables had little influence on the risk estimates, suggesting that other variables have to be strongly associated with both outcome and exposure in order to be important confounders. In Scandinavia, educational level or prepregnancy BMI appear not to be strongly associated with placental abruption (157, 161, 162).

Paper 4: It was important to demonstrate that ORs for 8 studied exposure-outcome associations were similar for participants and for the total population before and after adjustment for potential confounders. We selected a number of potential confounders that we knew had some influence on the associations: maternal age, marital status, parity and smoking at the end of pregnancy. For simplicity, all adjustment variables were included in each of the exposure-outcome analyses, although they were not evaluated for their actual confounding ability. The adjustment procedures had some

impact on the association estimates, but they did not alter systematically the deviations in ORs between the 2 populations.

5.3 External validity

External validity (or generalizability) refers to the ability to generalize results and conclusions from the population under study to people outside that population (151). To achieve high external validity of prevalence estimates, we need our study population to be representative of the larger population. However, representativeness is generally not a prerequisite when the scientific goal is to report generalizable effect estimates. For instance, in paper 4, we found no statistically significant relative deviation in ORs for eight studied exposure-outcome associations if they were based on the MoBa participants instead of the total population of Norwegian pregnant women, even though several exposures and outcomes were overrepresented or underrepresented in MoBa.

Paper 1: We included women who had completed a baseline questionnaire and a follow-up questionnaire at week 30. Thus, the patterns and predictors of folic acid supplement use may only be generalized to those who delivered after 30 weeks of gestation. Furthermore, since the percentage of vitamin users is markedly overrepresented in MoBa (as shown in paper 4) the prevalence estimates in paper 1 cannot be generalized to the total Norwegian pregnant population. The internal exposure-outcome associations, on the other hand, were shown in paper 4 to be generalizable to Norwegian pregnant women in general, suggesting that the predictors in paper 1 also are generalizable to pregnant women outside MoBa.

Paper 2: We only included women with singleton pregnancies. Furthermore, using limits from Norwegian guidelines for nutritional intake (163), we discovered that only 4 women had a daily folate intake below the lower limit (100 μg/day), and only 342 women (11.7 percent) had a daily folate intake below the limit of estimated average requirements (200 μg/day). Although folate-deficient women are present in our study

sample, the prevalence of folate-deficient women may be too small in order to generalize our null-findings in this paper to the total pregnant population in Norway.

Paper 3: We used data from the population-based MBRN. The study may therefore be generalizable to all women giving birth in Norway, except for those who had multiple pregnancies. Multiple pregnancies were excluded for 12,944 women (3.7 percent), because they might involve complex confounding mechanisms that differ from those in singleton gestations (164).

5.4 Effect modification

Effect modification is a situation in which the effect measure of an exposure on the outcome varies according to the levels of a third variable (151). While confounding is considered a confusion of effects, effect modification is a property of the effect under study. In this thesis, effect modification was evaluated by stratification and the inclusion of interaction terms in multiple logistic regression models.

Paper 2: We examined whether the effects of several folate indicators on SGA were different for smokers and non-smokers, but found no statistical evidence of effect modification, possibly due to low numbers of smokers and SGA cases (i.e., 49 women). The rationale for testing smoking as an effect modifier is that smokers tend to have less blood folate than non-smokers, independent of folate intake (140). Since smoking is associated with SGA, we hypothesized that folate could be more beneficial for smokers than non-smokers.

Paper 3: We examined effect modification of the association between vitamin use and placental abruption by smoking and preeclampsia, but differences in ORs between strata of these variables were statistically insignificant. Evaluating preeclampsia as an effect-modifier was initially done to rule out that the effect of folic acid supplement use on placental abruption was mediated through preeclampsia. Preeclampsia is a risk factor for placental abruption (121) and have been associated with vitamin use (165).

6. Conclusions and implications

In this thesis, we have used existing data from MoBa and MBRN to study several folate indicators, including folic acid supplement use, and their relation to birth outcomes in Norwegian pregnant women. The following conclusions can be drawn for each question:

1. **To which extent do Norwegian pregnant women follow the recommendations regarding periconceptional folic acid supplement use?**

   We found that 72 percent of the participants in 2000-2003 had used folic acid-containing supplements at some time before or during pregnancy. Of these, more than 70 percent had started use after becoming pregnant, the majority during the first and second month of pregnancy. Only 10 percent of participants had taken folic acid supplements regularly from 1 month before pregnancy throughout the first 3 months of pregnancy. The low use before conception indicates that women are unaware of the recommended timing of use.

2. **Which maternal factors predict periconceptional folic acid supplement use among Norwegian pregnant women?**

   Maternal age, marital status, smoking, maternal and paternal education, maternal income, parity, pregnancy planning, and fertility treatment, were all independent predictors of regular folic acid supplement use during the periconceptional period. Low education, living alone, and unplanned pregnancy were the strongest determinants for not taking folic acid. Notably, women who suffered from epilepsy did not use folic acid more often than others during the periconceptional period, despite the special recommendation for women on AED.

3. **Is infant birth size associated with second trimester folate status in Norwegian pregnant women?**

Second trimester folate indicators in terms of food folate, supplemental folic acid, total dietary folate intake, plasma folate, and plasma total homocysteine did not statistically influence the size of the infant, measured as gestational age, infant birth weight, head circumference, crown-heel length, or SGA. The absence of statistically significant associations might be explained by an adequate intake of folate from diet and supplements in most women in our study population.

4. Is periconceptional folic acid and multivitamin supplement use associated with placental abruption in Norway?

Use of folic acid and other vitamin supplements before and during pregnancy reduced the risk for placental abruption by up to 30 percent. The association between vitamin supplement use and placental abruption was strongest for women using both folic acid and multivitamin supplements.

5. Are prevalence estimates of exposure and outcome variables in MoBa different from those of all women giving birth in Norway?

Significant relative deviations in prevalence estimates were found in most of the 23 exposure and outcome variables studied, including vitamin use and smoking. Ten of the variables deviated with more than 15 percent.

6. Are exposure-outcome associations in MoBa different from those of all women giving birth in Norway?

We found no statistically significant relative deviation in OR estimates for 8 studied exposure-outcome associations if they were based on the MoBa participants instead of the total population.

In summary, many women use supplements containing folic acid in connection with pregnancy, but start too late with respect to NTD prevention. Still, increased intake of folate during pregnancy may have benefits. Our finding of a risk reduction in

placental abruption with folic acid supplementation supports findings from the 1960s and 1970s. If proven in trials, current recommendations to assure adequate intake of folate before and during early pregnancy to prevent NTDs may be extended to placental abruption as well.

7. Perspectives

7.1 Future studies

A general limitation of paper 2 is that information on folate exposures was collected only once during pregnancy. Particularly, we did not have information on plasma folate and folate consumption during late pregnancy, when the growth of the infant is greatest (166). Because plasma folate and growth rate may change during pregnancy, the cross-sectional nature of our study can lead to some exposure misclassification (152), which again may lead to some underestimation of possible effects of these folate indicators. To avoid this problem, future studies of infant birth size should include longitudinal data on vitamin exposures.

Studies have reported that women who have recently undergone a viable pregnancy may become folate deplete in the next pregnancy, especially if the inter-pregnancy interval is short (33). We further showed that high parity was a marked predictor for not taking folic acid. Hence, both inter-pregnancy interval and parity seem to be important covariates, and should always be included in future studies of pregnancy outcomes and folate values. Furthermore, because the utilization of folate depends on other B-vitamins in the folate metabolism, future studies of inter-pregnancy interval and nutritional status should also include these and related metabolites to obtain a more complete picture of one-carbon metabolism in pregnancy.

MoBa is a long-term follow-up study that includes more than 100,000 pregnancies between 1999 and 2008. The participants received 3 self-administered questionnaires by mail during the pregnancy, and another 4 during the infant's early childhood. In addition, the women were asked to give 1 blood sample during pregnancy and 1 blood sample immediately after delivery. Further questionnaires are planned during childhood and adolescence. Because not all women are able or willing to follow up on each event, some selection bias due to loss to follow-up can be introduced. A study of

loss to follow-up is thus needed, especially after delivery during which response rate for questionnaires are lower than that before pregnancy (113).

7.2 Recent developments

Recent studies of non-pregnant individuals, including randomized trials, indicate that high folate doses may have potentially adverse effects by accelerating the growth of premalignant and malignant lesions (106, 107). Furthermore, it is suggested that unmetabolized folic acid in blood is associated with reduced natural killer cell cytotoxicity, a component of the nonspecific immune response (167).

Recent scientific developments have raised concerns regarding folic acid supplement use also in pregnant women. A Norwegian study from 2009 provided some evidence that supplemental folic acid during pregnancy may increase the risk of certain infant respiratory diseases in early childhood (168). This finding was supported by a study from Australia (169), which showed that folic acid taken in supplement form in late pregnancy was associated with an increased risk of childhood asthma at 3.5 years (RR = 1.26; 95 percent CI: 1.08, 1.43) and with persistent asthma (RR = 1.32; 95 percent CI: 1.03, 1.69).

Although randomized controlled trials have proven the beneficial effect of folate supplementation on the prevention of NTDs we do not have the full picture of potential adverse effects of folic acid supplement use in pregnant women. Therefore, it is also important to monitor possible adverse effects of prenatal folic acid supplementation, especially in countries were also mandatory and voluntary food fortification with folic acid are practiced.

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Appendix   I

Notification form for MBRN (1998 -)

MFR — Melding om avsluttet svangerskap etter 12. uke – Fødsel, dødfødsel, spontanabort
Se utfyllingsinstruks for blanketten på baksiden

Sosial- og helsedirektoratet

A – Sivile opplysninger

Institusjonsnr:
Institusjonsnavn

Fødsel utenfor institusjon:
- Hjemme, planlagt
- Hjemme, ikke planlagt
- Under transport
- Annet sted

Mors fulle navn og adresse

Mors sivilstatus
- Gift
- Samboer
- Ugift/enslig
- Skilt/separert/enke
- Annet

Pikenavn (etternavn):

Slektskap mellom barnets foreldre?
- Nei
- Ja   Hvis ja, hvorledes:

Mors bokommune

Fars fødselsdato   Fars fulle navn

Mors fødselsnr:

B – Om svangerskap og mors helse

Siste menstr. 1. blødn.dag
- Sikker
- Usikker

Mors tidligere svangerskap/fødte
Levende-fødte | Dødfødte (24. uke og over) | Spontanabort/Dødfødte (12.–23. uke) | Spontanaborter (under 12. uke)

Ultralyd utført?
- Nei   UL
- Ja   termin:

Annen prenatal diagnostikk?
- Nei
- Ja, angi type:

Patologiske funn ved prenatal diagnostikk?
- Nei
- Ja, hvis bekreftet – spesifiser

Spesielle forhold før svangerskapet:
- Intet spesielt
- Astma
- Allergi
- Tidligere sectio
- Res. urinveisinfeksjon
- Kronisk nyresykdom
- Kronisk hypertensjon
- Reumatoid artritt
- Hjertesykom
- Epilepsi
- Diabetes type 1
- Diabetes type 2
- Annet, spesifiser i «B»

Regelmessig kosttilskudd:
- Nei   Før sv.sk.   I sv.sk.
- Multivitaminer
- Folat/Folsyre
- Annet, spesifiser i «B»

Spesifisering av forhold før eller under svangerskapet:
B

Spesielle forhold under svangerskapet:
- Intet spesielt
- Blødning < 13 uke
- Blødning 13–28 uke
- Blødning > 28 uke
- Glukosuri
- Svangerskapsdiabetes
- Hypertensjon alene
- Preeklampsi lett
- Preeklampsi alvorlig
- Preeklampsi før 34. uke
- HELLP syndrom
- Eklampsi
- Hb < 9.0 g/dl
- Hb > 13.5 g/dl
- Trombose, beh.
- Infeksjon, spes. i «B»

Legemidler i svangerskapet:
- Nei
- Ja – spesifiser i «B»

Røyking og yrke
Forutsetter mors samtykke – se rettledning på baksiden
- Skriftlig orientering gitt til mor
- Samtykker ikke for røykeoppl.

Røykte mor ved sv.sk. begynnelse?
- Nei   Daglig   Av og til   Ant. sig. dagl.:

– ved sv.sk. avslutning?
- Nei   Daglig   Av og til   Ant. sig. dagl.:

Mors yrke

- Samtykker ikke for yrkesoppl.
- Ikke yrkesaktiv
- Yrkesaktiv heltid
- Yrkesaktiv deltid

Mors yrke

Bransje:

C – Om fødselen

Leie/presentasjon:
- Normal bakhode
- Sete
- Tverrleie
- Avvikende hodefødsel
- Annet, spesifiser i «C»

Fødselsstart:
- Spontan
- Indusert
- Sectio

Ev. induksjonsmetode:
- Prostaglandin
- Oxytocin
- Amniotomi
- Annet, spesifiser i «C»

Indikasjon for inngrep og/eller induksjon
- Komplikasjoner som beskrevet nedenfor
- Fostermisdannelser
- Overtid
- Annet, spesifiser i «C»

Inngrep/tiltak
- Ingen
- Utskj. tang, hodeleie
- Annen tang, hodeleie
- Vakuumekstraktor
- Episitomi

Fremhj. ved setefødsel:
- Vanlig fremhjelp
- Uttrekning
- Tang på etterk. hode

Sectio:
Var sectio planlagt før fødsel?
- Nei   Ja
- Utført som elektiv sectio
- Utført som akutt sectio

Spesifikasjon av forhold ved fødselen/andre komplikasjoner
C

Komplikasjoner
- Ingen
- Vannavg. 12–24 timer
- Vannavg. > 24 timer
- Mekaniske misforhold
- Vanskelig skulderforløsning

- Placenta previa
- Abruptio placentae
- Perinealruptur (grad 1-2)
- Sphincterruptur (gr. 3-4)

- Blød.> 1500 ml, transf.
- Blødning 500–1500 ml
- Eklampsi under fødsel
- Navlesnorfremfall

- Truende intrauterin asfyksi
- Risvekkelse, stimulert
- Langsom fremgang
- Uterus atoni   Annet:

Anestesi/analgesi:
- Ingen
- Lystgass
- Petidin

- Epidural
- Spinal

- Pudendal
- Infiltrasjon

- Paracervical blokk
- Narkose   Annet:

Placenta:
- Normal
- Hinnerester
- Ufullstendig
- Infarkter

- Koagler
- Utskraping
- Manuell uthenting
- Placenta-vekt:

Navlesnor:
- Normal
- Velamentøst feste
- Marginalt feste
- Karanomalier

- Omslyng rundt hals
- Annet omslyng
- Ekte knute
- Navlesnor-lengde:

Fostervann:
- Normal
- Polyhydramnion
- Oligohydramnion

- Misfarget
- Stinkende, infisert
- Blodtilblandet

Komplikasjoner hos mor etter fødsel
- Intet spesielt
- Feber > 38.5˚
- Trombose
- Eklampsi post partum

- Mor overflyttet
- Mor intensivbeh.
- Sepsis
- Annet, spesifiser

D – Om barnet

Fødselsdato   **Klokken**

Pluralitet
- Enkeltfødsel
- Flerfødsel   Nr.   Av totalt

For flerfødsel:

Kjønn
- Gutt
- Pike
Ved tvil spesifiser i «D»
For dødfødte: Usikkert kjønn

Barnets vekt:

Hode-omkrets:

Total lengde:

Eventuelt sete-issemål:

Apgar score:
1 min

5 min

Barnet var:
- Levendefødt

For dødfødte:
- Dødfødt/sp.abort
Oppgi dødsårsak i «D»
- Død før fødsel
- Død under fødselen
- Ukjent dødstidpunkt

For dødfødte, oppgi også
- Død før innkomst
- Død etter innkomst

Levendefødt, død innen 24 timer
Livet varte: Timer   Min.

Død senere (dato):   **Klokken**

Overfl. barneavd.
- Nei   Ja   Dato:
Overfl. til

Indikasjon for overflytting:
- Respirasjonsproblem
- Prematur

- Medfødte misd.
- Perinatale infeksjoner
- Annet, spesifiser

Neonatale diagn.: (Fylles ut av lege/pediater)
- Intet spesielt
- Hypoglyk. (< 2 mmol/l)
- Medf. anemi (Hb < 13.5 g/dl)
- Hofteleddsdyspl. beh. m/pute
- Intrakraniell blødning

- Transit. tachypnoe
- Resp. distress syndr.
- Aspirasjonssyndrom
- Neonatale kramper

- Cerebral irritasjon
- Cerebral depresjon
- Abstinens

- Konjunktivitt beh.
- Navle./hudinf. beh.
- Perinat. inf. bakterielle
- Perinat. inf. andre

- Fract. claviculae
- Annen fraktur
- Facialisparese
- Plexusskade

Behandlingskoder:
- Systemisk antibiotika
- Respiratorbeh.
- CPAP beh.

Icterus behandlet:
- Lysbehandlet
- Utskifting
Årsak:

Tegn til medfødte misdannelser:
- Nei   Ja

Spesifikasjon av skader, neonatale diagnoser og medfødte misdannelser – utfylles av lege
D

- AB0 uforlik.
- RH immunisering
- Fysiologisk
- Annen årsak

IS-1002   23011. 07.06. Andvord Grafisk.

- Kryss av hvis skjema er oppfølgingsskjema

Jordmor v/fødsel:

Jordmor v/utskrivning:

Lege:

Lege barsel/barneavd:

Utskrivningsdato
Mor:
Barn:

Protokollnr.: ___ / ___

Appendix **II**

Data collection in MoBa

Time schedule of all MoBa questionnaires and blood samples by January 2009.

Time	Questionnaire (Q)[a]			Blood sample (B)[b]		
	Mother	Infant	Father	Mother	Infant	Father
Pregnancy week 18	**Q1**[b]		Q1	**B1**		B1
Pregnancy week 18/22[c]	**Q2**[b]					
Pregnancy week 30	**Q3**					
Delivery				B2	B1[d]	
Child age 6 months		Q4				
Child age 18 months		Q5				
Child age 36 years		Q6				
Child age 7 years		Q7				

[a] Q, questionnaire; B, blood sample. Numbers followed by Q or B indicate the order of questionnaires and blood samples. Bold face indicates data sources used in this thesis.

[b] Q1 is the baseline questionnaire and Q2 is the FFQ.

[c] Q2 was sent out at gestational week 22 from April 2004.

[d] B1 from the child at delivery was taken from the umbilical cord.

Webpage link for pdf-versions of the entire questionnaires:
www.fhi.no/artikler/?id=51491

Appendix **III**

Supplement use at baseline in MoBa

Page 7 of the baseline questionnaire returned around gestational week 18.

The complete questionnaire can be found at: **www.fhi.no/artikler/?id=51491**.

40. Do you have a congenital malformation/birth defect?
☐ No
☐ Yes
41. If yes, which? _____

42. Do your gums bleed when you brush your teeth at present?
☐ No, rarely or never
☐ Yes, sometimes
☐ Yes, often
☐ Yes, almost always

43. If you had diabetes before you became pregnant, what was your last long-term blood sugar level (HbA1c) before you became pregnant?
☐ Less than 7.5
☐ 7.5 - 12
☐ More than 12
☐ Don't Know

Other medicines

44. Have you used other medication not previously mentioned? If yes, which and when did you take them?

Use of medication during pregnancy weeks

Name of medication (e.g. Valium, Rohypnol, Paracetamol)	Last 6 months before pregnancy	0-4	5-8	9-12	13+	Number of days used
_____	☐	☐	☐	☐	☐	
_____	☐	☐	☐	☐	☐	
_____	☐	☐	☐	☐	☐	
_____	☐	☐	☐	☐	☐	
_____	☐	☐	☐	☐	☐	

Vitamins, minerals and dietary supplements

45. Do you take vitamins, minerals or other dietary supplements?
☐ No (proceed to question 49)
☐ Yes

46. If yes, fill in the table below for the vitamins and minerals found in the contents list on the vitamin package/bottle. (For instance, if you have taken cod liver oil for the last six months before becoming pregnant, enter a cross for each period under "When" (i.e. 7 crosses) and enter a cross in "Daily" under "How often").

	When did you take the supplements?							In this period how often did you take this?		
	Last 6 months before pregnancy			During pregnancy						
	26-9 weeks	8-5 weeks	4-0 weeks	0-4 weeks	5-8 weeks	9-12 weeks	13+ weeks	Daily	4-6 times a week	1-3 times a week
1 Folate/folic acid	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
2 Vitamin B1 (Thiamine)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
3 Vitamin B2 (Riboflavin)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
4 Vitamin B6 (Pyridoxine)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
5 Vitamin B12	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
6 Niacin	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
7 Pantothenic acid	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
8 Biotin	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
9 Vitamin C	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
10 Vitamin A	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
11 Vitamin D	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
12 Vitamin E	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
13 Iron	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
14 Calcium	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
15 Iodine	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
16 Zinc	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
17 Selenium	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
18 Copper	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
19 Chromium	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
20 Magnesium	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
21 Cod liver oil	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
22 Omega-3 fatty acid	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Appendix **IV**

Supplement use at follow-up in MoBa

Page 7 of the follow-up questionnaire returned around gestational week 30.

The complete questionnaire can be found at: **www.fhi.no/artikler/?id=51491**.

54. Have you taken other medication after the 13th week of pregnancy not previously mentioned, for example, sleeping tablets or sedatives? Give the name, when and how many days altogether the medication was taken **for.** (This applies to all types of medicines including alternative and herbal remedies, both regular and occasional use. Do not include vitamins and nutritional supplements as these are discussed elsewhere.)

Name of medication (e.g. Valium, Rohypnol, Paracetamol)	Use of medication in week of pregnancy 13–16 17–20 21–24 25–28 29+	No. of days taken
_____	☐ ☐ ☐ ☐ ☐	☐☐☐
_____	☐ ☐ ☐ ☐ ☐	☐☐☐
_____	☐ ☐ ☐ ☐ ☐	☐☐☐
_____	☐ ☐ ☐ ☐ ☐	☐☐☐

55. During this pregnancy have you been involved in an accident or been injured *(e.g. traffic accident, fall, hit in the stomach)?*
☐ No
☐ Yes

56. If yes, in which week of pregnancy?
☐☐

Vitamins, minerals and dietary supplements

57. Have you taken vitamins, minerals or other nutritional supplements after the 13th week of pregnancy?
☐ No (go to question 61)
☐ Yes
If you take supplements, please find the package/bottle.

58. Fill in the table below for the vitamins and minerals found on the vitamin package/bottle. Fill in when and approximately how often you have taken them.

	Week of pregnancy supplement taken? 13–16 17–20 21–24 25–28 29+	How often did you take this supplement? Daily / 4-6 times a week / 1-3 times a week
1 Folate/folic acid	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
2 Vitamin B1 (Thiamine)	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
3 Vitamin B2 (Riboflavin)	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
4 Vitamin B6 (Pyridoxine)	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
5 Vitamin B12	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
6 Niacin	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
7 Pantothenic acid	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
8 Biotin	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
9 Vitamin C	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
10 Vitamin A	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
11 Vitamin D	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
12 Vitamin E	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
13 Iron	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
14 Calcium	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
15 Iodine	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
16 Zinc	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
17 Selenium	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
18 Copper	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
19 Chromium	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
20 Magnesium	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
21 Cod liver oil	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐
22 Omega-3 fatty acid	☐ ☐ ☐ ☐ ☐	☐ ☐ ☐

Appendix **V**

Dietary habits in MoBa (FFQ)

Page 4 of the FFQ returned around gestational week 18 until April 2004. The complete questionnaire can be found at: **www.fhi.no/artikler/?id=51491**.

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5. Do you use butter/ margarine with your sandwiches?

☐ **Yes**   ☐ **No** (go to question 8)

6. If you use butter /margarine, on how many sandwiches on average and what kind do you use?

Number of slices

Type of butter/ margarine	13+	9-12	8	7	6	5	4	3	2	1	5-6	3-4	1-2	0
				per day							or	per week		
1. Butter/ /Bremyk	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
2. Hard margarine (Per, Melange)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
3. "Brelett"	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
4. Soft margarine (Soft, Vita, Olivero etc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
5. Light margarine (Soft light, Vita lett etc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

7. How much butter/ margarine do you use on you sandwiches?

Plenty ☐   **Medium** ☐   **Minimum** ☐

Cheese/ meat cold cuts/ fish/ spreads

8. How often do you have the following food items on your sandwiches?

Number of slices with this food item

Cheese	6+	5	4	3	2	1	5-6	3-4	1-2	3	2	1	0
			per day				or	per week		or	per month		
1. Whey cheese goat milk, regular	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
2. Whey cheese low fat, spread goat milk	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
3. Hard cheese, cream cheese	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
4. Hard cheese, cream cheese, low fat	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
5. Blue cheese (Camembert, Norzola etc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
6. Other kinds of cheese	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fish													
7. Roe spread	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
8. Mackerel/sardine in tomato sauce	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
9. Sardine in oil	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
10. Smoked salmon/trout/mackerel	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
11. Herring, pickled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
12. Shrimp, Northern	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
13. Crab	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
14. Tuna	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
15. Svolværpostei (spread of fish liver/roe)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
16. Other kinds of fish	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Meat													
17. Low fat cold cuts (ham, roast beef etc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
18. Medium fat cold cuts of lamb, calf etc.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
19. Salami, Swedish sausage etc.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
20. Cold cuts of turkey, chicken	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
21. Liver paste	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
22. Other kinds of meat	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Appendix **VI**

Dietary supplements in MoBa (FFQ)

Page 14 of the FFQ returned around gestational week 18 until April 2004. The complete questionnaire can be found at: **www.fhi.no/artikler/?id=51491**.

Supplements

39. Do you use, or have you used supplements during this pregnancy? ☐Yes ☐No

40. If yes, we ask you to name and quantify the supplements you have used/are using
(ts = teaspoon, bs = dessert spoon, ss = tablespoon)

Liquid supplements	Times per week									Amount		
	7	6	5	4	3	2	1	<1	0	1 ts	1bs	1ss
1. Cod liver oil	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
2. Omega-3 cod liver oil	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
3. Sanasol	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
4. Biovit	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
5. Liquid iron mixture (Floradix etc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other liquid supplements												
6. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
7.Corporation:												
8. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
9.Corporation:												

Capsules/tablets	Times per week									Number(s) at a time			
	7	6	5	4	3	2	1	<1	0	1	2	3	4+
10. Cod liver capsules	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
11. Cod liver capsules without A and D-vitamins	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
12. Vitaplex	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
13. Kostpluss/nyco plus multi	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
14. Nyco plus folic acid 0,4 mg	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
15.Spektro (Solaray)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
16. Hemofer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
17. Duroferon duretter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other supplements													
18. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
19. Corporation:													
20. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
21. Corporation:													
22. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
23. Corporation:													
24. Name:	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
25. Corporation:													

Please remember to fill out the date on page 2!

Thank you for your time and help!

Appendix **VII**

Vitamin supplement use in MBRN

Section of the MBRN form filled out during hospitalization at the time of birth.

Regular use of dietary supplement

		Before preg.	During preg.
☐ No			
Multivitamins		☐	☐
Folate/Folic acid		☐	☐

The complete notification form is shown in Appendix 1.

Appendix **VIII**

Additional table

Covariates and confounders included in papers 1 through 4.

Data source	Categorization	Paper 1	2	3	4
MBRN					
Year of delivery	Years	Yes	Yes	Yes	Yes
Maternal age	<25, 25-34, ≥35 years	Yes	Yes	Yes	Yes
Marital status	Married, cohabitation, single	Yes	Yes	Yes	Yes
Parity	0, 1, 2, ≥3 previous births	Yes	Yes	Yes	Yes
Maternal diseases[a]	No, yes (before pregnancy)	Yes		Yes	Yes
Previous stillbirths	0, 1, ≥2 previous births	Yes			Yes
Maternal smoking[b]	No, yes (daily or occasional)			Yes	Yes
In-vitro fertilization	No, yes				Yes
Medication use	No, yes (during pregnancy)				Yes
MoBa					
Maternal income	Low, medium, high (NOK)	Yes			
Maternal education	0-9, 10-12, ≥13 years	Yes	Yes		
Paternal education	0-9, 10-12, ≥13 years	Yes			
Prepregnancy BMI	<18.5, 18.5-24.9, 25-29.9, ≥30 kg/m^2	Yes[c]	Yes		
Pregnancy planning	No, yes	Yes			
In-vitro fertilization	No, yes	Yes			
Ovarian stimulation	No, yes	Yes			
Maternal smoking	No, yes (daily or occasional)	Yes[d]	Yes[e]		
Total energy intake	Quartiles in µg/day		Yes		

[a] Up to 8 diseases, including epilepsy, chronic hypertension, and pregestational diabetes.

[b] Any smoking during pregnancy (paper 3) or smoking at end of pregnancy (paper 4).

[c] The 2 first categories were pooled, i.e. prepregnancy BMI <25.0 kg/m^2 (paper 1).

[d] Smoking during pregnancy was assessed by the baseline and the follow-up questionnaires.

[e] Smoking during pregnancy was assessed by plasma cotinine around gestational week 18 (paper 2).