Folic acid supplement use in Danish Pregnancy Planners: The impact on menstrual cycle and fecundability

PhD dissertation

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Dedication

This dissertation is dedicated to all the women who participated in the Snart-Gravid study. Without your contributions and willingness to share your personal information this PhD would not be at all.

> *"…I have additional information for the study. As my husband has been posted in Paris for six months, we have only seen each other at the weekends. But we have only been together a few times during ovulation…"*
>
> *"…Unfortunately I cannot continue my participation in the study, because my husband and I are getting divorced and therefore, we have stopped trying to have more children…"*
>
> *"…I'm pregnant and I have a question. Before I knew that I was pregnant, I have been drinking beer. Could that harm the fetus? And what about vitamins and other things related to pregnancy?…"*

The quotations derive from women who participated in the Snart-Gravid study from 2007 through 2011. The quotations exemplify some of the challenges in the study and what some women experienced while participating in the study. Furthermore, it is a reminder that behind all the numbers and statistics presented in this report, are real women trying to have a baby.

Acknowledgements

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Heidi Theresa Ørum Cueto, 2014

Dissertation papers

Paper I

Predictors of Preconceptional Folic Acid or Multivitamin Supplement use: A Cross-sectional Study of Danish Pregnancy Planners.
Heidi T. Cueto, Anders H. Riis, Elizabeth E. Hatch, Lauren A. Wise, Kenneth J. Rothman, Ellen M. Mikkelsen. Clin. Epi. 2012; 4: 259-265.

Paper II

Folic Acid Supplement Use and Menstrual Cycle Characteristics: A Cross-sectional Study of Danish Pregnancy Planners.
Heidi T. Cueto, Anders H. Riis, Elizabeth E. Hatch, Lauren A. Wise, Kenneth J. Rothman, Henrik T. Sørensen, Ellen M. Mikkelsen. (S*ubmitted*)

Paper III

Folic Acid Supplementation and Fecundability: A Danish Prospective Cohort Study.
Heidi T. Cueto, Anders H. Riis, Elizabeth E. Hatch, Lauren A. Wise, Kenneth J. Rothman, Henrik T. Sørensen, Ellen M. Mikkelsen. (*Conditionally accepted in Eur. J. Clin. Nutr.)*

Abbreviations

CI	Confidence interval
CPR	Central Personal Registry
DNBC	Danish National Birth Cohort
FA	Folic acid
FR	Fecundability ratio
LMP	Last menstrual period
MTHFR	Methylentetrahydrofolate reductase
MV	Multivitamin
NTD	Neural tube defect
OR	Odds ratio
PP	Prevalence proportion
PPD	Prevalence proportion difference
SAM	S-adenosylmethionine
THF	Tetrahydrofolate
TTP	Time to pregnancy

Contents

1. Introduction

Folates are essential in DNA synthesis and various methylation reactions,[1-3] and are required for cell division and maintenance.[4] The preventive effect of folic acid (FA) on neural tube defects (NTDs) is considered one of the most important nutritional discoveries in the last four decades.[4] NTDs are severe congenital abnormalities of the nervous system that occur when the neural tube fails to close during early embryonic development.[4;5] The breakthrough emerged in the early 1990s, when the Medical Research Council's Vitamin Study (1991)[6] reported a 70% reduced risk of NTD recurrence with daily supplementation with 4.0 mg of FA, and the Hungarian randomized trial (1992)[7] demonstrated that periconceptional supplementation with multivitamins (MVs) containing 800 µg FA resulted in a 90% reduction in the first occurrence of an NTD.

These groundbreaking findings led numerous countries, including Denmark, to introduce periconceptional FA recommendations to women of childbearing age.[8;9;10] Since 1997, the Danish National Board of Health has recommended that women who are planning to conceive are supplemented with 400 µg FA per day until the 12th week of gestation.[11] Although NTDs are relatively rare birth outcomes, with an estimated rate of 76 cases per year in Denmark,[12] they are severe malformations. In addition, estimations imply that 75% of all NTDs could be prevented if 70% of women planning a pregnancy complied with the FA recommendation.[12] Despite campaigns promoting the FA recommendation in Denmark in 1999 and 2001,[13] compliance with the recommendation has reportedly been poor.[14;15] Between 2000 and 2002, the proportion of FA compliers among 22,000 participants in the Danish National Birth Cohort (DNBC) increased from 14% to only 22%, and thus remained low even after the campaigns.[13;15] Interestingly, a recently published study reported that 67% of 258 pregnant women attending their initial midwifery visit at Aalborg Midwifery Center during 2011-2012 complied with the preconceptional FA recommendation, indicating an increase in compliance during the 10-year time period.[14] Although national surveys of dietary habits also indicate insufficient dietary folate intake among women of childbearing age,[16] Denmark, like all other European countries,[17] has not introduced a mandatory national program to fortify flour and grain with FA.[5;18;19] Because FA supplementation is a simple and useful preventive

method, it is important to monitor compliance to evaluate to what extent the Danish population benefits from the knowledge that FA can prevent NTDs.

In vitro studies have also shown that folate may be involved in many aspects of human reproduction, including menstrual cycle hormones.[18;20;21] Among women of reproductive age, normal menstrual cycle function depends on the complex interaction between the hypothalamic-pituitary-ovarian axis and endogenous hormones.[22] Alterations in these hormones may affect menstrual cycle patterns, such as cycle regularity and length. In addition, women with abnormal menstrual cycles may have higher risk of infertility.[23] It is estimated that 10-15% of all couples experience infertility,[24] defined as the failure to conceive after 1 year of regular unprotected intercourse with the same partner, at some time during their reproductive life.[21;25] Therefore, epidemiological studies on the relation between preconceptional FA supplementation and menstrual cycle function and fecundability (defined as the probability of conceiving during one menstrual cycle assuming regular unprotected intercourse[25]) are warranted.

In this dissertation, we aimed to examine the prevalence of preconceptional FA supplement use, obtained through either single FA tablets or MVs, and to identify socio-demographic, lifestyle, reproductive, and medical predictors of such use. We also examined the association between FA supplement use and both menstrual cycle characteristics and fecundability among Danish pregnancy planners enrolled from 2007 to 2011 in the internet-based 'Snart-Gravid' prospective cohort study.[26-28]

2. Background

The background section briefly describes the properties of folate and FA, followed by definitions of fertility and fecundability and brief descriptions of the established risk factors of female infertility, and the role of FA in relation to follicular development. This section closes with a review of the existing literature leading to the aims of the dissertation.

2.1. Folic acid

Folate is the generic term for a group of compounds that includes FA and derivatives that have nutritional properties similar to FA.[29] Folate belongs to the group of water-soluble B vitamins and is one of the 13 known essential vitamins; this vitamin cannot be synthesized de novo in humans. Dietary sources are green leafy vegetables, beans, citrus fruits and juices, liver, and wheat.[4;29] Naturally occurring folates primarily consist of polyglutamate species, while synthetic FA, which can be consumed additionally as supplements and in fortified food, consists of the monoglutamate form only.[30] FA is absorbed directly in the upper small intestine, while folates are changed into monoglutamates before absorption.[4] Intracellularly, both folate and FA are converted to the metabolically active species dihydrofolate and tetrahydrofolate (THF) to participate in cellular metabolism.[4] Folate, in the form of THF, is an essential cofactor in various one-carbon-unit transfer reactions, including the biosynthesis of purines and thymidylate, which is essential for the synthesis and repair of DNA and RNA.[3;4;18] Folates are also suppliers of methyl groups to re-methylate homocysteine into methionine. The methionine derivative S-adenosylmethionine (SAM) is the most important methyl donor in the body for the methylation of lipids and proteins as well as the epigenetic regulation of gene activity.[5;18]

The influence of folate status on megaloblastic anemia has long been recognized.[18] The preventive effects of folate on cardiovascular diseases,[31-34] depression,[35-37] and cancer[38;39] have also been studied intensively; however, results have been inconclusive. Despite scarce evidence, high folate intake appears to be protective against colon cancer[40;41] and against breast cancer.[42-45] Although the FA recommendation applies solely to the preventive effect of FA on NTD, several observational studies have demonstrated

reduced risk of other congenital abnormalities, in particular cardiovascular abnormalities, after supplementation with FA-containing MVs or FA alone.[46-49] However, FA supplementation has been shown to be less effective against congenital abnormalities among some women with epilepsy treated with antiepileptic drugs.[4] FA supplementation has also been associated with reduced risk of preterm birth,[18;50-53] low birth weight,[53;54] and miscarriage,[18;54] although results have been conflicting.

2.1.1. Folic acid and homocysteine in relation to follicular development

The adverse effects of folate deficiency and subsequent homocysteine accumulation on female reproduction include impaired DNA and RNA synthesis,[55] inflammatory cytokinin production,[20] altered nitric oxide metabolism,[20;21] oxidative stress,[56;57] elevated apoptosis,[55] and altered methylation reactions.[3;20] In addition, cross-sectional studies of women undergoing assisted reproduction have reported an association between elevated homocysteine concentration in the follicular fluid and poor oocyte maturity,[58;59] while FA supplementation has been shown to increase folate concentration and decrease homocysteine concentration in the microenvironment of the maturing oocyte.[59;60] One study also suggested that women who were homozygous or heterozygous for a common gene mutation that led to decreased 5,10-methylenetetrahydrofolate reductase (MTHFR) activity (and the subsequent accumulation of homocysteine[1;61]) exhibited reduced ovarian responsiveness to follicle-stimulation hormone and produced fewer oocytes.[62] Furthermore, two prospective follow-up studies of women with no history of infertility respectively showed that regular use of MVs containing FA decreased the risk of ovulatory infertility[63] and a diet high in synthetic FA reduced the risk of anovulatory cycles,[64] suggesting that FA may play a essential role in ovulatory function.

2.2. Fertility and fecundability

Fertility is generally accepted as a person's or a couple's biologic capacity to produce a baby, but is also used to signify the actual production of a baby.[25;65] In epidemiology, fecundability is a quantitative term that describes a couple's capacity to conceive, defined as the probability of conceiving during one menstrual cycle, assuming that the couple is having regular unprotected intercourse.[25] Mean fecundability is

approximately 20%, with wide variation among couples.[24;25] A couple's given fecundability cannot be measured directly; however, the mean fecundability for a group of couples can be measured indirectly by counting the number of cycles that it takes a couple to become pregnant.[25]

2.2.1. Risk factors that affect female infertility

One of the most prevalent causes of female infertility is ovulatory dysfunction, which are most commonly caused by polycystic ovary syndrome.[24;66] The absence of menses is usually associated with anovulation, and irregular menses are associated with reduced fecundability.[25] Although women with variable cycle length may be fertile, both long and short cycle length have been associated with reduced ability to conceive.[23;25;67-70] Tubal obstruction is another major cause of infertility, often caused by infections such as pelvis inflammatory disease or chlamydia.[21;66] Other causes, such as endometriosis, cervical mucus defects, and uterine abnormalities, have also been identified.[21] Previously published reviews imply that, approximately 25% of infertility cases are unexplained.[21;24;71] As fertility naturally decreases with age, postponing of the first pregnancy until a greater age is a strong determinant of fecundability.[72-74] There is also increasing evidence that lifestyle factors, such as smoking[75-77] and obesity,[78-80] are associated with infertility. Other factors, such as alcohol consumption,[81-86] caffeine intake,[83;85;87] and physical activity,[88;89] have also been suggested, albeit without clear evidence. In contrast, modifiable lifestyle behaviors, such as the timing and frequency of intercourse, have been shown to offset the age-related decline in fecundability.[72] Although the underlying mechanisms that connect FA supplementation to menstrual function are unclear, they are likely caused by alterations in hormonal patterns, which in turn predict the occurrence and timing of ovulation and growth of the endometrial lining. Thus, FA supplementation may be another modifiable lifestyle factor of major public health interest in relation to fertility, because it can be targeted as a preventive measure at minimal costs and with no evidence of side effects even of high intakes.[5] The upper level of recommended FA intake has been set at 1000 µg per day.[29]

2.3. Literature review

To review the existing literature regarding the prevalence of preconceptional FA supplementation and predictors of compliance with the FA recommendations, as well as the potential associations between FA supplementation and cycle characteristics and fecundability, I searched Medline for English language human studies published before November 2014.

I used Medical Subject Headings (MeSH) first creating the search builder from "AND/OR" combinations of major MeSH topics: "Folic acid", "Multivitamins", "Preconceptional", "Prenatal care", "Dietary supplements", "Vitamins", "Vitamin B", "Fertility", "Infertility", "Pregnancy", "Time to pregnancy" "Menstrual cycle", "Ovulation", and "Hormones". If the search only revealed a few hits, non-major MeSH topics were used instead.

I also searched for publications by key authors and reviewed reference lists of the selected publications for other relevant papers. Additional sources of literature were found on the webpages of official health authorities and books. There was an overwhelming amount of literature on FA, NTD, and other birth outcomes as well as on the topic 'FA fortification-strategies'. There were also many hits on FA and oxidative stress and chemistry, as well as socio-demographic and lifestyle factors associated with FA or multivitamin supplement use in the general population. Thus, the literature in this review was limited to FA or MV supplement use in relation to pregnancy. An overview of the existing literature for these topics is provided in Table 1.

Table 1. Summary of literature

Study I: Studies presenting prevalence of compliance with preconceptional folic acid (FA) recommendations and predictors of compliance				
Author, year	**Design**	**Study-population, study period, and data collection**	**Definition of compliance**	**Results – compliance and predictors**
Backhausen et al.[14] Denmark, 2014	Cross-sectional study	Pregnant women attending their initial midwifery visit at Aalborg Midwifery Center, 2011-2012 Gestational week=12-16 Questionnaire N=258	FA use during the month prior to pregnancy	Compliance: 67% (very well planned pregnancies), 35% (fairly well planned pregnancies), 3% (unplanned pregnancies) Positive association between FA use and higher degree of planned pregnancy (p<0.001)
Hoyo et al.[90] USA, 2011	Cross-sectional study	Pregnant women interviewed at two obstetrics-care facilities in Durham, North Carolina, 2005-2008 Gestational week=19-42 Either interview or questionnaire N=539	Any FA or MV supplement use during 12 month prior to pregnancy (<1,000µg per day)	Compliance: 39% (95%CI: 35-43%) No information on planned pregnancies Predictors of FA supplement use(<1,000 µg/day): Caucasian vs. African American: (OR=2.00; 95% CI: 1.18, 1.38) Married or partner vs. No partner: (OR=2.66; 95% CI: 1.59, 4.45) Tendencies towards Increased odds for older maternal age and higher education level
Chuang et al.[91] USA, 2011	Cross-sectional study	Non-pregnant women participating in the Behavioral Risk Factor Surveillance System, 2004 Telephone interview N=9,279 (for FA analyses)	Daily FA or MV supplement	Compliance: 54.3% (among women who intend pregnancy < 12 months) OR of health behaviors associated with FA use: Pregnancy intention <12 months vs. No intention: (OR=1.57; 95% CI: 1.21, 2.04) 35-44 years vs. 18-24 years: (OR=1.46; 95% CI: 1.14, 1.86) Obese vs. Non-obese: (OR=0.78; 95% CI: 0.89, 1.27) <High school vs. College: (OR=0.50; 95% CI: 0.36, 0.70) Black race vs. White race: (OR=0.67; 95% CI: 0.51, 0.89)
Forster et al.[92] Australia, 2009	Cross-sectional study	Pregnant women attending the Mercy Hospital for Women in Melbourne, 2003-2004 Gestational week=36-38 Questionnaire N=588	FA use at least 4 weeks prior to pregnancy	Compliance: 23%, 95% CI (0.20,0.27) No information about whether the estimate included unplanned pregnancies
Brough et al.[93] UK, 2009	Cross-sectional study	Pregnant women attending the Homerton Hospital antenatal clinic and two community clinics in East London, 2002-2004 Gestational week<13 In-person interview Interview N=402	Any FA use before conception until gestational week 6	Compliance: 29% No information about planned pregnancies Predictors of preconceptional FA supplement use: Ethnicity (Caucasian vs. Ethnic minority): 19% vs. 5-12% (p<0.001) Social class (social class I and II vs. Students and unwaged): 31% vs. 5% (p<0.001) Age (≥30 years vs. 16-24 years): 17% vs. 7% (p=0.01)

Author, year	Design	Study-population, study period, and data collection	Definition of compliance	Results – compliance and predictors
Inskip et al.[94] UK, 2009	Cross-sectional data in a cohort study	Non-pregnant women recruited to the Southampton Women's Survey who became pregnant within 3 months, 1998-2002Home interview N=238	Taking ≥400 µg of FA per day during the 3 months preceding the interview	Compliance: 5.5% Any FA supplement use in the 3 months before the interview: 44% No information about intention of pregnancy
Conlin et al.[95] Australia, 2006	Cross-sectional study	Pregnant women attending an antenatal clinic at three Hospitals in South Australia, 2005 Gestational week>16 Questionnaire (not clear) N=362	Full compliance: 400 µg per day during pregnancy or 6 months before; Partial compliance: Any supplementation during this time period No clear definition of the time period	Full compliance: 30% (No information on whether the estimate included unplanned pregnancies) Partial compliance 43%
Goldberg et al.[96] USA, 2006	Cross-sectional study	Pregnant callers to the California Teratogen Information Service Center, 2003-2004 Mean gestational week±SD=12.5±7.6 Telephone interview N=327	Taking a MV with FA or a single FA supplement for any number of days prior to the date of conception.	Compliance: 44% (No information on whether the estimate included unplanned pregnancies) Predictors of delayed onset of FA supplement use and no use at all (reference groups not specified): Maternal BMI≥25 kg/m^2: (OR=1.73; 95% CI: 1.05, 2.85) Maternal age<25 years: (OR=5.72; 95% CI: 2.84, 11.53) Less than high school: (OR=8.75; 95% CI: 1.99, 38.41) Unplanned pregnancy: (OR=6.90; 95% CI: 4.16, 11.45) Non-white race ethnicity: (OR=2.17; 95% CI: 0.36, 3.45) Knowledge of benefits of FA supplementation: (OR=2.16; 95% CI: 1.26, 3.70)
Nilsen et al.[97] Norway, 2006	Cross-sectional data in a cohort study	Pregnant women in the Norwegian Mother and Child Cohort Study (MoBa), 2000-2003 Gestational week 17-18 Questionnaire N=22,500	FA use once or more per week starting 1 month before pregnancy and continuing throughout the first 3 months of pregnancy	Compliance: 11.9% ± 0.24 (planned pregnancies) Relative risks of behaviors in relation to periconceptional FA use: Maternal age (>34 years vs. <25 years): (RR=1.2; 95% CI: 1.3, 2.0) Marital status (Married vs. Single or other): (RR=2.4; 95% CI: 1.7, 3.5) Income (>300,000 NOK vs. <200,000 NOK): (RR=1.3; 95% CI: 1.2, 1.5) Education (University vs. Primary school): (RR=6.0; 95% CI: 3.4, 10.6) Parity (>2 vs. 0): (RR=0.6; 95% CI: 0.5, 0.7) Pregnancy planning (Yes vs. No): (RR=2.4; 95% CI: 2.1, 2.8) *In vitro* fertilization (Yes vs. No report): (RR=2.0; 95% CI: 1.7, 2.3) Smoking (Yes vs. No report): (RR=0.6; 95% CI: 0.5, 0.7) Chronic diseases (Any vs. No report): (RR=1.2; 95% CI: 1.1, 2.3)
Knudsen et al.[15] Denmark, 2004	Cross-sectional data in a cohort study	Pregnant women enrolled in Danish National Birth Cohort (DNBC), 2000-2002 Gestational week=10-15 Questionnaire and telephone interview N=13,680 (planned pregnancy)	Taking 80% of the recommended dose of 400 µg per day from 4 weeks prior to the date of last menstrual period until gestational weeks 6	Compliance: 16.4% (planned pregnancy), 5.6% (partly planned), 3.3% (not planned) OR for FA supplement use: Maternal age<19 years vs. ≥25 years: (OR=0.2; 95% CI: 0.05, 0.81) Multiparous vs. Nulliparous: (OR=0.76; 95% CI: 0.69, 0.84) Long education vs. Unskilled: (OR=1.89; 95% CI: 1.40, 2.54) Never vs. Ever smoking (OR=1.52; 95% CI: 1.34, 0.72)

Braekke and Staff[98] Norway, 2003	Cross-sectional study	Pregnant women attending antenatal ultrasound screening in the two main obstetric departments in Oslo, 2001 Gestational week 17-19 Questionnaire N=1,541	FA supplementation before the first day of the last menstrual period and used through the first 2 to 3 months of pregnancy	Compliance: 17% No information on unplanned pregnancies OR for FA supplement use: Non-Western immigrants vs. Norwegian: (OR=0.13; 95% CI: 0.01, 0.26) Maternal age 17-24 years vs. 35-45 years: (OR=0.01; 95% CI: 0.03, 0.21) Parous (2 children) vs. Nulliparous: (OR=0.31; 95% CI: 0.16, 0.61)
Study II: Studies evaluating the effect of multivitamins (containing folic acid) on menstrual cycle characteristics				
Author, year	**Design**	**Study-population**	**Exposure and outcome**	**Results**
Dudás and Czeizel[99] Hungary, 1995	Double-blind RCT*	Women from the Hungarian RCT database during 1989-1990 N=1,010	Exposure: ELEVIT pronatal® (0.8 mg FA) or placebo from 1 month before planned conception until 12 months (if no pregnancy) Outcome: Mean length of menstrual cycle; preovulatory phase; postovulatory phase; and mean duration of menstrual bleeding	Compared with placebo there was no differences in mean length of menstrual cycle; preovulatory phase; postovulatory phase; and mean duration of mensterual bleeding before and during multivitamin supplementation (days±SD): (no p-values reported between treatment groups) Menstrual cycle: 30.04±4.52 to 30.05± 4.86, in the multivitamin group vs. 29.88±5.13 to 29.57±5.28, in the placebo group Preovulatory phase: 15.33±4.06 to 15.09±4.06, in the multivitamin group vs. 15.38±4.38 to 14.94±4.14, in the placebo group Postovulatory phase: 14.71±2.88 to 14.96±3.23, in the multivitamin group vs. 14.50±3.04 to 14.63±3.34, in the placebo group Duration of menstrual bleeding: 4.99±1-39 to 5.00±1.33, in the multivitamin group vs. 4.92±1.25 vs. 5.00±1.26, in the placebo group
Westphal et al.[100] USA, 2006	Double-blind RCT*	Women aged 24-42 years with fertility disorders N=93	Exposure: FertilityBlend® (0.4 mg FA) or placebo for 3 menstrual cycles Outcome: Menstrual cycle length	There was no difference in mean cycle length between treatment groups Difference in short (<27days) and long (>32 days) cycle length within treatment groups: FertilityBlend® group: 24.2 vs. 27.6 days (p=0.001, short cycle length), and (41.6 vs. 31.7 days (p=0.017, long cycle length) Placebo group: 25.6 vs. 26.1 days (p=0.286, short cycle length), and (35.3 vs. 29.3 days (p=0.082, long cycle length)
Study III: Studies evaluating the effect of multivitamins (containing folic acid) on conception rates				
Author, year	**Design**	**Study-population**	**Exposure and outcome**	**Results**
Czeizel et al.[101] Hungary, 1996	Double-blind RCT*	Women aged <35 years without a history of infertility or delayed conception N=7,905	Exposure: ELEVIT pronatal® (0.8 mg FA) or placebo from 1 month before planned conception until at least 3 months and until 14 month (if no pregnancy) Outcome: Conception rate, time to pregnancy	Improved conception rates (71.3% vs. 67.9 %), and time to pregnancy (3,8 cycles vs. 4.0 cycles) in the multivitamin group compared with placebo, (OR=1.10, 95% CI: 1.00, 1.21)

Westphal et al.[100] USA, 2006	**Double-blind RCT***	**Women aged 24-42 years with fertility disorders N=93**	**Exposure: FertilityBlend® (0.4 mg FA) or placebo for 3 menstrual cycles Outcome: Pregnancy rates**	**Improved pregnancy rates in the multivitamin group compared with placebo: Pregnancy rates: 26% vs. 10% (p=0.012)**

*RTC, randomized controlled trial

2.4. Prevalence and predictors of preconceptional FA supplement use (Study I)

According to the National Survey of Dietary Habits from 2003-2008, only 5-10% of Danish women of fertile age have a sufficient intake of dietary folate.[16] As there is no FA food fortification in Denmark, supplementation with FA appears to be the only means to achieve the recommended daily dose of 400 µg of FA. In humans, dorsal folding of the neural plate is normally complete at 35-42 days of gestation, only a few weeks after the pregnancy is recognizable as such.[4] Thus, starting FA supplementation before conception is an important component of the effective prevention of NTDs.[4;7]

In Denmark, compliance with the preconceptional FA recommendation has been examined in two pregnancy-based cross-sectional studies. One study from the DNBC[15] reported that only 16% of the 14,000 women who planned their pregnancy between 2000 and 2002 fully complied with the FA recommendation, defined as taking 80% of the recommended FA dose per day from 4 weeks prior to the date of last menstrual period until gestational week 6.[15] In contrast, a more recent study of 258 pregnant women attending their initial midwifery visit at Aalborg Midwifery Center between 2011 and 2012 reported that 67% of the women who planned their pregnancy took FA supplements during the month prior to pregnancy.[14]

During the years of the first recommendations, several cross-sectional studies of already pregnant women in other European countries, Australia, and the United States reported a wide range of compliance estimates: 12%[97] and 17%[98] in Norway, 29% in the United Kingdom,[93] 23%[92] and 30%[95] in Australia, and 39%[90] and 44%[96] in the United States. In addition, two cross-sectional studies of non-pregnant women in the United Kingdom and the United States, one enrolling 238 women who became pregnant within 3 months of enrollment in the Southampton Women's Survey in 1998-2002, and the other enrolling 9,000 women who planned a pregnancy within 12 months of enrollment in the Behavioral Risk Factor Surveillance System in 2004, reported respective compliance estimates of 6%[94] and 54%.[91]

In addition to the concern that women with unplanned pregnancies do not have the possibility benefit from the FA recommendation,[14;15;102] social gradients in risk

behavior may also prevent some women from complying with the preconceptional recommendation.[19;103] Various socio-demographic, behavioral, and lifestyle factors have been associated with compliance with the FA recommendations. In Denmark, young age, low education, having given birth, and being a smoker have been identified as predictors of non-compliance.[15] In addition, Backhausen et al.[14] confirmed a positive association between FA supplement use and higher degree of planned pregnancy. These associations have also been reported in other countries.[90;93;96-98] Ethnicity has also been associated with compliance with the FA recommendation: being non-Caucasian was associated with non-compliance in the United States[90;91;96] and the United Kingdom,[93] and being a non-Western immigrant was associated with non-compliance in Norway.[98] Likewise, social class,[93] maternal income,[97] marital status,[90;97] and BMI[91;96] have also been identified as determinants of compliance. Finally, in a study by Nilsen et al.,[97] women who underwent *in vitro* fertilization or reported chronic diseases (e.g., diabetes and hypertension) were more likely to use preconceptional FA supplements.

2.4.1. Limitations of existing literature

The wide range of previously reported prevalences of preconceptional FA supplement use (6% to 67%) are possible because of differences in study designs, study populations, and methods of data collection. All studies, with exception of those by Chuang et al.[91] and Inskip et al.,[94] were focused on already pregnant women, which increased the risk of recall bias, as women who were in their first, second, or third trimester were asked to recall their FA supplement intake during the preconceptional period. Selection bias may have been another source of bias, as women were self-selected to all cross-sectional studies. In addition, data collection included in-person interviews, telephone interviews, or questionnaires, and may also have introduced information errors. With the exceptions of the studies from the DNBC,[15] The Norwegian Mother and Child Cohort,[97] and the study by Chang et al.,[91] the study populations were relatively small, ranging from 238 women interviewed in the Southampton Women's Survey[94] to 1,541 women attending an antenatal ultrasound screening in the two main obstetric departments in Oslo.[98] Consequently, estimates of the prevalence of FA use and predictors of such use reported in these studies may have been imprecise.

The definition of compliance period, as well as information about both frequency and dose of FA intake, differ widely among studies, and are likely to contribute to the wide range in prevalence estimates. In the study by Braekke and Staff,[98] 17% of the women began supplementation before the first day of their last menstruation. However, by extending the compliance period to 0-4 weeks of gestation, an additional 16% used FA supplements. These widely defined compliance periods also underscore the challenges of defining compliance during the preconceptional period.

As reported by Backhausen et al.[14] and Knudsen et al.,[15] women with unplanned pregnancies are less likely to use FA supplements prior to pregnancy, as indicated by the 3% prevalence of preconceptional FA supplement use among women with unplanned pregnancies, reported in both studies. Four of the studies did not include information about unplanned pregnancies.[90;93;96;98] Prevalence estimates of unplanned pregnancies were reported to be 19% and 39%, respectively, in Australian studies by Forster et al.[92] and Conlin et al.,[95] and 36% in an American study conducted by Goldberg et al.[96] However, it was not clear whether the reported prevalence estimates of FA use included women with unplanned pregnancies. Uncertainties about the inclusion of unplanned pregnancies may also have biased estimated associations in relation to preconceptional FA supplement use.

2.5. Preconceptional FA supplement use in relation to the menstrual cycle (Study II)

The most consistent predictor of menstrual cycle length is age,[104] as cycles become shorter when women get older.[105] Other predictors of short cycle length includes smoking,[77;104;106;107] alcohol intake,[106;108] and caffeine consumption,[108;109] whereas correlates of long cycle length include parity,[104] high BMI,[104;107] and vigorous physical activity,[106;110] and irregular cycles have been associated with smoking,[111] heavy alcohol intake,[108] and obesity.[104;107;112] To our knowledge, no study has examined the association between preconceptional FA supplementation and menstrual characteristics. In 1995, as a part of the Hungarian Periconceptional Service, a randomized trial of 1,010 women without a history of infertility, reported improved menstrual cycle regularity by means of lower variation of cycle length after supplementation with MVs that included 800 µg FA, compared with placebo.[99] However, mean menstrual cycle length before and during

supplementation did not differ significantly in either the MV group or the placebo group. The estimates were 30.04 days before and 30.05 days during supplementation in the MV group, and 29.88 days before and 29.57 days during supplementation in the placebo group.[99] In addition, there was no difference in duration of menstrual bleeding between treatment groups.

In 2006, in another randomized trial of 93 women who had tried unsuccessfully to conceive for 6 to 36 months, Westphal et al.[100] reported normalization of both short and long cycle lengths after supplementation for 3 months with MVs containing 400 µg FA.[100] During supplementation, cycle lengths of <27 days increased from 24.2 days to 27.3 days (p=0.001) and from 25.6 days to 26.1 days (p=0.268) in the MV and placebo groups, respectively. The corresponding estimates during supplementation for cycle lengths of >32 days were reductions from 41.6 days to 31.7 days (p=0.017) and from 35.3 days to 29.3 days (p=0.082) in the MV and placebo groups, respectively. MV supplementation was also associated with increased progesterone levels and an increased number of days with basal temperature >37°C during the luteal phase.[100]

2.5.1. Limitations of existing literature

Neither study employed relative estimates and confidence intervals to demonstrate the strength and precision of the associations. In the study by Westphal et al.,[100] the method used to calculate the statistical significance of cycle length and progesterone levels was not comprehensive; although menstrual cycle length changed during MV supplementation, there was no difference between treatment groups. The study also lacked information about the recruitment method and dropouts. Therefore, it remains unclear whether and to what extent FA supplementation might improve hormonal balance and menstrual cycle function.

2.6. Preconceptional FA supplement use in relation to fecundability (Study III)

In another randomized trial from the Hungarian Periconceptional Service of 7,905 women, Czeizel et al.[101] reported higher conception rates (71.3%) among women taking MV supplements that included 800 µg FA than among women taking placebo (67.9%), with a reported OR of 1.10 (95% CI: 1.00, 1.21) during a 14-month follow-up period. The

mean time to conception was 3.8 cycles in the MV group compared with 4.0 cycles in the placebo group.[101] In the trial of 93 women (see section 2.5), Westphal et al.[100] also reported increased pregnancy rates after 3 months of supplementation with MVs that included 400 μg FA compared with women taking placebo (26% vs. 10%; p=0.012).

2.6.1. Limitations of existing literature

In the trial by Czeizel et al.,[101] MV supplements were taken from 1 month prior to planned conception until at least 3 months or 14 months (depending on when conception occurred) , and compliance was reportedly verified by the women and by counting the remaining tablets in returned boxes. However, uncertainties remain about the dropout rate during the 14-month follow-up period. In the study by Westphal et al.,[100] there was no information regarding compliance with the prescribed supplementation (one tablet per day for three menstrual cycles). In addition, the study Westphal et al.[100] was excluded from a 2012 review because of uncertainties about the statistical method used (Fisher's exact test).[113] The authors of that review recalculated the statistics (using the same method) and found no significant effect on the pregnancy rate.[113] Thus, both the internal and the external validity of the study by Westphal et al.[100] may be limited. Finally, the independent effects of the different components in the MV supplements could not be determined in either study.

In conclusion, no study has examined the prevalence of preconceptional FA supplement use or health-related predictors of such use among pregnancy planners. Therefore, we lack knowledge regarding adherence to the FA recommendation and factors that contribute to social and behavioral gradients in compliance among the population of Danish women trying to conceive. In addition, while the involvement of FA metabolism in several developmental abnormalities and in pregnancy complications has given rise to a large amount of scientific work, evidence of a positive relationship between preconceptional FA supplementation and menstrual cycle function and fecundability is scarce.

3. Aims of the dissertation

Study I: To estimate the prevalence of preconceptional FA supplement use, obtained through either single FA tablets or MVs, and to identify socio-demographic, lifestyle, reproductive and medical predictors of such use among Danish pregnancy planners enrolled in the internet-based 'Snart-Gravid' prospective cohort study.

Study II: To examine the association between FA supplement use, obtained through either single FA tablets or MVs, and menstrual cycle regularity, cycle length, and duration and intensity of menstrual flow among Danish pregnancy planners enrolled in the internet-based 'Snart-Gravid' prospective cohort study.

Study III: To examine the association between FA supplement use, obtained through either single FA tablets or MVs, and fecundability among Danish pregnancy planners enrolled in the internet-based 'Snart-Gravid' prospective cohort study.

4. Methods

4.1. The Snart-Gravid study

The three studies that comprise this dissertation were based on data from the internet-based cohort study of Danish pregnancy planners 'Snart-Gravid.dk' (soon pregnant), which was conducted during 2007-2011. The Snart-Gravid study was initiated primarily to prospectively evaluate the relationships between several lifestyle and behavioral factors and fecundability among women trying to conceive.[26]

Participants were recruited mainly through a pop-up advertisement placed on a well-known health-related website (www.netdoktor.dk) and two press releases that attracted attention from print media, online news sites, television and radio. Participants were enrolled solely through the study website. Data were collected through e-mail and self-administered questionnaires.

Before enrollment, potential participants entering the study website were required to read a consent form and complete a screening questionnaire in order to confirm eligibility. Participants were also required to provide a valid e-mail address and their Central Personal Registry (CPR) number. Eligible women were invited to complete a baseline questionnaire and bimonthly follow-up questionnaires for 12 months or until conception occurred after which active follow up ended. Women who conceived were asked to complete one questionnaire during early pregnancy.[26] Participants were initially randomized to receive either a short- or a long-form baseline questionnaire to determine how the length of the baseline questionnaire affected enrollment and the completeness of data. Therefore, some questions were asked of only 50% of the cohort during the first 6 months of enrollment. Completion rates and missing data were similar for both questionnaire versions.[27]

4.2. Study designs

Studies I and II were designed as cross-sectional studies that used only baseline data. Study III was designed as a cohort study that included Snart-Gravid follow-up data.

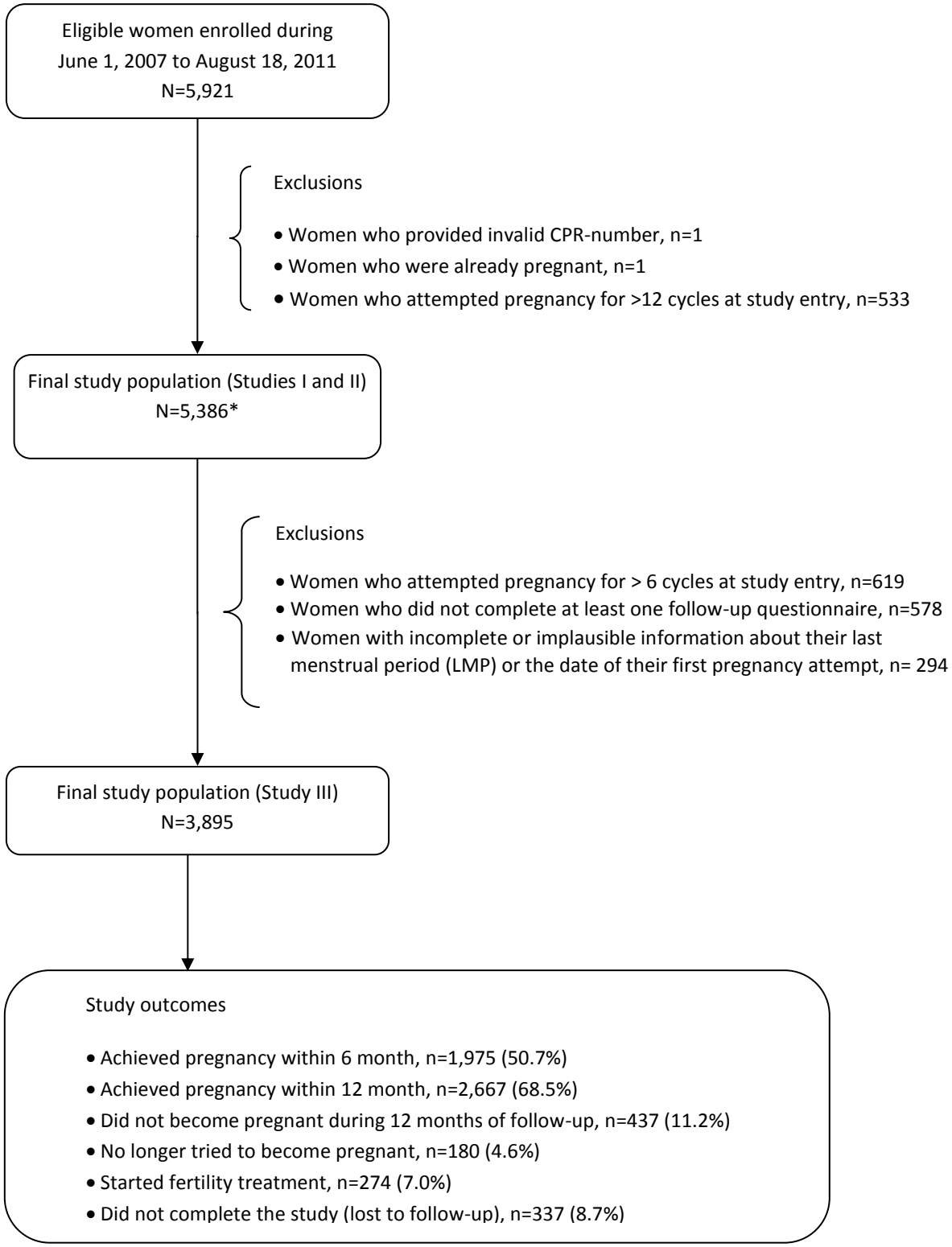
4.3. Study periods and study populations

The study periods were from 1 June 2007 to 3 August 2011 for Study I, and from 1 June 2007 to 18 August 2011 for Study II and Study III. Women were eligible for the Snart-Gravid study if they were Danish residents aged 18-40 years, in a stable relationship with a male partner, not using birth control, and not receiving any type of fertility treatment.

Exclusions

In all, 5,918 women enrolled from 1 June 2007 through 3 August 2011, and 5,921 women enrolled from 1 June 2007 through 18 August 2011 (Figure 1). We excluded one woman (0%) who did not provide a valid CPR number; one woman (0%) who was already pregnant; and 533 (9%) women who had been trying to conceive for more than 12 months at the time of study entry. Study I and Study II analyzed data from 5,383 and 5,386 women, respectively. In Study III, we additionally excluded 619 women (10%) who had been attempting to become pregnant for >6 cycles at baseline; 578 women (10%) who did not complete at least one follow-up questionnaire; and 294 (5%) women with incomplete or implausible information about their last menstrual period (LMP) or the date of their first pregnancy attempt. After these exclusions, 3,895 women were included in the analysis.

Figure 1. Flow chart for Studies I, II, and III



*For Study I, the study period was June 1, 2007 through August 3, 2011. 5,918 eligible women were enrolled and 5,383 comprised the final study population.

4.4. Data

The baseline questionnaire included questions about socio-demographic background, reproductive and medical history, and lifestyle and behavioral factors, including use of vitamins and other dietary supplements. Follow-up questionnaires collected information about the last menstrual period (LMP), pregnancy status, and lifestyle variables such as vitamin use, frequency of intercourse, and smoking status (i.e., variables that might change over time).

4.5. FA supplement use

The primary exposure for all three studies was FA supplement use obtained through either single FA tablets or MVs. In the baseline and follow-up questionnaires, women were asked, "Do you take vitamins on a regular basis - daily or almost every day?" If the response was positive, the women were asked to specify which of the following vitamins or minerals they were taking regularly: "Multivitamins, vitamin A, beta-carotene, vitamin B, vitamin C, vitamin D, vitamin E, folic acid, calcium, magnesium, selenium, and other". Participants who reported 'multivitamin' or listed a specific brand of MV in the "other" section were classified as "MV users". Similarly, participants who reported 'folic acid', or reported 'folate', 'folacin', or 'folic acid' in the "other" section, were classified as "FA users". The baseline questionnaire also collected information about the duration of supplement use in categories of <1 year, 1-5 years, >5 years, and 'don't know'.

Definition of FA supplement users

Most MV supplements marketed in Denmark contain 400 µg of FA, especially those intended for use during pregnancy. In addition, most women wrote the name of their MV product in the questionnaire, revealing whether the MV included FA. For study purposes, we created a single binary exposure variable defined as "FA supplementation", which was set to 1 for women who were FA users, MV users, or both. For women who used single vitamin or mineral supplements other than FA and women who did not take any dietary supplements, the exposure variable "FA supplement use" was set to zero and was defined as "non-use".

4.6. Socio-demographic variables

We assessed age (categorized as <25, 25-29, 30-34, and ≥35 years), schooling (primary and lower secondary school, high school, and other), vocational training [none, short (<3 years), medium (3-4 years), and long (>4 years)], and total monthly household income (<12,500, 12,500-24,999, 25,000-39,999, 40,000-64,999, and ≥65,000 DKK/month).

4.7. Life style and behavioral variables

Weight, height, physical activity, and smoking history were also reported at baseline. We calculated BMI from self-reported weight and height (kg/m^2). Total metabolic equivalents (METs) were estimated by summing the METs from moderate physical activity (hours per week multiplied by 3.5) and vigorous physical activity (hours per week multiplied by 7.0).[114;115] Self-reported smoking was categorized as pack-years of ever smoking, with 1 pack-year defined as smoking 20 cigarettes per day for 1 year, or smoking 7,305 cigarettes.

We assessed lifestyle and behavioral variables that could indicate intense efforts to conceive, such as frequency of intercourse (categorized as <1, 1-3, and ≥4 times per week) and months attempting pregnancy at study entrance (0-1, 2-4, 5-6, and 7-12 months). Compliance with other health recommendations, such as attending the national screening program for cervical cancer (Pap test) during the last 3 years (none vs. one or more), smoking history (never smoked, <5, 5-9, and ≥10 pack-years), current alcohol intake (none, 1-3, 4-7, 8-14, and ≥15 drinks/week), caffeine intake (<100, 100-199, 200-299, and ≥300 mg/day), BMI (<18.5, 18.5-24.9, 25-29.9, 30-34.9, and ≥35 kg/m^2), and engagement in physical activity (<10, 10-19, 20-39, and ≥40 METs/week), were also of interest.

4.8. Reproductive and medical variables

We assessed previous spontaneous abortion (yes vs. no), parity (nulliparous vs. parous), and last method of contraception (barrier methods, oral contraceptives, and other methods). Finally, we assessed medical conditions that might increase awareness of health in relation to pregnancy, including hypertension (yes vs. no),

diabetes (yes vs. no), thyroid disease (yes vs. no), pelvic inflammatory disease (yes vs. no), and infection with chlamydia (yes vs. no).

4.9. Menstrual cycle variables

The menstrual cycle variables included cycle regularity, cycle length, duration of menstrual bleeding, and intensity of menstrual bleeding. Women who responded 'yes' to the question "are your menstrual periods regular, e.g. you can usually predict about when your next period will start?" were considered to have regular cycles. We assessed cycle length, defined as "the number of days from the first day of a menstrual period to the first day of the next menstrual period" and categorized as short (<27 days), normal (27-29 days), or long (≥30 days).[23] Duration and intensity of menstrual bleeding were assessed among women with regular cycles by means of two questions: "How many days does your period usually flow (bleeding not spotting)?", in categories of short (<3 days), normal (3-4 days), long (5-6 days), and very long (>6 days); and "How would you classify the total amount of your menstrual flow?", in categories of light (≤10 pads or tampons/menstrual cycle), moderate (11-20 pads or tampons/menstrual cycle), heavy (21-30 pads or tampons), and very heavy (>30 pads or tampons/menstrual cycle).

4.10. Cycles at risk and TTP

Time to pregnancy (TTP) is the time interval from onset of sexual activity without contraception to the conception of a clinically recognized pregnancy, usually measured in either number of months or menstrual cycles.[25] Studies of fecundability based on measures of TTP have proven fruitful in identifying various exposures with effects on fertility.[116] Subsequently, the mean fecundability can be used to observe differences across exposure groups.[116;117] The main outcome of interest in Study III was the first reported pregnancy during the follow-up period (12 cycles), regardless of pregnancy outcome.

The total number of cycles at risk was calculated using data from the screening questionnaire regarding how many months study participants had been trying to conceive before enrollment in the study; the baseline questionnaire on usual cycle length; and the follow-up questionnaire on LMP, current pregnancy status, and any other pregnancy outcomes since the date of the last completed questionnaire, including

miscarriage, induced abortion, and ectopic pregnancy. Total cycles at risk were calculated as: (days of trying to conceive at study entry/cycle length) + [(LMP date from most recent follow-up questionnaire – date of baseline questionnaire completion)/usual cycle length) + 1].[23] One cycle was added to account for the average woman being at mid-cycle when she completed the baseline questionnaire.

To handle the problem of delayed entry into the study (i.e., women who entered the study after having tried to conceive for one or more cycles), we defined observed cycles at risk as those contributed after study entry.[65] For example, if a woman had been trying to conceive for two cycles before entering the study and then reported a pregnancy after 6 cycles of attempt time, she would contribute with four observed cycles, starting at cycle 3 when she was first observed in the study and at risk of pregnancy.[65;80] Women contributed cycles at risk until they reached a study endpoint: pregnancy, use of fertility treatment, loss to follow-up, or end of follow-up (12 cycles), whichever came first.

4.11. Study endpoints

Among the 3,895 women, 2,667 (69%) achieved a pregnancy within 12 cycles of follow-up. Four hundred thirty-seven (11%) women did not conceive after 12 cycles and were censored at 12 cycles, which is the typical time interval after which couples seek medical assistance for fertility.[118] The 180 women (5%) who changed their intention to become pregnant or actively resigned from the study and the 274 women (7%) who reported initiation of fertility treatment were censored at the time that they completed their last follow-up questionnaire. The 337 (9%) women with an unknown reason for not completing the study were considered lost to follow-up and also censored at the time that they completed their last follow-up questionnaire (Figure 1).

4.12. Statistical analysis

In all three studies, we calculated frequencies of FA supplement use exclusively, MV supplement use exclusively, combined FA and MV supplement use, use of single vitamins or minerals other than FA, and no use of dietary supplements. We used descriptive statistics, means, medians, and proportions to describe the study populations

in all three studies. Statistical analyses were performed using STATA® statistical software (version 11.2) and SAS statistical software (version 9.2, SAS Institute, Cary, NC).

4.12.1. Multivariate binomial regression (Study I)

To identify socio-demographic, lifestyle, reproductive, and medical predictors of FA supplement use, we estimated the prevalence proportion (PP) of FA users at each level of characteristic. Multivariate binomial regression was used to obtain prevalence proportion differences (PPDs) with 95% confidence intervals (CIs) for each predictor level in relation to the reference group. The multivariate analyses for the PP and PPD for each study predictor were adjusted for all other study predictors.

4.12.2. Logistic regression (Study II)

In Study II, we examined the association between FA supplement use and cycle regularity. We employed logistic regression to estimate odds ratios (ORs) with 95% CIs. After restriction to women with regular cycles, we used polytomous logistic regression to estimate ORs with 95% CIs for the association of FA supplement use with short (<27 days) and long (≥30 days) cycle length, compared with normal cycle length (27-29 days). We also estimated ORs with 95% CIs for the association of FA use with short (<3 days), long (5-6 days), and very long (>6 days) duration of menstrual bleeding, compared with normal (3-4 days) duration of bleeding, and with light (≤10 pads or tampons/menstrual cycle), heavy (21-30 pads or tampons/menstrual cycle), and very heavy (>30 pads or tampons/menstrual cycle) intensity of menstrual bleeding, compared with moderate (11-20 pads or tampons/menstrual cycle) intensity of bleeding. The multivariate analyses were adjusted for previously recognized correlates of menstrual cycle characteristics, such as age, parity, previous spontaneous abortions, BMI, pack-years of smoking, alcohol intake, caffeine intake, physical activity, and last method of contraception. In addition, the analyses of duration and intensity of menstrual flow were mutually adjusted.

Stratified analyses

In a sub-analysis, we stratified the data according to parity (parous and nulliparous) and age (18-30 and 30-40 years) at study entry. To eliminate the effect of previous use of hormonal contraceptives on menstrual cycle function, we also evaluated the associations

after restricting the study population to women who reported a non-hormonal method of last contraception.

4.12.3. Proportional probability regression (Study III)

In Study III, we computed fecundability ratios (FRs) and 95% CI for FA supplement users relative to non-users. To model probabilities of conception in a given cycle at risk of pregnancy, we fitted a proportional probabilities regression model, using discrete time to event.[119] This survival-analytic method allows censoring (e.g., if a couple changes their minds about conceiving) in order to contribute information appropriately, and allows for adjustment for confounding and exposures that may change from cycle to cycle.[65] Each menstrual cycle provides a single ovulatory opportunity for conception.[65] Thus, TTP is inherently discrete, with the menstrual cycle serving as the natural counting unit of time, and the FR represents the cycle-specific probability of conception among exposed women (FA users) divided by that among unexposed women (non-users). An FR >1.0 corresponds to increased fecundability among FA users relative to non-users. The primary analysis also modeled different categories of FA supplement use (FA and MV, FA exclusively and MV exclusively) to assess their independent effects.

Confounding

Potential confounders were selected based on review of the literature[66;120] and identified predictors of FA supplement use found in Study I.[121] In multivariate analyses (including Kaplan-Meier curves), we adjusted for age, education, BMI, physical activity, pack-years of smoking, alcohol intake, Pap tests, parity, timing of intercourse, history of spontaneous abortions, menstrual cycle regularity and cycle length, and last method of contraception used. Time-varying variables, such as timing and frequency of intercourse, current smoking status, and alcohol consumption, were updated using data reported on follow-up questionnaires.

Restricted analysis

After restricting the analysis to 2,289 FA supplement users who were not using any other vitamins or minerals and who had been trying to conceive for 0-3 months at study

entry, we evaluated the extent to which the FR differed between FA supplementation for <1 year vs. ≥1 year.

Stratified analyses

Because cycle regularity and cycle length may mediate the correlation between FA supplementation and fecundability, we stratified the data by cycle regularity (regular and irregular), and cycle length [short (<27 days), medium (27-29 days), and long (≥30 days)]. Because younger and parous women may have increased fecundability regardless of vitamin supplement use, we also stratified by age at study entry (18-30 years and 31-40 years) and by parity (parous and nulliparous).

4.12.4. Life table and Kaplan-Meier methods (Study III)

To estimate the cumulative probability of conceiving within the 12-cycle follow-up period, we used life table and Kaplan-Meier methods to handle delayed entry into the study, that some women stopped trying to conceive, and that not all women remained under follow-up for the entire 12 cycles.[65]

4.13. Missing values

In our study populations, 197 women (3.7% in Study I and Study II) and 127 women (3.3% in Study III) did not answer the initial vitamin question. Because 1,540 participants were initially randomized to receive the short-form baseline questionnaire, they did not receive the questions about medical diseases such as hypertension and diabetes or about the amount of menstrual flow. Therefore, the proportions of missing data on these variables were 29%. However, the participants that were missing this information were a completely random subset of enrollees by design. The amount of missing data for other covariates ranged between 0.1% (BMI) and 8.7% (total monthly household income).

We used multiple imputation methods[122-124] to impute missing exposure (vitamin supplement use) and covariate values. However, in Study I, because of the design of the initial question on vitamin intake, we found it reasonable to assume that women who skipped the initial question did so because they in fact were non-users. Therefore, in Study I we classified the 197 women with missing values on vitamin use as “non-users”. Preliminary analyses for Study II revealed no differences in estimates computed from

non-imputed or imputed exposure variables on FA use (unpublished data). The imputation was based on observed data regarding covariates, including outcome variables. We created five different plausible imputed datasets and combined the results obtained from each of them.[122] The estimated associations in each dataset were averaged together to provide overall estimated associations.

5. Results

5.1. Study I: Prevalence and predictors of preconceptional FA supplement use

Table 2 shows the distribution of dietary supplement use for Studies I and II. Overall, 412 women (7.7%) used FA supplements exclusively, 1,101 (20.4%) used MV supplements exclusively, and 1,831 (34.0%) used both, yielding 3,344 (62.1%) FA supplement users. Eighty-two women (1.5%) used single vitamins or minerals other than FA, and 1,960 (36.4%) did not use any dietary supplements, yielding 2,042 (37.9%) non-users.

Table 2. Dietary supplement use with respect to folic acid (FA) and multivitamins (MVs) among 5,386* pregnancy planners

	N	%
FA users	3,344	62.1
FA exclusive	412	7.7
MV exclusive	1,101	20.4
FA and MV	1,831	34.0
Non-users	2,042	37.9
No use	1,960	36.4
Other vitamins and minerals exclusive	82	1.5
Total	5,386*	100

*In Study I, 5,383 comprised the final study population. The overall distribution was: FA exclusive=412; MV exclusive=1,100; FA and MV=1,831; No use=1,958; Other vitamins and minerals exclusive=82.

Socio-demographic predictors

Predictors of FA supplement use are presented in Table 3. The median age was 28 years for both FA users and non-users. After mutual adjustment for other predictors, higher age, higher level of education, and household income were positively associated with FA supplement use. Compared with women 35 years and older, the PPD was -5.2% (95% CI: -11.6, 1.1) for women younger than 25 years of age. Compared with women who finished high school, PPD for women with less schooling was -5.9% (95% CI: -10.0, -1.8). Similarly, compared with women with a long vocational training period (>4 years), respective PPDs (95% CI) for women with no vocational training and a short vocational training period (<3 years) were -5.7% (-11.2, - 0.2) and -7.1% (-11.2, -3.0). Compared with a total monthly household income of ≥65,000 DKK/month, PPD for <12,500 DKK/month was -8.7% (95% CI: -19.8, 2.2).

Lifestyle and behavioral predictors

While intercourse frequency of more than once per week was associated with an increased prevalence of FA supplement use (PPD= -5.9%; 95% CI: -10.3, 1.5, for none versus one or more), we observed no clear association between months attempting to conceive at study entrance and FA use. Furthermore, women who had Pap tests once or more during the last 3 years were more likely to use FA supplements than women who did not have Pap tests (PPD= -9.7%; 95% CI: -13.2, -6.2, for none versus one or more). Smoking, alcohol use, and obesity (BMI ≥30 kg/m^2) were associated with reduced prevalence of FA supplement use. Compared with women who never smoked, PPD for ≥10 pack-years of smoking was -11.3%, (95% CI: -16.8, -5.8). Compared with no alcohol intake, PPD for ≥ 15 drinks/week was -18.4%, (95% CI: -29.1, -7.6). Compared with normal weight (BMI = 18.5-24.9 kg/m^2), respective PPDs (95% CI) for BMIs of 30-34.9 and ≥35 kg/m^2 were -5.1 (-10.1, -0.1) and -7.1% (-13.4, -0.8). Furthermore, women who engaged in physical activity were more likely to use FA supplements than sedentary women (<10 METs/week).

Reproductive and medical predictors

The PP for FA supplement use was 7.7% points (95% CI: 3.7, 11.8) higher among women who experienced a previous spontaneous abortion than among women who did not report having a previous spontaneous abortion. Being multiparous was not associated with FA supplement use. Finally, there was no clear association between preconceptional FA supplement use and diagnosis with hypertension, diabetes, thyroid disease, pelvic inflammatory disease, or chlamydia infection.

Table 3. Prevalence proportions (PPs) and prevalence proportion differences (PPDs) with 95% confidence intervals (CIs) of folic acid (FA) supplement use in relation to socio-demographic, lifestyle, reproductive, and medical characteristics in 5,383 women

	Non-users[a]	FA users[b]	Unadjusted	Adjusted[c]		
Characteristics	N (2,040)	N (3,343)	PP (%)	PP (%)	PPD	95% CI
Socio-demographic factors						
Age, years						
<25	461	507	52.4	58.8	-5.2	-11.6, 1.1
25-29	882	1,515	63.2	62.5	-1.6	-6.7, 3.5
30-34	518	1,037	66.7	66.7	2.7	-2.3, 7.6
≥35	179	284	61.3	64.1	Ref.	0
Schooling						
Primary/lower secondary	387	405	51.1	56.6	-5.9	-10.0,-1.8
High school	1,386	2,612	65.3	62.5	Ref.	0
Other	267	326	55.0	56.4	-6.1	-10.5, -1.8
Vocational training						
None	345	379	52.4	57.4	-5.7	-11.2, -0.2
Short (<3 years)	716	906	55.9	56.0	-7.1	-11.2, -3.0
Medium (3-4 years)	622	1,252	66.8	62.5	-0.6	-4.3, 3.0
Long (>4 years)	357	806	69.3	63.1	Ref.	0
Household income, DKK/month						
<12,500	64	64	50.0	51.8	-8.7	-19.8, 2.2
12,500-24,999	280	370	56.9	61.8	1.2	-4.8, 7.3
25,000-39,999	552	805	59.3	61.8	1.2	-3.5, 5.8
40,000-64,999	854	1,572	64.8	62.5	1.9	-3.5, 5.8
≥65,000	290	532	64.7	60.6	Ref.	0
Lifestyle factors						
Intercourse frequency, times/week						
<1	392	495	55.8	54.7	-5.9	-10.3,- 1.5
1-3	1,236	2,217	64.2	62.5	1.8	-1.5, 5.1
≥4	412	631	60.5	60.7	Ref.	0
Attempting pregnancy, months						
0-1	868	1,457	62.7	62.5	Ref.	0
2-4	569	994	63.6	65.0	2.5	-0.5, 5.5
5-6	252	372	59.6	61.3	-1.1	-5.4, 3.2
7-12	351	520	59.7	61.3	-1.1	-4.9, 2.6
Pap test[d], last 3 years						
None	545	556	50.5	62.5	-9.7	-13.2, -6.2
≥1	1,495	2,787	65.1	72.2	Ref.	0
Smoking status, pack-years						
Never smoked	1,052	2,097	66.6	62.5	Ref.	0
<5	526	726	58.0	58.1	-4.3	-7.6, -1.1
5-9	280	327	53.9	53.1	-9.4	-14.3, -4.7
≥10	182	193	51.5	51.2	-11.3	-16.8, -5.8

Continues

Table 3 continued

	Non-users[a]	FA users[b]	Unadjusted	Adjusted[c]		
Characteristics	N (2,040)	N (3,343)	PP (%)	PP (%)	PPD	95% CI
Alcohol intake, drinks/week						
None	601	1,097	64.6	67.0	Ref.	0
1-3	795	1,404	63.9	62.5	-4.5	-7.5, -1.5
4-7	466	602	56.4	55.4	-11.6	-15.4, -7.8
8-14	130	204	61.1	60.3	-6.7	-12.4, -1.0
≥15	48	36	42.9	48.6	-18.4	-29.1, -7.6
BMI, kg/m^2						
<18.5	91	128	58.5	65.2	-2.7	-9.4, 4.0
18.5-24.9	1,219	2,192	64.3	62.5	Ref.	0
25-29.9	424	651	60.6	63.4	-1.8	-5.1, 1.5
30-34.9	185	239	56.4	60.1	-5.1	-10.1, -0.1
≥35	121	133	52.4	58.1	-7.1	-13.4, -0.8
Physical activity, METs/week						
<10	388	476	55.1	56.5	Ref.	0
10-19	649	1,080	62.5	62.0	5.5	1.5, 9.4
20-39	666	1,230	64.9	62.5	5.9	2.0, 9.9
≥40	337	554	62.2	60.7	4.2	-0.5, 8.8
Reproductive history						
Previous spontaneous abortions (%)						
No	1,848	2,955	61.5	62.5	Ref.	0
Yes	192	388	66.9	70.2	7.7	3.7, 11.8
Parous, ever had live birth						
No	1,358	2,234	62.2	54.2	Ref.	0
≥1	682	1,109	61.9	52.9	-1.2	-4.2, 1.9
Medical history						
Hypertension (%)						
No	1,303	3,116	62.1	62.5	Ref.	0
Yes	137	227	62.4	63.4	1.1	-6.2, 8.4
Diabetes (%)						
No	2,020	3,300	62.0	54.2	Ref.	0
Yes	20	43	68.3	59.7	13.8	-1.0, 28.6
Thyroid disease (%)						
No	1,989	3,263	62.1	62.5	Ref.	0
Yes	51	80	61.1	59.4	-3.1	-15.4, 9.2
Pelvic inflammatory disease (%)						
No	1,733	2,905	62.6	62.5	Ref.	0
Yes	307	438	58.8	60.8	-1.7	-5.9, 2.6
Chlamydia (%)						
No	1,591	2,661	62.6	54.2	Ref.	0
Yes	449	682	60.3	54.0	0.1	-3.0, 3.3

[a]Non-users includes no supplement use and use of other single vitamin/mineral supplements than folic acid.
[b]FA users includes use of both FA and MV, use of MV exclusively, and use of FA exclusively.
[c]Mutually adjusted for all other covariates.
[d]Attending the national screening program for cervical cancer (Pap test).

5.2. Study II: Preconceptional FA supplement use and menstrual cycle characteristics

Menstrual cycle regularity

Of the 5,386 women included in the study, 1,345 (25.0%) reported irregular periods. Overall, we observed no association between FA supplement use and cycle regularity (adjusted OR=1.00, 95% CI: 0.88, 1.14). Compared with non-use, FA supplement use was associated with slightly reduced odds of having irregular cycles among parous women (adjusted OR=0.84, 95% CI: 0.67, 1.07; Table 4).

Table 4. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for irregular cycles compared with regular cycles, with respect to parity, age, and use of folic acid (FA) supplementation (N=5,386)

			All	Regular cycles (Ref.)	Irregular cycles	
			N (%)	n	n	OR (95% CI)
Overall	FA supplement use	Crude	3,344 (62.1)	2,516	828	0.97 (0.85-1.10)
		Adjusted[1]				1.00 (0.88-1.14)
	Non-use		2,042 (37.9)	1,525	517	1 (Ref.)
Parous	FA supplement use	Crude	1,111 (20.6)	871	240	0.83 (0.66-1.04)
		Adjusted[2]				0.84 (0.67-1.07)
	Non-use		682 (12.7)	514	168	1 (Ref.)
Nulliparous	FA supplement use	Crude	2,233 (41.5)	1,645	588	1.04 (0.89-1.21)
		Adjusted[2]				1.07 (0.91-1.26)
	Non-use		1,360 (25.2	1,011	349	1 (Ref.)
18-30 years	FA supplement use	Crude	2,294 (42.6)	1,661	633	1.01 (0.87-1.17)
		Adjusted[3]				1.02 (0.88-1.19)
	Non-use		1,485 (27.6)	1,077	408	1 (Ref.)
31-40 years	FA supplement use	Crude	1,050 (19.5)	855	195	0.93 (0.71-1.20)
		Adjusted[3]				0.89 (0.68-1.16)
	Non-use		557 (10.3)	448	109	1 (Ref.)

[1]Overall: Adjusted for age, BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

[2]Parity: Adjusted for age, BMI, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

[3]Age: Adjusted for BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

Menstrual cycle length

Among the 4,041 women that reported regular menstrual cycles, 597 (14.8%) reported cycles of <27 days, 2,214 (54.8%) reported cycles of 27-29 days, and 1,230 (30.4%) reported cycles of ≥30 days. Overall, compared with non-use, FA supplement use was associated with reduced odds of short cycle length (<27 days; adjusted OR=0.76, 95% CI: 0.63, 0.93) and a trend toward reduced odds of long cycle length (≥30 days; adjusted OR=0.94, 95% CI: 0.81, 1.10; Table 5).

Table 5. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for short and long cycle length compared with normal cycle length, with respect to parity, age, and folic acid (FA) supplementation, among women with regular cycles (N=4,041)

			Cycle length (days)					
			All	27-29 (Ref.)	<27		≥30	
			N (%)	n	n	OR (95 % CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,417	336	0.74 (0.61-0.90)	763	0.93 (0.80-1.07)
		Adjusted[1]				0.76 (0.63-0.93)		0.94 (0.81-1.10)
	Non-use		1,525 (37.7)	797	261	1 (Ref.)	467	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	494	128	1.02 (0.74-1.43)	249	0.96 (0.75-1.24)
		Adjusted[2]				1.14 (0.81-1.61)		1.01 (0.78-1.32)
	Non-use		514(12.7)	289	73	1 (Ref.)	152	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	923	208	0.63 (0.50-0.80)	514	0.90 (0.76-1.08)
		Adjusted[2]				0.63 (0.50-0.81)		0.92 (0.76-1.10)
	Non-use		1,011 (25.0)	508	188	1 (Ref.)	315	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	929	197	0.65 (0.51-0.83)	535	0.84 (0.71-1.00)
		Adjusted[3]				0.67 (0.52-0.85)		0.83 (0.70-0.99)
	Non-use		1,077 (26.7)	530	179	1 (Ref.)	368	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	488	139	0.93 (0.68-1.28)	228	1.24 (0.94-1.65)
		Adjusted[3]				1.00 (0.73-1.40)		1.27 (0.95-1.70)
	Non-use		448 (11.0)	267	82	1 (Ref.)	99	1 (Ref.)

[1]Overall: Adjusted for age, BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

[2]Parity: Adjusted for age, BMI, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

[3]Age: Adjusted for BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

In a sensitivity analysis, we observed a stronger association between FA supplementation and cycle length <25 days (adjusted OR=0.61, 95% CI: 0.45, 0.82; Table 6). Compared with women whose cycle length was 27-29 days, for women with cycle lengths of 25-26 days, 30-31 days, 32-33 days, and ≥34 days, the respective adjusted ORs (95% CI) were 0.84 (0.66, 1.07), 0.89 (0.76, 1.05), 0.91 (0.66, 1.23), and 1.14 (0.82, 1.60). After restricting the analyses to women who reported a non-hormonal method of last contraception (n=2,547), the overall associations were consistent (adjusted OR=0.76, 95% CI: 0.63, 0.93 for short cycle length, and adjusted OR=0.95, 95% CI: 0.81, 1.10 for long cycle length; data not shown).

Table 6. Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for cycle lengths of <25, 25-26, 30-31, 32-33, and ≥34 days compared with normal cycle length (27-29 days), with respect to folic acid (FA) supplementation, among women with regular cycles (N=4,041)

			Cycle length (days)									
	All	27-29 (Ref.)	<25		25-26		30-31		32-33		≥34	
	N (%)	n	n	OR[1] (95% CI)	n	OR[1] (95% CI)	n	OR[1] (95% CI)	n	OR[1] (95% CI)	n	OR[1] (95% CI)
Users	2,516 (62.3)	1,417	118	0.61 (0.45-0.82)	218	0.84 (0.66-1.07)	530	0.89 (0.76-1.05)	115	0.91 (0.66-1.23)	118	1.14 (0.82-1.60)
Non-users	1,525 (37.7)	797	104	1 (Ref.)	157	1 (Ref.)	337	1 (Ref.)	74	1 (Ref.)	56	1 (Ref.)

[1]Adjusted for age, BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

Stratified analyses (by age and parity)

In the stratified analyses, we observed a stronger association between FA supplementation and short cycle length among nulliparous women (adjusted OR=0.63, 95% CI: 0.50, 0.81) than among parous women (adjusted OR=1.14, 95% CI: 0.81, 1.61; Table 5). We also observed a stronger association between FA supplementation and short cycle length among women aged 18-30 years (adjusted OR=0.67, 95% CI: 0.52, 0.85) than among women aged 31-40 years (adjusted OR=1.00, 95% CI: 0.73, 1.40). Compared with non-users, FA users aged 18-30 years also had reduced odds of having long cycle length (adjusted OR=0.83, 95% CI: 0.70, 0.99). In contrast, FA users aged 31-40 years had slightly increased odds of having long cycle length (adjusted OR=1.27, 95% CI: 0.95, 1.70).

Duration and intensity of menstrual bleeding

Tables 7 and 8 present the associations between FA supplementation and the duration and intensity of menstrual flow, respectively. Overall, there were no clear associations between FA supplementation and duration or intensity of menstrual flow. The ORs indicated little association at the higher extremes of duration of flow (>6 days) and intensity of flow (>30 pads or tampons/menstrual cycle); however, the estimates were imprecise.

Table 7. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for durations of menstrual flow of <3, 5-6, and >6 days compared with normal duration of menstrual flow (3-4 days), with respect to parity, age, and folic acid (FA) supplementation, among women with regular cycles (n=4,041)

			Duration of menstrual flow (days)							
			All	3-4 (Ref.)	<3		5-6		>6	
			N (%)	n	n	OR (95 % CI)	n	OR (95% CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,324	234	1.10 (0.87-1.38)	859	1.06 (0.92-1.22)	99	1.27 (0.89-1.81)
		Adjusted[1]				1.13 (0.89-1.45)		1.04 (0.89-1.21)		1.20 (0.81-1.77)
	Non-use		1,525 (37.7)	834	133	1 (Ref.)	509	1 (Ref.)	49	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	430	61	0.98 (0.63-1.53)	332	1.07 (0.84-1.35)	48	1.54 (0.89-2.69)
		Adjusted[2]				1.11 (0.67-1.83)		1.05 (0.81-1.36)		1.46 (0.74-2.89)
	Non-use		514 (12.7)	264	37	1 (Ref.)	194	1 (Ref.)	19	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	894	173	1.15 (0.87-1.50)	527	1.07 (0.90-1.28)	51	1.08 (0.68-1.72)
		Adjusted[2]				1.11 (0.85-1.48)		1.09 (0.91-1.32)		1.15 (0.72-1.86)
	Non-use		1,011 (25.0)	570	96	1 (Ref.)	315	1 (Ref.)	30	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	861	141	1.02 (0.78-1.35)	594	1.11 (0.94-1.31)	65	1.12 (0.74-1.40)
		Adjusted[3]				1.07 (0.80-1.45)		1.08 (0.90-1.30)		1.06 (0.68-1.67)
	Non-use		1,077 (26.7)	582	93	1 (Ref.)	363	1 (Ref.)	39	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	463	93	1.24 (0.83-1.86)	265	0.98 (0.76-1.27)	34	1.84 (0.89-3.79)
		Adjusted[3]				1.30 (0.81-2.01)		0.91 (0.70-1.23)		2.07 (0.89-4.45)
	Non-use		448 (11.0)	252	40	1 (Ref.)	146	1 (Ref.)	10	1 (Ref.)

[1]Overall: Adjusted for age, BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

[2]Parity: Adjusted for age, BMI, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

[3]Age: Adjusted for BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

Table 8. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for menstrual flow intensities of ≤10, 21-30, and >30 pads or tampons/menstrual cycle compared with moderate flow (11-20 pads or tampons/menstrual cycle), with respect to parity, age, and folic acid (FA) supplementation, among women with regular cycles (n=4,041)

			Intensity of menstrual flow (pads or tampons/menstrual cycle)							
			All	11-20	≤10		21-30		>30	
			N (%)	n	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,519	585	0.85 (0.71-1.01)	361	0.91 (0.73-1.12)	51	1.25 (0.59-2.65)
		Adjusted[1]				0.81 (0.66-1.00)		0.92 (0.75-1.15)		1.37 (0.63-2.97)
	Non-use		1,525 (37.7)	867	408	1 (Ref.)	228	1 (Ref.)	22	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	544	123	0.76 (0.53-1.09)	172	0.91 (0.65-1.28)	32	1.37 (0.48-3.90)
		Adjusted[2]				0.78 (0.53-1.15)		0.91 (0.64-1.29)		1.41 (0.44-4.51)
	Non-use		514 (12.7)	300	96	1 (Ref.)	106	1 (Ref.)	12	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	975	462	0.88 (0.72-1.06)	189	0.90 (0.69-1.18)	19	1.09 (0.45-2.63)
		Adjusted[2]				0.82 (0.66-1.03)		0.92 (0.68-1.23)		1.68 (0.60-4.67)
	Non-use		1,011 (25.0)	567	312	1	122	1 (Ref.)	10	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	994	396	0.89 (0.72-1.10)	245	0.99 (0.78-1.25)	26	1.20 (0.59-2.45)
		Adjusted[3]				0.88 (0.70-1.12)		1.04 (0.81-1.32)		1.57 (0.72-3.45)
	Non-use		1,077 (26.7)	617	294	1 (Ref.)	153	1 (Ref.)	13	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	525	189	0.76 (0.54-1.06)	116	0.74 (0.50-1.10)	25	1.28 (0.28-5.67)
		Adjusted[3]				0.67 (0.45-0.98)		0.68 (0.45-1.03)		1.02 (0.19-5.42)
	Non-use		448 (11.0)	250	114	1 (Ref.)	75	1 (Ref.)	9	1 (Ref.)

[1]Overall: Adjusted for age, BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

[2]Parity: Adjusted for age, BMI, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

[3]Age: Adjusted for BMI, parity, previous spontaneous abortions, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

5.3. Study III: Preconceptional FA supplement use and fecundability

Characteristics of the study population regarding FA supplement use

The analysis for Study III included 3,895 women (317 users of FA exclusively, 824 users of MV exclusively, 1,419 users of both FA and MV, and 1,335 non-users) contributing 16,338 menstrual cycles and 2,667 confirmed pregnancies. In all, 2,560 women (65.7%) used FA supplements, obtained through either single FA tablets or MVs, and 1,335 women (34.3%) were non-users. Baseline characteristics with respect to supplement use are listed in Table 9. FA supplement use was associated with higher education level, having at least one Pap test during the last 3 years, doing something to time intercourse, and using barrier methods as the last method of contraception. These associations appeared to be stronger among users of both FA supplements and MVs.

Table 9. Baseline characteristics of 3,895 women with respect to supplement use at baseline

	Vitamin supplement use				
	FA supplementation		Subgroups of FA supplementation		
Characteristic	Non-use[1]	FA supplementation[2]	FA and MV	MV exclusive	FA exclusive
No. of women, n (%)	1 335 (34.3)	2 560 (65.7)	1 419 (36.4)	824 (21.2)	317 (8.1)
Age, years (mean)	28.0	28.6	28.8	28.5	27.8
Partner's age, years (mean)	30.6	30.9	31.0	30.8	30.4
Level of education (%)					
Short (none, semi-skilled,<3 years)	50.8	37.2	34.2	41.6	38.8
Medium (3-4 years)	29.8	37.0	37.0	36.4	38.5
Long (>4 years)	19.4	25.9	28.8	22.0	22.7
Body mass index (mean)	24.7	23.9	23.8	23.9	24.0
Physical activity, MET hrs/wk (median)	21.0	21.0	22.8	21.0	19.3
Pack-years of ever smoking (mean)	2.6	1.9	1.8	2.0	1.8
Alcohol intake, drinks/wk (mean)	2.8	2.4	2.3	2.6	2.8
Pap test[3], ≥1time last three years (%)	74.8	83.6	84.6	83.4	79.5
Last method of contraception, (%)					
Oral contraceptives	62.8	60.9	60.5	62.0	59.1
Barrier methods	25.3	28.8	29.5	27.8	27.8
Parous, ever had live birth (%)	32.7	33.2	33.9	33.9	28.1
Previous spontanous abortion, yes (%)	8.6	10.4	11.4	9.6	7.9
Doing something to time intercourse, yes (%)	36.1	51.4	55.0	44.9	52.1
Frequency of intercourse, ≥4 times/wk (%)	19.3	19.5	19.2	18.9	22.4
Attempt time before study entry, (%)					
0-1 cycles	54.5	53.1	50.7	56.1	55.5
2-3 cycles	25.4	26.3	26.8	26.8	22.4
4-6 cycles	20.1	20.7	22.5	17.1	22.1
Irregular cycles, yes (%)	23.1	24.5	23.3	25.4	27.4
Cycle length, days (mean)	30.5	30.7	30.5	30.9	30.9

FA=folic acid; MV=multivitamin; MET=total metabolic equivalents.

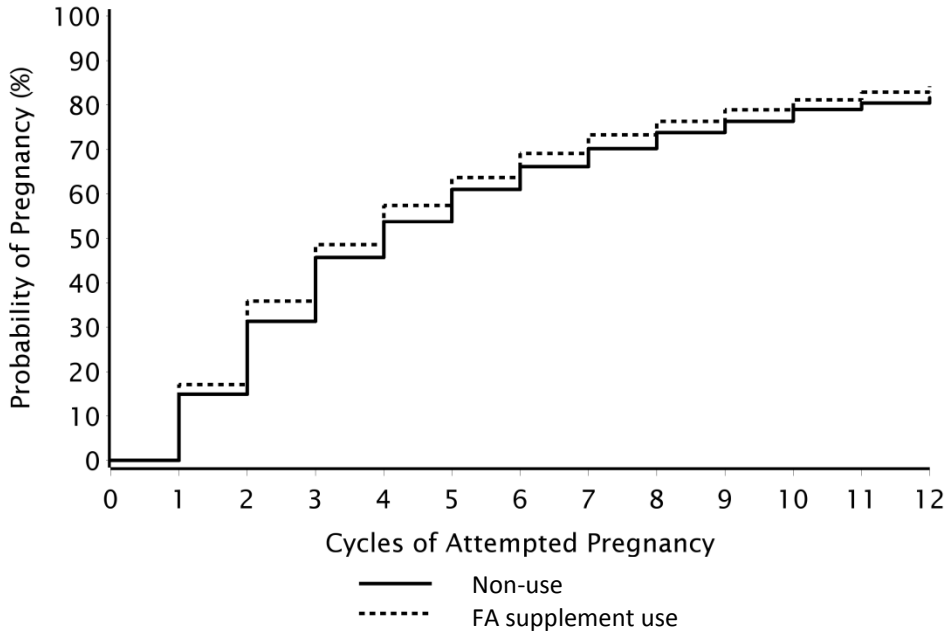
[1]Non-use includes no supplement use 1 273 (32.7%) and use of other single vitamin/mineral supplements than folic acid 62 (1.6%); [2]FA supplementation includes use of both FA and MV, use of MV exclusively, and use of FA exclusively; [3]Attending the national screening program for cervical cancer (Pap test).

The 337 women (9%) with an unknown reason for not completing the study and subsequently considered lost to follow-up were, on average, less likely to use FA supplements than cohort participants (49% vs. 66%). However, the baseline characteristics of women lost to follow-up were similar to those of cohort participants, with only small differences in education level, Pap tests, doing something to time intercourse, and cycle regularity (Appendix I).

Cumulative probability of conceiving

The crude cumulative probability of conception (69%) did not adjust for the facts that some women stopped trying to conceive and not all women participated in follow-up for the entire 12 cycles. When life-table methods were used to handle this issue (including delayed entry), we found that the estimated probability of becoming pregnant within 12 cycles was 83%. The adjusted Kaplan-Meier curve (Figure 2) shows that the 25th, 50th, and 75th percentiles for the cumulative probability of conceiving were 2, 4, and 8 cycles, respectively, among FA supplement users and 2, 4, and 9 cycles, respectively, among non-users. The curves indicate that the associations between FA supplement use and fecundability were relatively constant across 12 cycles of attempted pregnancy.

Figure 2. Kaplan-Meier pregnancy probability curves with respect to folic acid (FA) supplement use. The curves are adjusted for age at baseline, vocational training, cycle regularity, parity, previous spontaneous abortion, Pap test, physical activity level, pack-years of smoking, alcohol intake, timing and frequency of intercourse, BMI, and last method of contraception used (N=3,895)



Fecundability ratios

Compared with non-use, FA supplement use was associated with increased fecundability (adjusted FR=1.15; 95% CI: 1.06, 1.25; Table 10). Use of FA and MV, MV exclusively, and FA exclusively exhibited similar associations, with respective adjusted FRs (95% CI) of 1.12 (1.03, 1.23), 1.20 (1.08, 1.32), and 1.15 (1.00, 1.31) compared with non-use.

When restricted to women who had tried to conceive for 0-3 months at study entry (n=2,289), the adjusted FR for FA supplementation for <1 year was 1.15 (95% CI: 1.03, 1.28) compared with non-use. The corresponding FR was 1.04 (95% CI: 0.92, 1.19) for FA supplementation for >1 year compared with non-use.

Table 10. Fecundability with respect to folic acid (FA) supplement use among 3,895 women

	Pregnancies	Cycles	Unadjusted Model		Adjusted Model[2]	
			FR	95% CI	FR	95% CI
Vitamin supplement use						
Non-use[1]	732	4 997	1.00	Ref.	1.00	Ref.
FA supplementation	1 935	11 341	1.22	1.13-1.32	1.15	1.06-1.25
FA and MV	1 069	6 340	1.21	1.11-1.33	1.12	1.03-1.23
MV exclusive	602	3 404	1.24	1.12-1.37	1.20	1.08-1.32
FA exclusive	264	1 597	1.20	1.05-1.37	1.15	1.00-1.31

FR=fecundability ratio; CI=confidence interval; FA=folic acid; MV=multivitamin.
[1] Non-use includes no supplement use and use of other single vitamin/mineral supplements than folic acid.
[2] Models for FA supplementation, FA and MV use, MV exclusive, and FA exclusive are adjusted for age at baseline, vocational training, parity, previous spontaneous abortion, Pap test, physical activity level, pack-years of smoking, current smoking status, alcohol intake, timing and frequency of intercourse, BMI, and last method of contraception used.

Stratified analysis

After we stratified the data by menstrual cycle regularity and cycle length, the adjusted FRs for FA supplementation relative to non-use were 1.35 (95% CI: 1.12, 1.65) for women with irregular periods and 1.11 (95% CI: 1.01, 1.22) for women with regular periods. The FRs were 1.36 (95% CI: 0.95, 1.95) for women with short cycles (<27 days), 1.10 (95% CI: 0.98, 1.22) for women with medium cycle length (27-29 days), and 1.24 (95% CI: 1.10, 1.41) for women with long cycles (≥30 days). We found little effect of age and parity on the association between FA use and fecundability. The adjusted FRs (95% CI) were 1.18 (1.07, 1.30) and 1.09 (0.94, 1.27) for women aged 18-30 years and 31-40 years, respectively, and 1.14 (1.00, 1.30) and 1.16 (1.05, 1.29) for parous women and nulliparous women, respectively (Table 11).

Table 11. Fecundability with respect to folic acid (FA) supplement use, stratified by selected factors (N=3,895)

	Pregnancies	Cycles	Unadjusted Model		Adjusted Model[2]	
			FR	95% CI	FR	95% CI
Age 18-30 y						
Non-use[1]	531	3 640	1	Ref.	1	Ref.
FA supplement use	1 380	7 903	1.24	1.13-1.36	1.18	1.07-1.30
Age 31-40 y						
Non-use	201	1 357	1	Ref.	1	Ref.
FA supplement use	555	3 438	1.17	1.00-1.36	1.09	0.93-1.27
Nulliparous						
Non-use	452	3 503	1	Ref.	1	Ref.
FA supplement use	1 232	8 119	1.23	1.11-1.36	1.16	1.05-1.29
Parous						
Non-use	280	1 494	1	Ref.	1	Ref.
FA supplement use	703	3 222	1.22	1.07-1.39	1.14	1.00-1.30
Irregular periods						
Non-use	141	1 130	1	Ref.	1	Ref.
FA supplement use	435	2 634	1.39	1.16-1.66	1.35	1.12-1.65
Regular periods						
Non-use	591	3 867	1	Ref.	1	Ref.
FA supplement use	1 500	8 707	1.18	1.08-1.29	1.11	1.01-1.22
Cycle length, short (<27 d)						
Non-use	89	704	1	Ref.	1	Ref.
FA supplement use	227	1 317	1.47	1.17-1.85	1.36	0.95-1.95
Cycle length, medium (27-29 d)						
Non-use	430	2 785	1	Ref.	1	Ref.
FA supplement use	1 090	6 599	1.14	1.02-1.26	1.10	0.98-1.22
Cycle length, long (≥30 d)						
Non-use	302	2 212	1	Ref.	1	Ref.
FA supplement use	845	4 742	1.35	1.19-1.52	1.24	1.10 -1.41
Doing something to time intercourse						
Non-use	280	1,845	1	Ref.	1	Ref.
FA supplement use	943	5,311	1.19	1.06-1.35	1.17	1.03-1.32
Not doing something to time intercourse						
Non-use	488	3,335	1	Ref.	1	Ref.
FA supplement use	956	5,847	1.16	1.05-1.29	1.12	1.01-1.24
Intercourse frequency, ≥ 4 times/wk						
Non-use	155	882	1	Ref.	1	Ref.
FA supplement use	374	2,064	1.11	0.94-0.32	1.08	0.91-1.28
Intercourse frequency,< 4 times/wk						
Non-use	613	4,298	1	Ref.	1	Ref.
FA supplement use	1,525	9,094	1.22	1.12-1.33	1.17	1.07-1.28
Last method of contraception, OC						
Non-use	471	3,262	1	Ref.	1	Ref.
FA supplement use	1,138	7,112	1.15	1.05-1.27	1.10	1.00-1.22
Last method of contraception, barrier						
Non-use	202	1,278	1	Ref.	1	Ref.
FA supplement use	581	2,890	1.31	1.13-1.51	1.25	1.07-1.45

Continues

Table 11 Continued

	Pregnancies	Cycles	Unadjusted Model		Adjusted Model[2]	
			FR	95% CI	FR	95% CI
Duration of FA supplement use						
Non-use	877	5,923	1	Ref.	1	Ref.
FA supplement use use < 1 year	801	4,331	1.22	1.12-1.33	1.14	1.04-1.25
FA supplement use ≥ 1 year	315	1,842	1.16	1.03-1.31	1.04	0.92-1.17

FR=fecundability ratio; CI=confidence interval; FA=folic acid; MV=multivitamin.

[1] Non-use includes no supplement use and use of other single vitamin/mineral supplements than folic acid.

[2] Models for the stratified analysis are adjusted for vocational training, previous spontaneous abortion, Pap test, physical activity level, pack-years of smoking, current smoking status, alcohol intake and BMI. Further, each model includes adjustment for the other covariates stratified for, with the exception of cycle regularity and cycle length.

6. Discussion

6.1. Main conclusions

We found that more than one-third of the Snart-Gravid cohort did not comply with the preconceptional FA recommendation. Predictors of non-compliance included risk behaviors such as smoking, alcohol use, obesity, and being physically inactive. We found a modest inverse association between FA supplement use and irregular cycles among parous women. We also observed modest respective inverse associations between preconceptional FA supplement use and short cycle length (<27 days) and long cycle length (≥30 days). These associations were strongest among 18- to 30-year-old and nulliparous women. Finally, preconceptional FA supplementation was associated with increased fecundability, and this association appeared to be stronger among women with irregular cycles and among women with either short or long cycle length. We observed no appreciable differences in fecundability among subgroups of FA or MV use, and longer duration of FA supplementation (≥1 year) did not increase fecundability.

6.2. Comparison with existing literature

6.2.1. Prevalence and predictors of FA supplement use (Study I)

No previous Danish study has examined the prevalence of FA supplement use and predictors of such use, based on prospectively collected data during the preconceptional period. The compliance estimate of 62%[121] observed in our study, is in line with the recently reported estimate of 67%[14] by Backhausen et al. The two estimates show a 4-fold increase in compliance compared with the disturbingly low compliance estimate of 16%[15] reported by the DNBC in 2004. This may partly reflect an increase over the past 10 years in health awareness in the general population, an increase in information available about pre-pregnant recommendations, as well as differences in study designs and study populations.

Our findings agree with previous reports that maternal age,[15;96;98] education,[15;93;96] income,[93] smoking,[15;97] and BMI[91;96] were associated with preconceptional FA use. Although a history of childbirth may increase awareness of the preconceptional FA

recommendation, we found only a weak association between parity and FA supplementation in our study. Previous studies reported that previous childbirth reduced the likelihood of FA use in subsequent pregnancies.[15;98] The positive respective associations between FA use and previous spontaneous abortion and intercourse frequency observed in our study have not been reported elsewhere. These findings may indicate a relationship between a high desire to conceive and increased knowledge about the preconceptional FA guidelines. Finally, being diagnosed with a chronic disease has previously been associated with increased use of dietary supplements in general.[125] Nilsen et al., found that users of FA supplements more frequently reported chronic diseases, especially diabetes and heart disease.[97] Thus, pregnancy planners with a chronic illness might be more prone to use FA supplements because of increased health awareness in relation to the disease; however, we found little association between diagnoses of hypertension, diabetes, thyroid disease, pelvic inflammatory disease, or chlamydia infection and preconceptional use of FA.

In addition to the FA recommendation, Danish women planning a pregnancy are advised to avoid alcohol altogether.[11] Thirty-two percent reported no alcohol intake in our study, which was slightly more than the 12% previously reported by the DNBC.[81] We also found the lowest proportion of FA users among women with the highest alcohol intake. Together with the concern that excessive alcohol intake impedes the normal bioavailability and metabolism of folate,[126] these results underscore the need to target women at high risk of not following preconceptional guidelines.

6.2.2. Preconceptional FA supplement use and menstrual cycle characteristics (Study II)

Although previous follow-up and cross-sectional studies have reported associations between menstrual cycle function and various reproductive[104;107;127] and lifestyle[104;106;110-112;127] factors, only two studies have evaluated supplementation with FA-containing MVs and menstrual cycle characteristics.[99;100] In contrast to our findings, Dudás and Czeizel[99] reported improved cycle regularity among women who reported irregular cycles at study entry, but found no change in cycle length during supplementation with MVs containing 800 µg FA. In agreement with our results, Westphal et al.[100] reported normalization of both long and short cycles among women with fertility problems during 3 months of

supplementation with MVs containing 400 µg FA. Still, it is difficult to compare the two studies with our study because of differences in study designs, and study populations.

6.2.3. Preconceptional FA supplement use and fecundability (Study III)

The FA-related increase in FRs found in our study, are in line with previous randomized trials that reported higher pregnancy rates among women who were taking MV supplements that included 800 µg FA[101] or 400 µg FA,[100] indicating that FA supplementation might increase fecundability to some extent. Our findings were also supported by previous studies relating FA supplementation with ovulatory function among women without history of infertility. In the Nurse's health study, Chavarro et al.[63] reported an inverse association (RR=0.69, 95%CI: 0.51, 0.95) between regular use of MVs that included FA and ovulatory infertility among 18,555 healthy women.[63] In another follow-up study of 259 women enrolled in the BioCycle study, Gaskins et al.[64] reported that a diet high in synthetic FA reduced the risk of anovulatory cycles (OR= 0.36, 95%CI: 0.14, 0.92).[64]

In our study, the associations between FA supplementation and fecundability were stronger among women with irregular menstrual cycles and among women with short (<27 days) or long (≥30 days) cycles. This finding suggests that the biological effect of FA on fecundability may be mediated in part by menstrual cycle hormones. FA supplementation might influence fecundability through several different mechanisms, such as alterations in DNA biosynthesis, multiple methylation reactions, and accumulation of homocysteine. However, our data did not include biological specimens to investigate the biological mechanisms behind our findings. Gaskins et al.[64] reported that women in the highest tertile of synthetic FA intake had 15.7% (95% CI: 0.1%, 33.8%) higher luteal progesterone levels and decreased odds of anovulation (OR=0.36, 95% CI: 0.14, 0.92) compared with women in the lowest tertile, suggesting that the effect of FA on anovulation was explained in part by its effect on menstrual cycle hormones.

Because most participants used FA supplements in combination with MVs, we were unable to determine the effect attributable to FA alone versus the synergistic effect of MVs. Interestingly, a randomized controlled trial of 56 women undergoing ovulation

induction revealed that supplementation with an MV product during ovulation increased pregnancy rates compared with supplementation with FA alone.[128]

Although the exact duration of preconceptional FA supplementation was not assessed in our study, we found no evidence of increased fecundability among women who used FA supplements for ≥1 year compared with those who used FA for <1 year. A previous study showed an increase in plasma folate levels after 3 weeks among folate-depleted women who were receiving 300 µg of dietary folate per day,[129] suggesting that folate deficiency is remedied quite quickly after supplementation. Another study reported a significant increase in red-cell folate concentrations after 3 months of supplementation with 400 µg FA per day.[130] Thus, it seems plausible that the biological effect of FA supplementation on fecundability might occur within a short time after the onset of supplementation, emphasizing the importance of daily FA supplementation and indicating that longer preconceptional supplement use may not contribute to increased fecundability.

6.3. Methodological considerations

This dissertation includes both descriptive and analytic measures. The prevalence estimates presented in Study I represent descriptive measures, while the estimates of association presented as PPDs (Study I), ORs (Study II), and FRs (Study III) represent the analytic measures. Before inferring associations and causal relationships, internal validity must be evaluated to assess the potential risk of systematic and random errors that may have affected the descriptive measures and estimates of association.[131]

6.3.1. Selection bias

Our study population comprised self-selected volunteers enrolled via the Internet. Our study participants may have been more health-conscious than women planning pregnancy in general; as such, we most likely have observed a higher prevalence of FA supplement use compared with the general population of Danish pregnancy planners. Likewise, the absolute estimates of baseline characteristics may be biased.

Selection bias is a systematic error that stems from the procedure used to select subjects and from factors that influence study participation.[131] It arises when the association between FA supplement use and outcome differs between study participants and non-participants.[131] A limitation of Studies I and II was the cross-sectional design. Information on FA supplement use was ascertained simultaneously with participant characteristics and menstrual cycle characteristics; therefore, we cannot determine the exact temporal sequence between FA supplementation and participant characteristics or infer causal relations in Study II. Self-selection of study participants via the Internet may also be considered a threat to validity in Study III, because the reason for self-referral may be associated with underlying fertility.[131] Because unplanned pregnancies (and hence the most fertile women) were not included in the cohort, we potentially observed an overrepresented proportion of subfertile women in our study population.[65] In addition, because FA supplement use appears more prevalent among pregnancy planners, it may have led us to underestimate the association between FA supplementation and fecundability. However, we excluded women who had been trying to conceive for more than 6 cycles at study entry, and the cumulative probability of

conceiving within 12 cycles was 83% in our study population. Thus, selection of the less fertile women may not be a major problem in this study. Furthermore, because close to 80% of pregnancies in DK are planned, the pregnancy planners in our study may not actually represent a highly selected group compared with the general population of pregnancy planners.[14;15]

If the associations between FA supplementation and baseline characteristics and between FA supplementation and fecundability differed for study volunteers versus other women because of an underlying health factor, the generalizability of our findings might have been limited. However, because women were enrolled without regard to FA supplement use, and because all of our comparisons were made within our population of study participants (rather than between study participants and pregnancy planners who did not participate in the study), it is unlikely that selection bias would meaningfully distort the respective associations between FA supplement use and baseline characteristics, menstrual cycle characteristics, and fecundability. The main strength of Study III was its prospective study design. Because women who volunteered for the study did so before pregnancy occurred, it seems unlikely that selection was affected by underlying fertility. Thus, we see no reason why the FA-related increase in fecundability would differ with respect to whether the women had Internet access, and the internal validity of the study should not be affected by differences between study participants and the general population.[132]

Even though the rate of loss to follow-up was low (9%), it may still have introduced selection bias. To address this issue, we compared baseline characteristics of the entire cohort with those of participants that were lost to follow-up. Although the prevalence of FA supplement use was higher among cohort participants than among those lost to follow-up, we observed no major differences in other baseline characteristics between the two groups. Thus, selection bias caused by loss to follow-up does not appear to be a major problem in our study.

6.3.2. Information bias

Information bias occurs when exposure or outcome data are measured erroneously.[131] If misclassification of FA supplement use or study outcomes (predictors of FA use, menstrual cycle characteristics, and TTP) were dependent on the presence of its counterpart, then it would be considered *differential misclassification*, and the direction of bias would be less predictable.[131] *Non-differential misclassification* occurs when the misclassification of FA use is independent of the outcome and vice versa, which most often biases the results towards null.[131]

Misclassification of baseline characteristics, including preconceptional FA supplement use and menstrual cycle variables

The collection of self-reported data may have introduced misclassification into our studies. Some participants may have reported being "FA users" simply because they were asked or because they were planning to begin supplementation within a short time. In addition, the questionnaire did not provide information regarding the exact dose of FA ingested or the brand name of the FA or MV product, and 20% of the study participants used MVs exclusively. Because MV supplements are not subject to standardized definitions,[29;133-135] it is uncertain to what extent the MVs contained FA. However, because most MVs on the Danish market contain 400 µg FA, it seems unlikely that misclassification of MVs posed a major problem in our study. We cannot rule out the possibility of some misclassification of socio-demographic, lifestyle and behavioral, and reproductive and medical variables. In Study II, we instructed participants to report their usual menstrual cycle characteristics when not using hormonal contraceptives, but still we lack defined reference periods for menstrual cycle outcomes. Also, the accuracy of menstrual cycle length reported by participants was not validated. However, one study suggested that sexually active women who are trying to conceive may be more accurate in their reports.[136] In addition, a previous sub-analysis of participants in the Snart-Gravid study found modest agreement between reported menstrual cycle length on the baseline questionnaire and on a subsequent follow-up questionnaire.[23]

Because participants reported FA supplement use without knowledge about the hypothesis behind the studies and the characteristics to be studied, we see no reason

why misclassification of FA supplement use would differ between subgroups of participants. It also seems unlikely that misclassification of baseline characteristics would differ systematically between FA supplement users and non-users. Thus, systematic bias in reporting FA supplement use and baseline characteristics should bias results toward the null, and cannot explain the associations observed in Studies I and II.

Misclassification of TTP

In Study III, the prospectively reported data on TTP did not rely on recall. Although women are able to recall their TTP with high validity,[137] recall bias may be a problem for women with long TTP.[25] As it was not possible to restrict participation to women who were just about to discontinue contraception, we assessed the prior pregnancy attempt time in months, so that the entry of study participants could be delayed with respect to the appropriate risk set for their first observed menstrual cycle in the study.[65] Thus, calculation of cycles at risk of pregnancy from self-reported months of trying to conceive before study entry may have created some misclassification. In addition, some women who conceived immediately after study entry may have been classified as "0 cycles" and others as "1 cycle", because of the assumption that all women were mid-cycle at study entry. Some misclassification may also have occurred because we collected pregnancy status bimonthly. However, because FA supplementation was assessed before the occurrence of pregnancy, misclassification of pregnancy status and cycles at risk of pregnancy were unlikely to be related to FA supplement use. In addition, the proportional probability regression model accounted for variables that might change with time, including FA supplement use; this method reduced the risk of introducing bias related to behaviors that were only measured at baseline and that did not account for possible changes in behaviors over time because the women did not conceive in the first couple of cycles.[65] Hence, systematic bias in assessing FA supplement use and TTP seems unlikely, and any misclassification errors should bias our results towards the null and would not be able to explain the observed association between FA supplementation and fecundability.

6.3.3. Confounding

Adjustment for covariates in Study I and Study II

In Study I, we identified various demographic, lifestyle, and behavioral differences between FA supplement users and non-users (e.g., users were less likely to smoke and consume alcohol).[121] Such health behaviors may also be related to menstrual cycle function, which may confound the associations between FA supplement use and menstrual cycle characteristics.[104;106-108] In the regression models, we adjusted for several lifestyle factors. Thus, it seems unlikely that an underlying health factor would meaningfully affect the associations. It is possible that recent hormonal contraception use could impact the observed menstrual patterns. However, we found similar estimates with and without controlling for the last method of contraception. In addition, we stratified by age and parity in the analysis to avoid confounding by factors related to age or pregnancy and childbirth. However, we have no information about the length of the inter-pregnancy interval among parous women. Thus, menstrual cycle length may be influenced by recent child birth or breast feeding, which may explain some of the differences in the estimates between parous and nulliparous women in Study II.

Adjustment for confounding in Study III

Confounding can be thought of as confusion or distortion of the association between exposure and outcome, because the association is mixed with the effect of another variable.[131] A confounder must be an independent cause or a marker for the cause, imbalanced across exposure categories, and not on the causal pathway between exposure and study outcomes.[131] Because of the non-randomized nature of the study design, the observed association between FA supplement use and fecundability may be influenced by confounding. Several factors associated with fecundability that are not in the causal pathway between the FA supplementation and fecundability may be unequally distributed across FA users and non-users, and were therefore potential confounders.[131] In the proportional probabilities regression model, we adjusted for several lifestyle factors; however, some residual confounding remains possible because of roughly categorized variables in the questionnaires. Diet, including dietary folates, may be a source of unmeasured confounding. However, the bioavailability of FA is generally higher in supplements than in dietary folate;[130;138;139] synthetic FA is more stable and

absorbable.[140] Thus, it seems unlikely that a dietary factor would meaningfully confound the effect of FA supplementation on fecundability.

6.3.4. Precision

We used 95% CIs to estimate the precision of the estimates, and thus to evaluate whether or not our results were affected by random error (chance).[131] Despite the rather large study population in our three studies, some subgroups were small and the estimates were imprecise, as is indicated by the widths of the CIs. Thus, our results may have been affected by random error, particularly regarding the stratified analysis and the analyses of the duration and intensity of menstrual flow. These findings must be interpreted with caution.

6.3.5. Generalizability

Although the absolute estimates of baseline characteristics, including FA supplement use, may not be generalizable to the general population of Danish pregnancy planners, the internal validity of our study should not be affected by bias. Thus, assuming high internal validity, the associations observed in our study are likely generalizable to the general population of Danish pregnancy planners, as well as to most other Western societies with comparable lifestyle, risk behaviors, and socio-demography.

6.4. Perspectives

This dissertation adds to the increasing body of evidence that compliance with the preconceptional FA recommendation of 400 µg per day presents a challenge for women. Our results underscore the need for ongoing strategies for regular and systematic initiatives to promote the FA guidelines. Such initiatives should clarify the knowledge of the beneficial effects of FA on NTDs, including the importance of timing FA supplementation, because the neural tube closes just a few weeks after conception. Therefore, initiation of supplementation, even early in pregnancy, will not provide the same protection against NTDs as supplementation initiated before conception.

Also, the somewhat abstract definition of "thinking of planning a pregnancy" appears to pose a challenge regarding interpretation of the FA recommendation. Would "the day you stop using contraception" be easier to understand? Nevertheless, in order to achieve the optimal protection against NTDs, there will be a challenge in defining the preconceptional period. Finally, future initiatives should focus on means to target vulnerable women (e.g., young women with low level of education and general risk behaviors, such as smoking). Still, there are bound to remain some women who will not benefit from the recommendation; likewise, the recommendation has no effect on unplanned pregnancies. Thus, the inevitable question arises; is FA supplementation the most appropriate strategy, or are additional initiatives, such as FA fortification of wheat flour and other foods, a possibility in Denmark? To our knowledge, FA fortification has not been a topic of discussion nationwide since 2003.[19] The primary concern in relation to FA fortification was the risk of masking the hematological symptoms caused by deficiency of vitamin B12 with high intakes of FA. Although there is little evidence of folate-induced masking or exacerbation of neuropathies in humans,[5] assessment of the possible negative effects of FA doses exceeding the upper recommended level of 1000 µg per day should be considered.

Because the exact biological nature of the associations between FA supplementation and menstrual cycle function and fecundability remain unclear, it is too early to make recommendations; more evidence is warranted. For ethical reasons, randomized controlled trials of preconceptional FA use cannot be undertaken in today's

society. Thus, we rely on well-designed large cohort studies to examine the relation between preconceptional FA use and fecundability in greater depth. It would be possible though, to conduct a randomized controlled trial to assess the effect of FA supplementation on menstrual cycle function among women who are not planning a pregnancy.

Finally, challenges related to subfertility may increase with the tendency to postpone motherhood until later in life. In Denmark, from 1970 to 2013, mean maternal age for first pregnancy has increased from 24 to 29 years.[141] In addition, The Danish National Board of Health reported that 8% of all children, born in 2010, were conceived by assisted reproduction.[142] Therefore, increased knowledge about cost-effective lifestyle and behavioral factors that might optimize the likelihood of conception is of major public health interest.

7. Summary

Folates are essential co-enzymes in DNA synthesis and various methylation reactions. The preventive effect of preconceptional folic acid (FA) supplementation on neural tube defects is well known, and FA may also affect many other aspects of reproduction. Little is known regarding compliance as well as predictors of compliance with the Danish preconceptional recommendation; women who are planning to conceive should be supplemented with 400 µg FA per day until the 12th week of gestation. Further, potential associations between FA supplementation and menstrual cycle function and fecundability have scarcely been explored.

We examined the prevalence of preconceptional FA supplement use, obtained either through single FA tablets or multivitamins (MVs), and identified socio-demographic, lifestyle, reproductive and medical predictors of such use. In addition, we examined the association between FA supplement use and menstrual cycle characteristics such as menstrual cycle regularity, cycle length, and duration and intensity of menstrual cycle bleeding, and fecundability, respectively, among Danish pregnancy planners enrolled in the internet-based prospective cohort study 'Snart-Gravid'.

We conducted two cross-sectional studies (I and II) and one prospective cohort study (III). Preconceptional FA supplement use was identified from the self-administered baseline questionnaire, which also included questions on socio-demographic background, reproductive and medical history, and lifestyle and behavioral factors. Bi-monthly follow-up questionnaires collected information on last menstrual period, pregnancy status, and lifestyle variables that may change over time, during the 12 month follow-up period or until conception.

In Study I, we included 5,383 women enrolled in the Snart-Gravid study between 1 June, 2007 and 3 August, 2011. Overall, 62% of the women used FA supplements. Characteristics such as higher age, higher level of education and household income, and intercourse frequency of ≥1 time/week, Pap test ≥1 during the last three years, and a previous spontaneous abortion were associated with increased prevalence of FA supplement use, whereas smoking, alcohol use, sedentary lifestyle, and obesity (BMI ≥30 kg/m^2), were associated with a decreased prevalence of FA use.

In Study II, we included 5,386 women enrolled in the Snart-Gravid study between 1 June, 2007 and 18 August, 2011. Compared with non-use, FA supplement use was associated with slightly reduced odds of irregular cycles (adjusted OR=0.84, 95% CI: 0.67, 1.07) among parous women. Overall, compared with non-use, FA use was associated with reduced odds of short cycle length (<27 days; adjusted OR=0.76, 95% CI: 0.63-0.93), and long cycle length (≥30 days; adjusted OR=0.94, 95% CI: 0.81-1.10). These associations were strongest among 18-30 year-old and nulliparous women. There was no clear association between FA supplementation and duration, and intensity of menstrual flow.

In Study III, we included 3,895 women enrolled in the Snart-Gravid study between 1 June, 2007 and 18 August, 2011. Using life-table methods, the estimated probability of becoming pregnant within 12 cycles was 83%. Compared with non-use, FA supplementation was associated with increased fecundability (fecundability ratio (FR) = 1.15; 95% CI: 1.06, 1.25). Use of FA and MV, MV exclusively, and FA exclusively exhibited similar associations, with respective FRs (95% CI) of 1.12 (1.03, 1.23), 1.20 (1.08, 1.32), and 1.15 (1.00, 1.31), compared with non-use. This association appeared stronger among women with irregular menstrual cycles and among women with short or long cycles, with respective FRs (95% CI) of 1.35 (1.12, 1.65), 1.36 (0.95, 1.95), and 1.24 (1.10, 1.41).

Although our study participants may have been more health-conscious than pregnancy planners in general, we found that more than one third of the women did not comply with the preconceptional FA recommendation. Because women were enrolled before the occurrence of pregnancy, without regard to FA supplement use, and because all our comparisons were made within the study population, it is unlikely that the inverse associations between FA supplement use and menstrual cycle characteristics, and fecundability, respectively, are meaningfully biased. In conclusion, preconceptional FA supplementation was inversely associated with short and long cycle length and associated with increased fecundability.

8. Dansk resumé

Folsyre er et B-vitamin, der blandt andet fungerer som co-enzym i DNA syntese og adskillige methyleringsreaktioner. Folsyre er essentiel for celledeling og bidrager til forebyggelse af neuralrørsdefekter, som er medfødte misdannelser, der opstår som følge af mangelfuld lukning af neuralrøret hos fostret 22 til 29 dage efter befrugtning. I Danmark har Sundhedsstyrelsen siden 1997 anbefalet kvinder, der planlægger at blive gravide, at indtage et dagligt kosttilskud med folsyre indtil 12. graviditetsuge. Det er dog uvist, i hvilken grad anbefalingen følges, og hvad der karakteriserer de kvinder, som følger anbefalingerne i forhold til kvinder, som ikke gør. Derudover er det ukendt, om der er en sammenhæng mellem brug af prækonceptionel folsyretilskud og henholdsvis menstruationskarakteristika og fertilitet.

Formålet med denne afhandling var at undersøge prævalensen af brug af prækonceptionel folsyretilskud (enten via folsyretabletter eller multivitaminer) og at identificere socioøkonomiske, livstils- og medicinske prædiktorer for et sådan indtag. Derudover undersøgte vi, om der var en sammenhæng mellem brug af prækonceptionel folsyretilskud og regelmæssighed og længde af menstruationscyklus, samt blødningsmængde og blødningsvarighed. Ligeledes undersøgte vi, om der var sammenhæng mellem brug af prækonceptionel folsyretilskud og fekundabilitet (den cyklusspecifikke sandsynlighed for at blive gravid).

Studierne i denne afhandling var baseret på det internetbaserede kohortestudie 'Snart-Gravid'. Fra 2007 til 2011 inkluderede Snart-Gravid-studiet 5921 danske kvinder mellem 18 og 40 år, som forsøgte at blive gravide. Kvinderne udfyldte ét selvadministreret baseline spørgeskema umiddelbart efter inklusion i studiet. Derudover udfyldte de op til 6 opfølgningsspørgeskemaer, som blev sendt hver anden måned indtil graviditet eller i ét år. Afhandlingen består af to tværsnitsstudier (I og II) og ét kohortestudie (III). Vi identificerede forbrug af folsyretilskud via baseline spørgeskemaet, som også indeholdt spørgsmål om kvindernes socioøkonomiske status, livstil, helbred og tidligere graviditeter. Opfølgningsspørgeskemaerne indeholdt information om seneste menstruationsperiode, graviditetsstatus og ændringer i livsstil.

I det første studie inkluderede vi 5383 kvinder fra Snart-Gravid-kohorten. Vi fandt, at 62% af kvinderne tog folsyretilskud, og at kvinder, som tog tilskud, generelt var ældre,

havde en længerevarende uddannelse og højere indkomst end kvinder, der ikke tog tilskud. Derudover var kvinder, der tog folsyre, karakteriseret ved tidligere at have haft en spontan abort, at være blevet screenet for livmoderhalskræft mindst én gang inden for de seneste 3 år, eller at have samleje mere end 1 gang om ugen. Derimod havde kvinder, med et øget alkoholforbrug, stillesiddende livstil, som røg eller havde et BMI på 30 kg/m^2 eller derover, lavere sandsynlighed for at tage folsyretilskud.

I studie II fandt vi, at blandt kvinder, der tidligere havde født, havde kvinder, der tog folsyretilskud, lavere sandsynlighed for at have uregelmæssig menstruationscyklus (odds ratio (OR)=0.84, 95% CI: 0.67-1.07), sammenlignet med kvinder, som ikke tog folsyretilskud. Kvinder, som tog tilskud, havde desuden lavere sandsynlighed for at have kort cykluslængde (<27 dage; OR=0.76, 95% CI: 0.63-0.93) og lang cykluslængde (≥30 dage; OR=0.94, 95% CI: 0.81-1.10), sammenlignet med kvinder, som ikke tog folsyretilskud. Denne sammenhæng var stærkest blandt de 18 til 30-årige kvinder, som ikke tidligere havde født. Vi fandt ingen klar sammenhæng mellem folsyretilskud og henholdsvis blødningsmængde og blødningsvarighed.

I studie III inkluderede vi 3895 kvinder fra Snart-Gravid-kohorten. Indenfor de 12 måneders opfølgning, blev 83% af kvinderne gravide. Sammenlignet med kvinder, som ikke tog folsyretilskud, havde kvinder, som tog folsyretilskud, 15% (95% CI: 1.06 to 1.25) øget fekundabilitet. Denne stigning i fekundabilitet syntes stærkere blandt kvinder, der havde henholdsvis uregelmæssig menstruationscyklus, kort cykluslængde og lang cykluslængde.

Selvom vores studiepopulation sandsynligvis var sundere og mere helbredsbevidst sammenlignet med den generelle danske befolkning af kvinder, som planlægger at blive gravide, viste vores studie, at mere end en tredjedel af kvinderne ikke fulgte Sundhedsstyrelsens anbefaling vedrørende folsyretilskud. Samlet set viste vores studier en sammenhæng mellem brug af folsyretilskud og normalisering af menstruationscyklus. Desuden fandt vi, at brugere af folsyretilskud havde en øget sandsynlighed for at blive gravide.

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10. Appendices

Appendix I: Table of baseline characteristics of study participants and women lost to follow-up

Appendix II: Paper I

Appendix III: Paper II

Appendix IV: Paper III

Appendix I

Appendix I. Baseline characteristics of the entire cohort of study participants and those lost to follow-up (N=3,895)

	Entire cohort		Lost to follow-up	
Characteristic	Non-use	FA use[1]	Non-use	FA use[1]
No. of women, n (%)	3,895 (100.0)		337 (8.7)	
No. of women, n (%)	1, 335 (34.3)	2 560 (65.7)	166 (50.7)	171 (49.3)
Age, years (mean)	28.0	28.6	27.6	28.1
Partner's age, years (mean)	30.6	30.9	31.0	30.7
Vocational training (%)				
Short (none, semi-skilled,<3 years)	50.8	37.2	63.3	42.7
Medium (3-4 years)	29.8	37.0	21.7	38.6
Long (>4 years)	19.4	25.9	15.1	18.7
Body mass index (mean)	24.7	23.9	25.6	24.2
Physical activity, MET hrs/wk (median)	21.0	21.0	19.3	19.3
Pack-years of ever smoking (mean)	2.6	1.9	3.5	2.1
Alcohol intake, drinks/wk (mean)	2.8	2.4	3.0	2.3
Pap test[2], ≥1times last three years (%)	74.8	83.6	71.1	77.8
Last method of contraception, (%)				
Hormonal contraceptives	62.8	60.9	66.9	59.7
Barrier methods	25.3	28.8	22.9	25.7
Parous, ever had live birth (%)	32.7	33.2	26.5	30.4
Previous spontanous abortions, yes (%)	8.6	10.4	7.8	8.2
Doing something to time intercourse, yes (%)	36.1	51.4	33.7	48.5
Frequency of intercourse, ≥4 times/wk (%)	19.3	19.5	27.7	15.2
Attempt time before study entry, (%)				
0-1 cycles	54.5	53.1	50.0	59.7
2-3 cycles	25.4	26.3	28.8	23.4
4-6 cycles	20.1	20.7	21.1	17.0
Irregular cycles, yes (%)	23.1	24.5	29.5	29.8
Cycle length, days (mean)	30.5	30.7	30.9	31.7

[1]FA use includes use of both FA and MV, use of MV exclusively, and use of FA exclusively
[2]Attending the national screening program for cervical cancer (Pap test)

Appendix II: Paper I

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ORIGINAL RESEARCH

Predictors of preconceptional folic acid or multivitamin supplement use: a cross-sectional study of Danish pregnancy planners

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Purpose: Compliance with the Danish preconceptional folic acid (FA) recommendation – a daily supplement of 400 µg – is reported to be poor. Uncertainty remains, however, about the prevalence of compliers and health-related predictors of compliance in the preconceptional period.

Methods: We used self-reported baseline data from 5383 women, aged 18–40 years, enrolled in an Internet-based prospective cohort study of Danish pregnancy planners during 2007–2011. We estimated the prevalence proportions of FA or multivitamin (MV) use in relation to selected sociodemographic, lifestyle, reproductive, and medical characteristics. Multivariate binomial regression was used to obtain prevalence proportion differences with 95% confidence intervals for each level of study predictors, adjusted for all other predictors.

Results: Overall, 7.7% of women used FA supplements, 20.4% used MV supplements, 34.0% used both, 1.5% used other single vitamins or minerals, and 36.4% did not use any dietary supplements. The prevalence of FA or MV supplement use was higher among older women, women with higher education and income, and women with healthy lifestyle factors such as being a nonsmoker, nondrinker, physically active, maintaining a normal body mass index and having regular pap smears. Greater intercourse frequency and a history of spontaneous abortion were also positively associated with FA or MV supplement use. We found no clear association between use of FA or MV supplements and a diagnosis of hypertension, diabetes, thyroid disease, pelvic inflammatory disease, or chlamydia.

Conclusion: A large proportion of pregnancy planners do not use FA or MV supplements. Pregnancy planners with generally risky lifestyle behaviors are less likely to comply with the FA recommendation.

Keywords: pregnancy, preconceptional supplement use, vitamins, folic acid

Introduction

In Denmark, women planning to conceive are advised to take a daily supplement of 400 µg folic acid (FA) until the 12th week of gestation to reduce the risk of neural tube defects (NTDs).[1] Starting FA supplementation before conception is an important component of effective prevention of NTDs, since the neural tube closes at gestational week 6, only a few weeks after the pregnancy may be recognized.[2] FA is primarily taken as single supplements or as a component of multivitamins (MVs) and other vitamin preparations made for pregnancy planners or pregnant women.

Despite campaigns promoting the FA recommendation in Denmark in 1999 and 2001, women in the preconceptional period appear to have neither adequate dietary intake of folate nor adequate supplementation from FA tablets or MV supplements.

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According to the National Survey of Dietary Habits from 2003 to 2008, only 5–10% of Danish women of fertile age have a sufficient intake of dietary folate.[3] The most recent data on periconceptional use of FA supplements in Denmark are from the Danish National Birth Cohort (DNBC). Between 2000 and 2002, the proportion of FA compliers among DNBC participants who planned their pregnancy increased from 14% to only 22%, and thus remained low even after the campaigns.[4,5] Several retrospective studies of pregnant women in other countries also reported poor compliance, with 12% of women in the UK,[6] 17% in Norway,[7] 23–30% in Australia[8,9] and 44% in the US[10] reporting FA supplement use in the preconception period. In addition, a prospective cohort study reported that only 6% of the women who became pregnant within 3 months of being interviewed in a general woman's survey in the UK followed the FA recommendation.[11]

In previous studies, young age, low education and income, smoking, overweight, and parity were reported to be predictors of noncompliance.[5–8,10] To date, no study has examined other health-related predictors of compliance during the preconceptional period, and we lack recent estimates of compliance with the FA recommendation reported from pregnancy planners during the preconceptional period. In the current study, we estimated the prevalence of FA and MV supplement use and identified sociodemographic, lifestyle, reproductive, and medical predictors of such use among Danish pregnancy planners.

Methods

Design of the present study

The present study was a cross-sectional analysis of baseline data from an Internet-based prospective cohort study of Danish pregnancy planners – Snart-Gravid (Soon Pregnant).The study design has been described in detail elsewhere.[12,13] Briefly, the study was initiated in June 2007. Participants were enrolled via the study website, and data were collected by email and self-administered questionnaires. Recruitment was achieved by a pop-up advertisement placed on a well-known health-related website (http://www.netdoktor.dk) and two press releases. Before enrolment, potential participants entering the study website were required to read a consent form and fill in a screening questionnaire in order to confirm eligibility. Further, participants were required to provide a valid email address and their personal civil registry (CPR) number, which is a unique ten-digit personal identification number allowing linkage to a number of nationwide registries. Eligible women were invited to complete a baseline questionnaire and bimonthly follow-up questionnaires for 12 months or until conception occurred, after which active follow-up ended. Participants were initially randomized to receive either a short- or a long-form baseline questionnaire. Completion rates and missing data were similar for both questionnaire versions.[13]

Study population and study period

Eligible women were Danish residents aged 18–40 years, living in a stable relationship with a male partner, not using birth control, not receiving any type of fertility treatment, and attempting to conceive for no more than 12 months. From June 1, 2007 to August 3, 2011, 5918 women completed the screener and the baseline questionnaire. Of these, one woman did not provide a valid CPR number in the screener, one woman was excluded because she was already pregnant (14 weeks), and 533 women were excluded because they had been attempting to conceive for more than 12 months. In total, 5383 eligible women enrolled in the study during the 4-year study period.

Data collection

Data on preconceptional use of FA and MV supplements and sociodemographic, lifestyle, reproductive, and medical variables were assessed at baseline.

Assessment of FA and MV supplement use

In the baseline questionnaire, women were asked "Do you take vitamins on a regular basis – daily or almost every day?", "How long have you been taking vitamins on a regular basis – less than one year, 1–5 years, more than 5 years, or don't know?", and "Which of the following vitamins or minerals do you take on a regular basis – MVs, vitamin A, beta-carotene, vitamin B, vitamin C, vitamin D, vitamin E, FA, calcium, magnesium, selenium, or other?" Participants who reported "multivitamin" or wrote the name of an MV product were defined "MV users." Similarly, participants who reported "folic acid" or wrote "folate" were defined "FA users." "Users" were defined as women who used either FA supplements, MV supplements, or both. Women who used single-vitamin or mineral supplements other than FA and women who did not take any dietary supplements were defined "nonusers." A total of 197 (3.7%) women did not answer the initial vitamin question and were also defined as nonusers.

Assessment of potential predictors of FA or MV use

Participants reported weight, height, physical activity, and smoking history in the baseline questionnaire, allowing

the calculation of body mass index (BMI), total metabolic equivalents (METs), and pack-years of smoking. BMI was calculated by dividing weight (kg) with height squared (m^2). METs were estimated by summing the METs from moderate and vigorous physical activity (hours per week multiplied by 3.5 and hours per week multiplied by 7.0, respectively).[14] Data on smoking were categorized as pack-years of ever-smoking where 1 pack-year was defined as smoking 20 cigarettes per day in 1 year.

To assess sociodemographic predictors of compliance among pregnancy planners, we examined the association between preconceptional FA or MV supplement use and the following variables: age (categorized as <25, 25–29, 30–34, and ≥35 years), schooling (primary and lower secondary school, high school, and other), vocational training (none, short [<3 years], medium [3–4 years], and long [>4 years]) and total monthly household income (<12,500, 12,500–24,999, 25,000–39,999, 40,000–64,999, and ≥65,000 DKK/month). We also examined lifestyle factors that could indicate intense efforts to conceive, such as intercourse frequency (categorized as <1, 1–3, and ≥4 times per week) and months attempting pregnancy at study entrance (0–1, 2–4, 5–6, and 7–12 months). Furthermore, compliance with other health recommendations, such as attending the national screening program for cervical cancer (pap smear) during the last 3 years (none vs one or more), smoking history (never smoked, <5, 5–9, and ≥10 pack-years), current alcohol intake (none, 1–3, 4–7, 8–14, and ≥15 drinks/week), BMI (<18.5, 18.5–24.9, 25–29.9, 30–34.9, and ≥35 kg/m^2), and engagement in physical activity (<10, 10–19, 20–39, and ≥40 METs/week), were also of interest. Finally, we studied the associations with previous spontaneous abortion (yes vs no), parity (nulliparous vs parous), and medical conditions that may increase health awareness in relation to pregnancy, which included hypertension (yes vs no), diabetes (yes vs no), thyroid disease (yes vs no), pelvic inflammatory disease (yes vs no), and infection with chlamydia (yes vs no).

Data analysis

We estimated the prevalence proportion (PP) of FA or MV users in each level of study predictors. Multivariate binomial regression was used to obtain prevalence proportion differences (PPDs) with 95% confidence intervals (CIs) for each predictor level in relation to the reference group. The multivariate analyses for the PP and PPD for each study predictor were adjusted for all other covariates. Stata statistical software (version 11.2; College Station, Texas) was used for all analyses.

Missing values ranged between 0.1% (schooling) and 8.7% (total monthly household income). Because 1540 (28.6%) participants were initially randomized to receive the short-form baseline questionnaire, they did not receive the questions about hypertension, diabetes, thyroid disease, and pelvic inflammatory disease. For the analyses, we used multiple imputation methods to impute all missing values.[15,16]

Results

Overall, 412 (7.7%) women used FA supplements exclusively, 1100 (20.4%) used MV supplements exclusively, and 1831 (34.0%) used both, yielding 62.1% users. A total of 82 (1.5%) used single vitamins or minerals other than FA, and 1958 (36.4%) did not use any dietary supplements, yielding 2040 (37.9%) nonusers of FA or MV supplements (Table 1). Among users, 57% had used supplements for less than 1 year.

Median age was 28 years for both users and nonusers. After mutual adjustment for covariates, higher age was associated with increased prevalence of FA or MV use (Table 2). Our data also indicate that women who finished high school were more likely to use FA or MVs compared with women with less schooling. Similarly, compared with women with a long vocational training (>4 years), PPDs for having no vocational training and a short (<3 years) vocational training were −5.7% and −7.1%, respectively, and compared with total monthly household of ≥65,000 DKK/month, PPD for <12,500 DKK/month was −8.7%.

While intercourse frequency ≥ 1 times/week was associated with an increased prevalence of FA or MV use, there was no clear association between months attempting to conceive at study entrance and FA or MV use. Furthermore, women who had pap smears once or more during the last 3 years were more likely to use FA or MV supplements compared with women who did not have pap smears (PPD was −9.7% for none versus one or more).

Smoking, alcohol use, and obesity (BMI ≥ 30 kg/m^2) were associated with a decreased prevalence of FA or MV use. Compared with women who never smoked, PPD for ≥10 pack-years of smoking was −11.3%. Compared with

Table 1 Dietary supplement use among 5383 pregnancy planners

	n	(%)
Users		
Folic acid exclusive	412	(7.7)
Multivitamins exclusive	1100	(20.4)
Folic acid and multivitamins	1831	(34.0)
Nonusers		
No use	1958	(36.4)
Other vitamins and minerals exclusive	82	(1.5)

Table 2 Prevalence proportions (PPs), prevalence-proportion differences (PPDs) with 95% confidence intervals (CIs) of folic acid or multivitamin supplement use in relation to sociodemographic, lifestyle, reproductive, and medical characteristics, respectively in 5383 women

Characteristics	Nonusers	Users	Unadjusted	Adjusted[a]		
	n (2040)	n (3343)	PP (%)	PP (%)	PPD (%)	95% CI
Sociodemographic factors						
Age, years						
<25	461	507	52.4	58.8	–5.2	–11.6, 1.1
25–29	882	1515	63.2	62.5	–1.6	–6.7, 3.5
30–34	518	1037	66.7	66.7	2.7	–2.3, 7.6
≥35	179	284	61.3	64.1	Ref	0
Schooling						
Primary/lower secondary school	387	405	51.1	56.6	–5.9	–10.0, –1.8
High school	1386	2612	65.3	62.5	Ref	0
Other	267	326	55.0	56.4	–6.1	–10.5, –1.8
Vocational training						
None	345	379	52.4	57.4	–5.7	–11.2, –0.2
Short (<3 years)	716	906	55.9	56.0	–7.1	–11.2, –3.0
Medium (3–4 years)	622	1,252	66.8	62.5	–0.6	–4.3, 3.0
Long (>4 years)	357	806	69.3	63.1	Ref	0
Household income, DKK/month						
<12,500	64	64	50.0	51.8	–8.7	–19.8, 2.2
12,500–24,999	280	370	56.9	61.8	1.2	–4.8, 7.3
25,000–39,999	552	805	59.3	61.8	1.2	–3.5, 5.8
40,000–64,999	854	1572	64.8	62.5	1.9	–3.5, 5.8
≥65,000	290	532	64.7	60.6	Ref	0
Lifestyle factors						
Intercourse frequency, times/week						
<1	392	495	55.8	54.7	–5.9	–10.3, –1.5
1–3	1236	2217	64.2	62.5	1.8	–1.5, 5.1
≥4	412	631	60.5	60.7	Ref	0
Attempting pregnancy, months						
0–1	868	1457	62.7	62.5	Ref	0
2–4	569	994	63.6	65.0	2.5	–0.5, 5.5
5–6	252	372	59.6	61.3	–1.1	–5.4, 3.2
7–12	351	520	59.7	61.3	–1.1	–4.9, 2.6
Pap smears[b], last 3 years						
None	545	556	50.5	62.5	–9.7	–13.2, –6.2
≥1	1495	2787	65.1	72.2	Ref	0
Smoking status, pack–years						
Never smoked	1052	2097	66.6	62.5	Ref	0
<5	526	726	58.0	58.1	–4.3	–7.6, –1.1
5–9	280	327	53.9	53.1	–9.4	–14.3, –4.7
≥10	182	193	51.5	51.2	–11.3	–16.8, –5.8
Alcohol intake, drinks/week						
None	601	1097	64.6	67.0	Ref	0
1–3	795	1404	63.9	62.5	–4.5	–7.5, –1.5
4–7	466	602	56.4	55.4	–11.6	–15.4, –7.8
8–14	130	204	61.1	60.3	–6.7	–12.4, –1.0
≥15	48	36	42.9	48.6	–18.4	–29.1, –7.6
BMI, kg/m^2						
<18.5	91	128	58.5	65.2	–2.7	–9.4, 4.0
18.5–24.9	1219	2,192	64.3	62.5	Ref	0
25–29.9	424	651	60.6	63.4	–1.8	–5.1, 1.5
30–34.9	185	239	56.4	60.1	–5.1	–10.1, –0.1
≥35	121	133	52.4	58.1	–7.1	–13.4, –0.8

(*Continued*)

Table 2 (*Continued*)

Characteristics	Nonusers	Users	Unadjusted	Adjusted[a]		
	n (2040)	n (3343)	PP (%)	PP (%)	PPD (%)	95% CI
Physical activity, METs/week						
<10	388	476	55.1	56.5	Ref	0
10–19	649	1080	62.5	62.0	5.5	1.5, 9.4
20–39	666	1230	64.9	62.5	5.9	2.0, 9.9
≥40	337	554	62.2	60.7	4.2	−0.5, 8.8
Reproductive history						
Previous spontaneous abortions (%)						
No	1848	2955	61.5	62.5	Ref	0
Yes	192	388	66.9	70.2	7.7	3.7, 11.8
Parous, ever had live birth						
No	1358	2234	62.2	54.2	Ref	0
≥1	682	1109	61.9	52.9	−1.2	−4.2, 1.9
Medical history						
Hypertension (%)						
No	1303	3116	62.1	62.5	Ref	0
Yes	137	227	62.4	63.4	1.1	−6.2, 8.4
Diabetes (%)						
No	2020	3300	62.0	54.2	Ref	0
Yes	20	43	68.3	59.7	13.8	−1.0, 28.6
Thyroid disease (%)						
No	1989	3263	62.1	62.5	Ref	0
Yes	51	80	61.1	59.4	−3.1	−15.4, 9.2
Pelvic inflammatory disease (%)						
No	1733	2905	62.6	62.5	Ref	0
Yes	307	438	58.8	60.8	−1.7	−5.9, 2.6
Chlamydia (%)						
No	1591	2661	62.6	54.2	Ref	0
Yes	449	682	60.3	54.0	0.1	−3.0, 3.3

Note: [a]Mutually adjusted for all other covariates. [b]National screening program for cervical cancer.

no alcohol intake PPD for ≥15 drinks/week was −18.4%. Compared with normal weight (BMI = 18.5–24.9 kg/m^2), PPDs of FA or MV use for BMIs of 30–34.9 and ≥35 kg/m^2 were −5.1% and −7.1%, respectively. Furthermore, women who engaged in physical activity were more likely to use FA or MVs than sedentary women (<10 METs/week).

Among women who had a previous spontaneous abortion, the PPD of FA or MV use was 7.7% higher than women who did not report having a previous abortion. However, being multiparous was not associated with increased FA or MV use.

Finally, there was no clear association between being diagnosed with either hypertension, diabetes, thyroid disease, pelvic inflammatory disease, or infections with chlamydia and preconceptional FA or MV supplement use.

Discussion

Prevalence proportions

Our data indicate that 62% of the 5383 pregnancy planners in this study adhere to the Danish FA recommendation. By interpreting "users" as compliers, we may overestimate the proportion of compliers following the FA recommendation. The questionnaire provided information on whether the women took an FA or MV supplement or not, but there was no information on the exact dose of FA ingested or the brand name of the FA or MV product. In addition, 20% of the women used MVs exclusively. Since MV supplements have no standard definitions,[17–20] it cannot be assumed that all MVs contained FA. However, most MVs on the Danish market contain 400 μg FA, especially those made for use during pregnancy.

No previous study has collected data on FA and MVs during the preconception period. The prevalence among pregnancy planners in our study is higher than the prevalence of preconception FA (12%–44%)[5–10] and MV use (11%)[8] reported by pregnant women in previous studies. However, these studies collected data during the second and third trimesters, which may decrease the recall of FA and MV use during the preconceptional period. In addition, the studies were relatively small, with 588 women attending an antenatal

clinic in Melbourne in gestational weeks 36–38,[8] 1541 women attending an antenatal ultrasound screening in gestational weeks 17–19 in Oslo,[7] and 327 pregnant women calling the California Teratogen Information Service in gestational week 13 in the US.[10] Consequently, prevalence estimates reported in these studies may have been imprecise.

The definition of compliance with the FA recommendation differs among studies. In the study by Forster et al,[8] 29% took prepregnancy FA supplements, but only 23% reported taking FA for at least 4 weeks before pregnancy. In the study by Braekke and Staff,[7] 17% of the women started the supplementation before the first day of the last menstruation. However, an additional 16% started FA supplementation at 0–4 weeks of gestation. Thus, 33% of the women started FA within 4 weeks of gestation. This proportion may be more comparable with our findings, since some "users" in our study may have been in gestational weeks 0–4 at baseline.

Women with unplanned pregnancies are less likely to use FA supplements before pregnancy. In the studies by Forster et al[8] and Goldberg et al,[10] the prevalence of unplanned pregnancy was 19% and 36%, respectively. These studies estimated FA use among all pregnancies (planned and unplanned), making their results difficult to compare with ours. In the study by Braekke and Staff,[7] there was no information about unplanned pregnancies or contributions of FA from MV supplements, both increasing the possibility of underestimation of the proportions of FA users in this study.

In Denmark, the largest and most recent study of lifestyle factors in relation to compliance with the FA recommendation was conducted among 18,294 pregnant women (gestational weeks 10–12) enrolled in the DNBC during 2000–2002.[5] In this study, FA supplements were derived from single FA tablets, MV tablets, and vitamins made for pregnant or lactating women. Compliance was defined as taking 320 μg FA per day, from 4 weeks before the date of the last menstrual period until gestation week 6. A total of 76% of the pregnancies were planned, and the overall proportion of women who complied with the recommendation was only 16% among planners. During the 10-year time period to the present study, there may have been an increase in the awareness as well as more available information about prepregnant recommendations, which may partly explain the observed increase in the prevalence of compliers with the FA recommendation found in this study. In contrast to the DNBC, our data on supplement use and potential predictors were reported during the preconceptional period, and information was recorded prospectively, which increases the validity of the study. On the other hand, Snart-Gravid study participants may be more health-conscious than the general population of pregnancy planners, possibly overestimating the prevalence of FA or MV use. Even so, it is unlikely that an overestimation of supplement use would meaningfully distort the associations between predictors and FA or MV use. In addition, differences in sociodemographic factors among Snart-Gravid study participants compared with the general population may also contribute to an overestimation of FA or MV use.

Predictors of FA or MV use

Our study agrees with previous studies that found that maternal age,[5,7,10] education,[5,6,10] income,[6,8] smoking,[5,8] and BMI[10] were associated with preconception FA use. Although a history of childbirth may increase awareness of preconceptional FA use, previous studies found that a previous childbirth either decreased the likelihood of FA use in subsequent pregnancies or was not related to FA use.[5,7,8] We found only little association between parity and FA use in our study.

The positive associations between previous spontaneous abortion and intercourse frequency and FA or MV use found in our study have not been reported previously. These findings may indicate a relation between a high desire to conceive and increased knowledge about the preconceptional FA guidelines.

While lifestyle recommendations during pregnancy are widely available, less advice is available for women trying to conceive. In addition to the FA recommendation, Danish women planning a pregnancy are advised to avoid alcohol altogether.[1] A total of 32% reported no alcohol intake in our study, which was slightly more than the 12% reported from the DNBC previously.[21] In addition, we found the lowest proportion of FA or MV users among women with the highest alcohol intake. Together with the concern that excessive alcohol intake impedes the normal bioavailability and metabolism of folate,[22] these results underscore the need to target women at high risk of not following the FA recommendation, as well as those not following other preconceptional guidelines.

Being diagnosed with a chronic disease has previously been associated with increased dietary supplement use in general.[23] Pregnancy planners with a chronic illness might be more prone to use FA or MVs because of increased health awareness, but we found little association between a diagnosis of hypertension, diabetes, thyroid disease, pelvic inflammatory disease or infections with chlamydia and preconceptional use of FA or MVs.

Conclusion

Our findings indicate that more than one-third of the pregnancy planners in this study do not follow the preconceptional FA recommendation. We found that pregnancy planners with other risky behaviors, such as smoking and alcohol use, obesity, and being physically inactive, are less likely to comply with the FA recommendation or use MVs.

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Disclosure

The authors report no conflicts of interest in this work.

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Appendix III: Paper II

Folic acid supplement use and menstrual cycle characteristics: a cross-sectional study of Danish pregnancy planners

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Abstract

Purpose To examine the association between folic acid (FA) supplement use obtained through either single FA tablets or multivitamins (MVs) and menstrual cycle characteristics among 5,386 women aged 18-40 years, enrolled in an Internet-based study of Danish women attempting pregnancy during 2007-2011.

Methods In a cross-sectional study, we used logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of FA supplementation with menstrual cycle regularity, short (<27 days) and long (≥30 days) cycle length, and duration and intensity of menstrual flow.

Results Compared with non-use, FA supplements use was associated with reduced odds of short cycle length [OR=0.76, 95% CI: 0.63-0.93] and a trend towards reduced odds of long cycle length [OR=0.94, 95% CI: 0.81-1.10] compared with cycle length of 27-29 days. The associations were stronger among 18-30 year-old women [OR=0.67, 95% CI: 0.52-0.85 for short cycle length and OR=0.83, 95% CI: 0.70-0.99 for long cycle length] and among nulliparous women [OR=0.63, 95% CI: 0.50-0.81 for short cycle length and OR=0.92, 95% CI: 0.76-1.10 for long cycle length]. We found no clear association between FA supplementation and cycle regularity.

Conclusion Pre-conception FA supplementation was inversely associated with both short and long menstrual cycle length.

Key words folic acid, menstrual cycle characteristics, vitamin supplementation and preconceptional supplements.

Abbreviations

FA	folic acid
MV	multivitamin
OR	odds ratio
CI	confidence interval

Introduction

The menstrual cycle is mediated by endogenous hormones produced by feedback loops of the hypothalamic-pituary-ovarian axis.[1] Previous studies have associated cycle irregularity,[2, 3] and both long[2, 3] and short[3-5] cycle length with reduced ability to conceive. However, the causes of cycle variability are incompletely understood.[6]

Epidemiologic follow-up and cross-sectional studies have reported associations between menstrual cycle function and various reproductive[6-8] and lifestyle[6, 8-12] factors. The most consistent predictor of cycle length is age,[8] as cycles become shorter when women get older.[13] Other identified predictors of short cycle length, such as smoking,[7, 8, 11, 14] alcohol consumption,[11] and caffeine consumption[15] are modifiable behaviors that may affect the hormonal balance. Another modifiable behavior closely related to planning a pregnancy is preconception supplementation with 400 µg folic acid (FA), taken either as folic acid tablets or in multivitamin supplements (MVs).[16] Folate status may play an essential role in hormonal balance and follicular development, possibly through the metabolism of homocysteine.[17-19] Previous studies have associated FA supplementation with reduced risk of ovulatory infertility[20] and decreased homocysteine in the follicular fluid, with a subsequent higher degree of follicular development and oocyte maturity in women undergoing IVF.[21-23] In addition, FA supplementation has been associated with improved ovarian responsiveness to follicle-stimulation hormone (FSH) in infertility patients with the MTHFR 677C>T mutation. This mutation leads to elevated concentrations of homocysteine and decreased availability of methyl groups for DNA, protein, and lipid methylation.[24]

To the extent that FA supplementation, obtained through either single FA supplements or MVs improves hormonal balance and follicular development, it may enhance menstrual cycle function. Only one study, a randomized trial has assessed the association between preconception FA-containing MV supplementation and menstrual cycle characteristics among women without

recognized fertility problems. Dudás and Czeizel[25] demonstrated improved cycle regularity during supplementation with FA-containing MVs compared with trace element supplementation among 1,000 women enrolled in a Hungarian study of periconceptional FA-containing MV use for prevention of neural tube defects during 1989 and 1990.

In the current study, we used cross-sectional data from women enrolled in a prospective cohort study of pregnancy planners to examine the association between FA supplementation obtained through single FA tablets or MVs and menstrual cycle regularity, cycle length, as well as duration and intensity of menstrual flow.

Methods

Study design

We performed a cross-sectional analysis of baseline data from the Danish Pregnancy Planning Study ("Snart-Gravid"), an internet-based prospective cohort study of women planning a pregnancy between 2007 and 2011. Recruitment methods have been described previously.[26-28] Before enrolment, participants read a consent form and completed an online screening questionnaire to confirm eligibility. Participants provided a valid e-mail address and their Civil Personal Registration (CPR) number. Eligible women were invited to complete an internet-based baseline questionnaire and bimonthly follow-up questionnaires for 12 months or until conception occurred. The baseline questionnaire collected information on socio-demographic factors, reproductive and medical history, and lifestyle behaviors. Participants were initially randomized to receive either a short- or a long-form baseline questionnaire, with some questions asked of only 50% of the cohort during the first six months of enrollment. Completion rates and missing data proportions were similar for both questionnaire versions.[27]

Study population and study period

Eligible women were Danish residents aged 18-40 years, in a stable relationship with a male partner, attempting to conceive for no more than 12 months, and not receiving fertility treatment at study entry. From June 2007 to August 2011, 5,387 women enrolled in the study. One woman who was already pregnant (14 weeks) was excluded. In total, 5,386 eligible women enrolled in the study.

Assessment of menstrual cycle characteristics

The menstrual cycle characteristics examined in the current study were cycle regularity, cycle length, duration of menstrual bleeding, and intensity of menstrual bleeding. Women who responded 'yes' to the question "are your menstrual periods regular, e.g. you can usually predict about when your next period will start?" were considered to have regular cycles.

Among the 4,041 women with regular cycles, we assessed cycle length, defined as "the number of days from the first day of a menstrual period to the first day of the next menstrual period" and categorized as short [<27 days], normal [27-29 days], or long [≥30 days].[5] Duration and intensity of menstrual bleeding were assessed among women with regular cycles by means of the questions, "How many days does your period usually flow (bleeding not spotting)?", in categories of short [<3 days], normal [3-4 days], long [5-6 days], and very long [>6 days], and "How would you classify the total amount of your menstrual flow?", in categories of light [≤10 pads or tampons/menstrual cycle], moderate [11-20 pads or tampons/menstrual cycle], heavy [21-30 pads or tampons/menstrual cycle], and very heavy [>30 pads or tampons/menstrual cycle].

Assessment of preconception FA and MV use

In the baseline questionnaire respondents were asked, "Do you take vitamins on a regular basis - daily or almost every day"?, "How long have you been taking vitamins on a regular basis - less than one year, 1-5 years, more than 5 years and 'don't know'"?, and "Which of the following vitamins or

minerals do you take on a regular basis - MVs, vitamin A, beta-carotene, vitamin B, vitamin C, vitamin D, vitamin E, FA, calcium, magnesium, selenium and 'other'"? Participants who reported 'multivitamin' or wrote the name of a MV product were classified as "MV users". Similarly, participants who reported 'folic acid' or wrote 'folate' were classified as "FA users". Most MVs marketed in Denmark contain 400 μg of FA, especially those made for use during pregnancy. Therefore, we created a single binary exposure variable defined as "FA supplementation", which was set to 1 for women who were FA users, MV users, or both. For women who used single vitamin or mineral supplements other than FA and women who did not take any dietary supplements, the exposure variable "FA supplementation" was set to zero and was defined as "non-use".

Assessment of covariates

From the baseline questionnaire, we obtained data on previously recognized correlates of menstrual cycle characteristics or correlates of FA or MV supplement use, including age, education, intercourse frequency, participation in the national screening program for cervical cancer (pap smear) during the last three years, history of miscarrige, parity, smoking, alcohol use, body mass index (BMI), physical activity level, caffeine intake, and last method of contraception. We calculated BMI from self-reported weight and height (kg/m^2). Total metabolic equivalents (METs) were estimated by summing the METs from moderate physical activity (hours per week multiplied by 3.5) and vigorous physical activity (hours per week multiplied by 7.0).[29] Self-reported smoking was categorized as pack-years of ever smoking, with one pack-year defined as smoking 20 cigarettes per day for one year.

Data analysis

We examined the association between FA supplementation and cycle regularity using logistic regression to estimate odds ratios (ORs) with 95% confidence intervals (CIs). For the analysis of cycle length and duration and intensity of menstrual bleeding, we restricted the analysis to women with regular cycles. We used polytomous logistic regression to estimate ORs with 95% CIs for the association of FA supplementation with short (<27 days) and long (≥30 days) cycle length, compared with normal cycle length (27-29 days). We also estimated ORs with 95% CIs for the association of FA supplementation with short (<3 days), long (5-6 days), and very long (>6 days) duration of menstrual bleeding, compared with normal (3-4 days) duration of bleeding, and with light (≤10 pads or tampons/menstrual cycle), heavy (21-30 pads or tampons/menstrual cycle), and very heavy (>30 pads or tampons/menstrual cycle) intensity of menstrual bleeding, compared with moderate (11-20 pads or tampons/menstrual cycle) intensity of bleeding. The multivariate analyses were adjusted for age (<25, 25-29, 30-34, ≥35 years), parity (parous vs. nulliparous), previous miscarriage (yes vs. no), BMI (<18.5, 18.5-24.9, 25-29, 30-34.9, ≥35 kg/m^2), pack-years of smoking (never smoked, <5, 5-9, ≥10 pack-years), alcohol intake (none, 1-3,4-7, 8-14, ≥15 drinks/week), caffeine intake (<100, 100-199, 200-299, ≥300 mg/day), physical activity (<10, 11-19, 20-39, ≥40 METs/week) and last method of contraception (barrier methods, oral contraceptives, other methods). For the analyses of duration of menstrual flow, we also adjusted for intensity of flow, and vice versa.

In a subanalysis, we stratified the data according to parity (parous and nulliparous) and age (18-30 and 31-40 years) at study entry. To eliminate the effect of hormonal contraceptives on menstrual cycle function, we also evaluated the associations after restricting the study population to women who reported a non-hormonal method of last contraception.

Because 1,540 (28.6%) participants were randomized to receive the short-form baseline questionnaire, they did not receive the questions about the amount of menstrual flow. Therefore, the proportion of missing data for the amount of menstrual flow was 28.8%, but those missing this information were a random subset of enrollees by design. One hundred ninety-seven (3.7%) women did not answer the initial vitamin question. The amount of missing data for covariates and menstrual cycle characteristics ranged between 0.1% and 2.9%. We used multiple imputation methods to impute missing values.[30, 31] STATA® statistical software (version 11.2) was used for all analyses.

Results

Overall, 3,344 (62.1%) women used either FA supplements exclusively (7.7%), MV supplements exclusively (20.4%), or both (34.0%). Another 2,042 (37.9%) women did not use any dietary supplements (36.4%) or used single vitamins or minerals other than FA (1.5%). Among users, 37.7% took supplements for one year or more. Characteristics of the study population according to FA supplementation are presented in Table 1. Users were more likely to be older, have higher education, be non-smokers, be physically active, consume less alcohol and caffeine, and have lower BMI. Users were also more likely to have greater intercourse frequency, a history of miscarriage, regular pap smears, and to have used barrier methods as their last method of contraception.

Association between FA supplementation and cycle regularity

A total of 1,345 (25.0%) women reported irregular periods. Overall, we found no association between FA supplementation and cycle regularity (adjusted OR=1.00, 95% CI: 0.88-1.14). Compared with no use, FA supplementation was associated with slightly reduced odds of having irregular cycles among parous women (adjusted OR=0.84, 95% CI: 0.67-1.07) (Table 2).

Association between FA supplementation and cycle length

Among the 4,041 regularly-cycling women, 597 (14.8%) reported cycles of <27 days, 2214 (54.8%) reported cycles of 27-29 days, and 1,230 (30.4%) reported cycles of ≥30 days (Table 3). Overall, compared with non-use, FA supplementation was associated with reduced odds of short cycle length (<27 days) (adjusted OR=0.76, 95% CI: 0.63-0.93) and a trend toward reduced odds of long cycle length (≥30 days) (adjusted OR=0.94, 95% CI: 0.81-1.10). In the stratified analyses, we found a stronger association between FA supplementation and short cycle length among nulliparous women (adjusted OR=0.63, 95% CI: 0.50-0.81) than among parous women (adjusted OR=1.14, 95% CI: 0.81-1.61). Also, we found a stronger association between FA supplementation and short cycle length among women aged 18-30 years (adjusted OR=0.67, 95% CI: 0.52-0.85) than among women aged 31-40 years (adjusted OR=1.00, 95% CI: 0.73-1.40). Compared with non-use, FA supplementation among women aged 18-30 years also had reduced odds of having long cycle length (adjusted OR=0.83, 95% CI: 0.70-0.99), whereas users aged 31-40 years had slightly increased odds of having long cycle length (adjusted OR=1.27, 95% CI: 0.95-1.70). The overall associations were consistent after restricting the analyses to women who reported a non-hormonal method of last contraception (N=2,547) (adjusted OR=0.76, 95% CI: 0.63; 0.93 for short cycle length and adjusted OR=0.95, 95% CI: 0.81; 1.10 for long cycle length).

In secondary analyses, we found an even stronger association between FA supplementation and cycle length of <25 days (adjusted OR=0.65, 95% CI: 0.48-0.89). Adjusted OR with 95% CI of having cycle length of 25-26 days, 30-31 days, 32-33 days and ≥34days were 0.84 (0.65-1.07), 0.92 (0.77-1.08), 0.86 (0.63-1.18), and 1.21 (0.86-1.70), respectively compared with cycle length of 27-29 days, data not shown.

Association between FA supplementation and duration and intensity of menstrual bleeding

Among the 4,041 regularly-cycling women, 367 (9.1%) reported a menstrual flow of <3 days, 2,158 (53.4%) reported a menstrual flow of 3-4 days, 1,368 (33.9%) reported a menstrual flow of 5-6 days, and 148 (3.7%) reported a menstrual flow of >6 days (Table 4). Among these women, 993 (24.6%) reported 'light' menstrual bleeding (≤10 pads or tampons/menstrual cycle), 2,386 (59.0%) reported 'moderate' menstrual bleeding (11-20 pads or tampons/ menstrual cycle), 589 (14.6%) reported 'heavy' bleeding (21-30 pads or tampons/ menstrual cycle), and 73 (1.8%) reported 'very heavy' bleeding (>30pads or tampons/ menstrual cycle) (Table 5). Overall, there was no clear association between FA supplementation and duration and intensity of menstrual flow. For the extremes of duration of flow (>6 days) and intensity of flow (>30 pads or tampons/menstrual cycle), the ORs indicated little association, but the estimates were imprecise.

Discussion

Main findings

In this cross-sectional study of Danish pregnancy planners aged 18-40 years, FA supplementation was associated with reduced odds of short cycle length (<27 days) and a trend towards reduced odds of long (≥30 days) cycle length. The associations were strongest among 18-30 year-old and nulliparous women.

Strengths and limitations

The main limitation of our study is the cross-sectional nature of the baseline data. Information on supplement use was ascertained simultaneously with menstrual cycle characteristics. Although 38% of users in our study reported using supplements for at least one year, we have no information on duration of use of the specific supplements, the exact dose of FA ingested, or brand name of all FA or MV products. Thus, we cannot determine the exact temporal sequence FA supplementation in relation to menstrual cycle characteristics.

Some participants may have reported themselves as "users" simply because they were asked or were planning to begin supplementation within a short time. Therefore, some users in this study may be misclassified. In addition, MV supplements have no standard definitions and it is uncertain whether all MVs contained FA, which may also lead to some misclassification. However, most MVs marketed in Denmark contain 400 μg FA, especially those made for use during pregnancy. We see no reason, however, why misclassification of information on vitamin use would differ between subgroups of women with different menstrual cycle characteristics or why information on cycle length would differ between FA supplement users and non-users. Therefore, any misclassification errors in assessing supplement use should bias results toward the null. Also, because most participants used FA supplements in combination with MVs, the effect attributable to FA alone or to the synergistic effect of MVs could not be determined in this study.

We did not validate the accuracy of menstrual cycle length reported by participants in this study. However, a sub-analysis of participants from the Snart-Gravid study population found modest agreement between menstrual cycle length reported on the baseline questionnaire and on a subsequent follow-up questionnaire. [5]

Previously identified predictors of FA or MV use indicate various demographic, lifestyle, and behavioral differences between FA supplement users and non-users, *e.g.*, users were less likely to smoke and consume alcohol.[32] Such health behaviors also may be related to menstrual cycle function which may confound the estimates.[7, 8, 11, 14] In the regression models we adjusted for several lifestyle factors, and it seems unlikely that an underlying health factor would meaningfully affect the associations. In addition, we stratified by age and parity in the analysis to avoid confounding by factors related to age or pregnancy and childbirth. However, we have no information about the length of the interpregnancy interval among parous women. Thus, menstrual

cycle length may be influenced by recent child birth or breast feeding, which may explain some of the differences in the estimates between parous and nulliparous women.

Because Snart-Gravid participants may have been more health-conscious than women planning pregnancy in general, we may have observed a higher prevalence of FA and MV users compared with the general population of Danish pregnancy planners. However, our comparisons were made within the population of study participants (rather than between study participants and pregnancy planners who did not participate in the study), and the internal validity of the study should not be affected by differences between the study participants and the general population.[33] In addition, participants in the Snart-Gravid study were enrolled without regard to supplement use or menstrual cycle characteristics. Thus, at study entry participants had no knowledge that the association between supplement use and menstrual cycle characteristics would be examined, reducing the possibility of bias.

Interpretation

To our knowledge, only two studies have evaluated FA-containing MV supplementation and menstrual cycle characteristics.[25, 34] In agreement with our results, Westphal *et al*. [34] found normalization of both long and short cycles after three months of supplementation with MVs containing 400 µg FA among 93 women who had tried unsuccessfully to conceive for 36 months. Still, it is difficult to compare the two studies because of differences in study designs, definitions of short and long cycle length, and study populations. The study by Westphal *et al*. was a double-blind trial examining changes in menstrual cycle characteristics during supplementation with a known MV product. Our study population was somewhat larger and had no known fertility problems.

In a randomized trial of 1000 women, Dudás and Czeizel[25] found no change in cycle length during supplementation with MVs containing 800 µg FA. Women took supplements until

conception occurred or for 12 months, allowing observation of changes in menstrual cycle characteristics during supplementation. Little association was found between MV supplementation and length of menstrual bleeding. In contrast to our findings, MV supplementation appeared to improve cycle regularity among women who reported irregular cycles at study entry.

Elevated concentrations of homocysteine as a consequence of low folate status have been suggested to contribute to reduced ovarian responsiveness to FSH in infertility patients undergoing simulation with FSH.[24] In addition, results from the Nurses' Health Study have suggested that FA contributed to the association between MV use and reduced risk of ovulatory infertility.[20] Furthermore, Westphal *et al.*[34] demonstrated increased progesterone levels in the luteal phase after supplementation with MVs.

Conclusion

In conclusion, it appears plausible that homocysteine metabolism and hormonal balance may have a role in accounting for the inverse associations between FA supplementation and cycle length.

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Details of ethics approval

The Snart Gravid study was approved by the Danish Data Protection Board (2006-41-6864) and no further approval is required according to the Danish Ethical Review System. The study was approved by the Institutional Review Board at Boston University, and consent was obtained from all participants via the internet.

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Table 1. Baseline characteristics of 5,386 women according to folic acid (FA) supplementation.

Characteristic	Vitamin supplement use	
	FA supplement use	Non-use[a]
Number of women	3344 (62.1%)	2042 (37.9%)
Age, years (mean)	28.6	27.9
BMI, kg/m^2 (mean)	24.1	24.7
Pap smear[b], once or more during the last three years (%)	83.3	73.2
Higher education, >4 years (%)	24.2	17.4
Parous, ever had live birth (%)	33.2	33.4
Previous miscarriage, yes (%)	11.7	9.5
Intercourse frequency, ≥1 times/week (%)	85.3	80.8
Caffeine intake, ≥300 mg/day (%)	11.5	14.2
Current smoker, yes (%)	9.9	21.9
Pack-years of smoking (mean)	5.2	5.9
Alcohol intake, drinks/week (mean)	2.5	3.1
Physical activity, h/week (mean)	25.0	23.8
Last method of contraception		
Hormonal contraceptives (%)	52.2	53.5
Barrier methods (%)	28.0	24.2

[a]Non-use includes no supplement use (36.4%) and use of single vitamin/mineral supplements other than FA acid (1.5%).
[b]Attending the national screening program for cervical cancer (pap smear).

Table 2. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for irregular cycles compared with regular cycles, by parity, age, and folic acid (FA) supplementation (N=5,386).

			All	**Regular cycles (Ref.)**		**Irregular cycles**
			N (%)	n	n	OR (95% CI)
Overall	FA supplement use	Crude	3,344 (62.1)	2,516	828	0.97 (0.85-1.10)
		Adjusted*				1.00 (0.88-1.14)
	Non-use		2,042 (37.9)	1,525	517	1 (Ref.)
Parous	FA supplement use	Crude	1,111 (20.6)	871	240	0.83 (0.66-1.04)
		Adjusted*				0.84 (0.67-1.07)
	Non-use		682 (12.7)	514	168	1 (Ref.)
Nulliparous	FA supplement use	Crude	2,233 (41.5)	1,645	588	1.04 (0.89-1.21)
		Adjusted*				1.07 (0.91-1.26)
	Non-use		1,360 (25.2	1,011	349	1 (Ref.)
18-30 years	FA supplement use	Crude	2,294 (42.6)	1,661	633	1.01 (0.87-1.17)
		Adjusted*				1.02 (0.88-1.19)
	Non-use		1,485 (27.6)	1,077	408	1 (Ref.)
31-40 years	FA supplement use	Crude	1,050 (19.5)	855	195	0.93 (0.71-1.20)
		Adjusted*				0.89 (0.68-1.16)
	Non-use		557 (10.3)	448	109	1 (Ref.)

*Overall: Adjusted for age, BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

*Parity: Adjusted for age, BMI, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

*Age: Adjusted for BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity and last contraception method used.

Table 3. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for short and long cycle length compared with normal cycle length, by parity, age, and folic acid (FA) supplementation among women with regular cycles (N=4,041).

				Cycle length (days)				
			All	27-29 (Ref.)	<27		≥30	
			N (%)	n	n	OR (95 % CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,417	336	0.74 (0.61-0.90)	763	0.93 (0.80-1.07)
		Adjusted*				0.76 (0.63-0.93)		0.94 (0.81-1.10)
	Non-use		1,525 (37.7)	797	261	1 (Ref.)	467	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	494	128	1.02 (0.74-1.43)	249	0.96 (0.75-1.24)
		Adjusted*				1.14 (0.81-1.61)		1.01 (0.78-1.32)
	Non-use		514(12.7)	289	73	1 (Ref.)	152	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	923	208	0.63 (0.50-0.80)	514	0.90 (0.76-1.08)
		Adjusted*				0.63 (0.50-0.81)		0.92 (0.76-1.10)
	Non-use		1,011 (25.0)	508	188	1 (Ref.)	315	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	929	197	0.65 (0.51-0.83)	535	0.84 (0.71-1.00)
		Adjusted*				0.67 (0.52-0.85)		0.83 (0.70-0.99)
	Non-use		1,077 (26.7)	530	179	1 (Ref.)	368	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	488	139	0.93 (0.68-1.28)	228	1.24 (0.94-1.65)
		Adjusted*				1.00 (0.73-1.40)		1.27 (0.95-1.70)
	Non-use		448 (11.0)	267	82	1 (Ref.)	99	1 (Ref.)

*Overall: Adjusted for age, BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

*Parity: Adjusted for age, BMI, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

*Age: Adjusted for BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, and last contraception method used.

Table 4. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for duration of menstrual flow of <3, 5-6, and <6 days compared with normal duration of menstrual flow (3-4 days), by parity, age, and folic acid (FA) supplementation among women with regular cycles (n=4,041).

				Duration of menstrual flow (days)						
			All	3-4 (Ref.)	<3		5-6		>6	
			N (%)	n	n	OR* (95 % CI)	n	OR (95% CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,324	234	1.10 (0.87-1.38)	859	1.06 (0.92-1.22)	99	1.27 (0.89-1.81)
		Adjusted*				1.13 (0.89-1.45)		1.04 (0.89-1.21)		1.20 (0.81-1.77)
	Non-use		1,525 (37.7)	834	133	1 (Ref.)	509	1 (Ref.)	49	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	430	61	0.98 (0.63-1.53)	332	1.07 (0.84-1.35)	48	1.54 (0.89-2.69)
		Adjusted*				1.11 (0.67-1.83)		1.05 (0.81-1.36)		1.46 (0.74-2.89)
	Non-use		514 (12.7)	264	37	1 (Ref.)	194	1 (Ref.)	19	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	894	173	1.15 (0.87-1.50)	527	1.07 (0.90-1.28)	51	1.08 (0.68-1.72)
		Adjusted*				1.11 (0.85-1.48)		1.09 (0.91-1.32)		1.15 (0.72-1.86)
	Non-use		1,011 (25.0)	570	96	1 (Ref.)	315	1 (Ref.)	30	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	861	141	1.02 (0.78-1.35)	594	1.11 (0.94-1.31)	65	1.12 (0.74-1.40)
		Adjusted*				1.07 (0.80-1.45)		1.08 (0.90-1.30)		1.06 (0.68-1.67)
	Non-use		1,077 (26.7)	582	93	1 (Ref.)	363	1 (Ref.)	39	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	463	93	1.24 (0.83-1.86)	265	0.98 (0.76-1.27)	34	1.84 (0.89-3.79)
		Adjusted*				1.30 (0.81-2.01)		0.91 (0.70-1.23)		2.07 (0.89-4.45)
	Non-use		448 (11.0)	252	40	1 (Ref.)	146	1 (Ref.)	10	1 (Ref.)

*Overall: Adjusted for age, BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

*Parity: Adjusted for age, BMI, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

*Age: Adjusted for BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and intensity of menstrual flow.

Table 5. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for intensity of menstrual flow of ≤10, 21-30 and 30 pads or tampons/menstrual cycle compared with moderate flow (11-20 pads or tampons/day), by parity, age, and folic acid (FA) supplementation among women with regular cycles (n=4,041).

				Intensity of menstrual flow (pads or tampons/menstrual cycle)						
			All	11-20 (Ref.)	≤10		21-30		>30	
			N (%)	n	n	OR* (95% CI)	n	OR (95% CI)	n	OR (95% CI)
Overall	FA supplement use	Crude	2,516 (62.3)	1,519	585	0.85 (0.71-1.01)	361	0.91 (0.73-1.12)	51	1.25 (0.59-2.65)
		Adjusted*				0.81 (0.66-1.00)		0.92 (0.75-1.15)		1.37 (0.63-2.97)
	Non-use		1,525 (37.7)	867	408	1 (Ref.)	228	1 (Ref.)	22	1 (Ref.)
Parous	FA supplement use	Crude	871 (21.6)	544	123	0.76 (0.53-1.09)	172	0.91 (0.65-1.28)	32	1.37 (0.48-3.90)
		Adjusted*				0.78 (0.53-1.15)		0.91 (0.64-1.29)		1.41 (0.44-4.51)
	Non-use		514 (12.7)	300	96	1 (Ref.)	106	1 (Ref.)	12	1 (Ref.)
Nulliparous	FA supplement use	Crude	1,645 (40.7)	975	462	0.88 (0.72-1.06)	189	0.90 (0.69-1.18)	19	1.09 (0.45-2.63)
		Adjusted*				0.82 (0.66-1.03)		0.92 (0.68-1.23)		1.68 (0.60-4.67)
	Non-use		1,011 (25.0)	567	312	1	122	1 (Ref.)	10	1 (Ref.)
18-30 years	FA supplement use	Crude	1,661 (41.1)	994	396	0.89 (0.72-1.10)	245	0.99 (0.78-1.25)	26	1.20 (0.59-2.45)
		Adjusted*				0.88 (0.70-1.12)		1.04 (0.81-1.32)		1.57 (0.72-3.45)
	Non-use		1,077 (26.7)	617	294	1 (Ref.)	153	1 (Ref.)	13	1 (Ref.)
31-40 years	FA supplement use	Crude	855 (21.2)	525	189	0.76 (0.54-1.06)	116	0.74 (0.50-1.10)	25	1.28 (0.28-5.67)
		Adjusted*				0.67 (0.45-0.98)		0.68 (0.45-1.03)		1.02 (0.19-5.42)
	Non-use		448 (11.0)	250	114	1 (Ref.)	75	1 (Ref.)	9	1 (Ref.)

*Overall: Adjusted for age, BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

*Parity: Adjusted for age, BMI, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

*Age: Adjusted for BMI, parity, previous miscarriage, pack-years of smoking, alcohol and caffeine intake, physical activity, last contraception method used, cycle length, and duration of menstrual flow.

Appendix IV: Paper III

Original article

Folic Acid Supplementation and Fecundability: A Danish Prospective Cohort Study

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Conflicts of interest

The authors declare to have no conflicts of interest.

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Abstract

Background/Objectives: Periconceptional folic acid (FA) supplementation reduces the risk of neural tube defects and has been associated with ovulatory function. However, only two studies have associated supplementation with multivitamins (MVs) that contained FA with increased pregnancy rates. We aimed to examine the association between FA supplementation (obtained either through single FA tablets or MVs) and fecundability.

Subjects/Methods: A prospective cohort study of 3 895 Danish women who were planning a pregnancy between 2007 and 2011.We estimated fecundability ratios (FR) and 95% confidence intervals (CI) in relation to FA supplementation (either through single FA tablets or MV) using a proportional probabilities regression model, with adjustment for potential socio-demographic, reproductive, and lifestyle confounders. In stratified analyses, we also estimated FR with 95% CI in relation to FA supplementation for women with regular and irregular cycles, respectively, and for women with short [<27 days], medium [27-29 days], and long cycles [≥30 days], respectively.

Results: FA supplementation was associated with increased fecundability (FR = 1.15, 95% CI: 1.06 to 1.25), compared with non-use. The adjusted FR for FA supplement use relative to non-use were1.35 (95% CI: 1.12 to 1.65) and 1.11 (95% CI: 1.01 to 1.22) for women with irregular and regular cycles, respectively, and 1.36 (95% CI: 0.95 to 1.95), 1.10 (95% CI: 0.98 to 1.22), and 1.24 (95% CI: 1.10 to 1.41) for women with short [<27 days], medium [27-29 days], and long cycles [≥30 days], respectively.

Conclusions: FA supplementation was associated with increased fecundability and this association appeared to be stronger among women with irregular cycles and among women with either short or long cycle length.

Key words: Fecundability, fertility, vitamin supplement use, cohort study

INTRODUCTION

Periconceptional folic acid (FA) supplementation is known to reduce the risk of neural tube defects[1,2] and other congenital malformations.[3,4] In many countries, including Denmark, women planning to conceive therefore are advised to take a daily supplement of 400 µg of FA.[5]

Folates in the form of tetrahydrofolates are essential cofactors for several one-carbon units transfer reactions, including the biosynthesis of methionine from homocysteine, and are required for the biosynthesis of purines, thymidylate, and DNA.[6,7] Although little is known about possible beneficial effects of FA supplementation on fecundability (defined as the probability of conceiving during a single menstrual cycle with unprotected intercourse), inadequate intake of dietary folate or FA supplements may play an essential role in the hormonal balance and follicular development.[6-10] Cross-sectional studies of women undergoing *in vitro* fertilization have associated FA supplementation with increased folate and decreased homocysteine concentrations in the follicular fluid and with a higher degree of oocyte maturity.[11,12] In a follow-up study of women without a history of infertility, Chavarro *et al.*,[13] reported that regular use of multivitamins (MV) (including FA) was inversely associated with ovulatory infertility. Also, Gaskins *et al.*,[14] reported that a diet high in synthetic FA reduced the risk of anovulatory cycles among women without a history of infertility. Finally, preconceptional FA and MV use has been associated with increased progesterone levels in the luteal phase,[14,15] improved menstrual cycle regularity,[16] and normalization of cycle length,[15] which all have been associated with fecundability.[17,18]

Two randomized trials, one enrolling 35 women with fertility problems[15] and one enrolling 7 905 women without fertility problems,[19] reported higher pregnancy rates among users of MV (including FA) compared with placebo tablets, indicating that FA supplementation to some extent increase fecundability. Although national surveys of dietary habits have indicated insufficient dietary folate intake among women of childbearing age,[20] Denmark has not introduced a mandatory national

program to fortify food with FA. Thus, whether fecundability can be improved by FA supplementation is of particular public health interest. In this study, we evaluated the association between FA supplementation, obtained either through single FA tablets or MV and fecundability among women enrolled in a Danish internet-based pregnancy planning study.

SUBJECTS AND METHODS

The Danish Pregnancy Planning Study

Data for this study were collected as part of the Danish Pregnancy Planning Study ('Snart-Gravid.dk'), an internet-based prospective cohort study of women planning a pregnancy. Recruitment methods have been described previously.[21-23] Briefly, enrolment and data collection via self-administered questionnaires were conducted on the study website (www.snart-gravid.dk). Before enrolment, potential participants read a consent form and completed an online screening questionnaire to confirm eligibility. Participants also provided a valid e-mail address and their Civil Registration Number.

Eligible women were invited to complete a baseline questionnaire and bimonthly follow-up questionnaires for 12 months or until conception occurred. The baseline questionnaire included questions on socio-demographic background, reproductive and medical history, and lifestyle and behavioral factors, including use of vitamins and other supplements. Follow-up questionnaires collected information on the date of last menstrual period (LMP), pregnancy status, and lifestyle variables such as vitamin use, frequency of intercourse and smoking status, *i.e.*, variables that may change over time.

Study population and study period

The Snart-Gravid.dk study included women who met the following criteria: Danish residents, aged 18-40 years, in a stable relationship with a male partner, attempting to conceive, and not receiving fertility treatment. From 1 June 2007 to 18 August 2011, 5,920 eligible women enrolled in the study. In the present analysis, we excluded women who had tried to conceive for more than six cycles at the time of study entry (n=1 152) as women may change their lifestyle behaviors the longer they have been trying to conceive. In addition, we excluded women who did not complete at least one follow-up questionnaire (n=579) and women who provided insufficient or implausible information about the date of their LMP or the date of their first pregnancy attempt (n=294). Thus, the final study population comprised 3 895 women (Figure 1).

Assessment of preconceptional FA and MV use

On the baseline and follow-up questionnaires, women were asked, "Do you take vitamins on a regular basis - daily or almost every day?" If the response was positive, the women were asked to specify which of the following vitamins or minerals they were taking regularly: "Multivitamins, vitamin A, beta-carotene, vitamin B, vitamin C, vitamin D, vitamin E, folic acid, calcium, magnesium, selenium, and other". Participants who reported 'multivitamin' or listed a specific brand of MV in the "other" section were classified as "MV users". Similarly, participants who reported 'folic acid' or reported 'folate' 'folacin', or 'folic acid' in the "other" section were classified as "FA users". The baseline questionnaire also collected information on duration of supplement use in categories of <1 year, 1-5 years, >5 years, and 'don't know'.

Most MV marketed in Denmark contain 400 μg of FA, especially those intended for use during pregnancy. In addition, most women wrote the name of their MV product in the questionnaire, revealing if the MV included FA. Therefore, we created a single binary exposure variable defined

as “FA supplementation”, which was set to one for women who were FA users, MV users, or both. For women who used single vitamin or mineral supplements other than FA and women who did not take any dietary supplements, the exposure variable “FA supplementation” was set to zero and was defined as “non-use”.

Assessment of pregnancies and cycles at risk

The main outcome of interest was the first reported pregnancy during the follow-up period, regardless of pregnancy outcome. The follow-up questionnaires included questions on the date of LMP, current pregnancy status and other pregnancy outcomes since the date of the last completed questionnaire, including miscarriage, induced abortion, or ectopic pregnancy. Total number of cycles at risk was calculated as: (days of trying to conceive at study entry/cycle length) + [(LMP date from most recent follow-up questionnaire – date of baseline questionnaire completion)/usual cycle length) + 1].[18] We added one cycle to account for the average woman being at mid-cycle when she filled out the baseline questionnaire. The observed cycles at risk were defined as those contributed after study entry. For example if a woman had been trying to conceive for five cycles before entering the study and then reported a pregnancy after 10 cycles of attempt time, she would contribute only five cycles.[24] Participants who were lost to follow-up (n=337, 8.7%), changed their intention to become pregnant or actively resigned from the study (n= 180, 4.6%), or did not conceive after 12 cycles (n=437, 11.2%) were censored at their last date of response. Participants who reported use of fertility treatment (n=274, 7.0%) were censored at the date of reporting fertility treatment (Figure 1).

Assessment of covariates

Weight, height, physical activity, and smoking history were reported at baseline, allowing the calculation of body mass index (BMI), total metabolic equivalents (MET) and pack-years of

smoking. Total MET was estimated by summing the MET from moderate physical activity (hours per week multiplied by 3.5) and vigorous physical activity (hours per week multiplied by 7.0).[25,26] We obtained data on other covariates, including age, level of education, history of spontaneous abortion, parity, timing and frequency of intercourse, alcohol consumption, attending the national screening program for cervical cancer (pap smear) during the last three years, menstrual cycle regularity and cycle length, and most recent method of contraception.

Data analysis

At baseline, 127 (3.3%) women did not answer the initial vitamin question. The amount of missing data for covariates ranged between 0.1% (BMI) and 7.8% (alcohol intake). Based on all information collected, including outcome variables, we used multiple imputation methods to impute missing exposure and covariate values.[27,28]

We examined the association between FA supplementation (obtained either through single FA tablets or MV) and fecundability by calculating fecundability ratios (FR) and 95% confidence intervals (CI) using a proportional probabilities regression model.[29] For the purpose of a sub-analysis, we also created three mutually exclusive categories of vitamin supplement use: FA and MV, MV exclusively, and FA exclusively. The FR represents the cycle-specific probability of conception among exposed women divided by that among unexposed women. A FR above one indicates enhanced fecundability among FA supplement users relative to non-users. We also assessed the cumulative probability of pregnancy among FA supplement users and non-users across the 12-month follow-up period, using Kaplan-Meier curves.

In multivariate analyses (including Kaplan-Meier curves), we adjusted for potential confounders selected on the basis of the literature and clinical relevance (Table 2). After restricting the analysis to FA supplement users, who were not using any other vitamins or minerals and who had been

trying to conceive for 0-3 months at study entry (n=2 289), we evaluated the extent to which the FR differed between FA supplementation for less than 1 year vs. 1 year or more.

Because cycle regularity and cycle length may mediate the relation between FA supplementation and fecundability, we stratified the data by cycle regularity (regular and irregular), and cycle length (short [<27 days], medium [27-29 days], and long [≥30 days]). Because younger and parous women may have increased fecundability regardless of vitamin supplement use, we also stratified by age at study entry (18-30 years and 31-40 years), and by parity (parous and nulliparous).

RESULTS

Overall, 2 560 respondents (65.7%) used FA supplements, obtained either through single FA tablets or MV, 62 (1.6%) used single vitamins or minerals other than FA, and 1 273 (32.7%) did not use any dietary supplements (Table 1). FA supplement use was associated with higher level of education, having at least one pap smear during the last three years, timing of intercourse, and using barrier methods as the last method of contraception.

Among the 3 895 study participants, 2 667 achieved a pregnancy within 12 cycles of follow-up, resulting in a crude cumulative probability of conception of 69%. This crude figure does not adjust for the fact that some women stopped trying to conceive and not all women remained under follow-up for the entire 12 cycles. Using life-table methods to handle this issue including delayed entry, the estimated probability of becoming pregnant within 12 cycles was 83%. Women lost to follow-up were less likely to use FA supplements (49% compared with 67% among women with complete follow-up). However, baseline characteristics of women who were lost to follow-up were similar to women with complete follow-up (data not shown).

FA supplementation and fecundability

After adjustment for potential confounders, FA supplementation was associated with increased fecundability [FR = 1.15 (95% CI: 1.06 to 1.25)], compared with non-use. Use of FA and MV, MV exclusively, and FA exclusively showed similar associations, with adjusted FR of 1.12 (95% CI: 1.03 to 1.23), 1.20 (95% CI: 1.08 to 1.32), and 1.15 (95% CI: 1.00 to 1.31), respectively, compared with non-use (Table 2). Among the 2 289 women who had tried to conceive for 0-3 months at study entry, the adjusted FR for FA supplementation for less than 1 year was 1.15 (95% CI: 1.03 to 1.28) compared with non-use. The corresponding FR was 1.04 (95% CI: 0.92 to 1.19) for FA supplementation for more than 1 year compared with non-use.

Stratified analysis

After stratifying the data by menstrual cycle regularity and cycle length, the adjusted FR for FA supplementation relative to non-use were 1.35 (95% CI: 1.12 to 1.65) for women with irregular periods and 1.11 (95% CI: 1.01 to 1.22) for women with regular periods. The FR were 1.36 (95% CI: 0.95 to 1.95) for women with short cycles [<27 days], 1.10 (95% CI: 0.98 to 1.22) for women with medium cycle length [27-29 days], and 1.24 (95% CI: 1.10 to 1.41) for women with long cycles [≥30 days]. We found little effect of age or parity on the association between FA or MV use and fecundability. The adjusted FR were 1.18 (95% CI: 1.07 to 1.30) and 1.09 (95% CI: 0.94 to 1.27) for women aged 18-30 years and 31-40 years, respectively, and 1.14 (95% CI: 1.00 to 1.30) and 1.16 (95% CI: 1.05 to 1.29) for parous women and nulliparous women, respectively (Table 3).

The adjusted Kaplan-Meier curve (Figure 2) shows that the 25^{th}, 50^{th}, and 75^{th} percentiles for the cumulative probability of conceiving were 2, 4 and, 8 cycles, respectively, among FA supplement users and 2, 4, and 9 cycles, respectively, among non-users. These curves indicate that the associations between FA supplement use and fecundability is relatively constant in our cohort across the 12 cycles of attempted pregnancy.

DISCUSSION

In this prospective cohort study of Danish pregnancy planners, we found higher fecundability among users of FA supplements. Our findings agree with previous randomized trials reporting higher pregnancy rates among women who were taking MV supplements including 800 μg FA[19] or 400 μg FA.[15] We found no appreciable differences in FR among subgroups of FA or MV use.

Some selection bias could occur if vitamin supplement use is related to underlying fertility. Our study population consists of women planning a pregnancy, who may be less fertile than women experiencing unplanned pregnancies. In addition, women planning a pregnancy may be more aware of FA recommendations. These factors could have led us to underestimate the association between FA supplementation and fecundability. To address these issues, we excluded women who had tried to conceive for more than 6 cycles at study entry. Although there was a higher prevalence of FA supplement use among study participants who completed the study compared with those lost to follow-up, we found no major differences in other baseline characteristics between the two groups. Thus, it seems unlikely that this difference would cause bias.

Some participants may have reported being "FA users" simply because they were asked or were planning to begin supplementation within a short time. This may have caused some misclassification. However, systematic bias in reporting vitamin supplement use seems unlikely because participants reported supplement use at baseline, before the occurrence of pregnancy. As we collected pregnancy status bimonthly, some misclassification of the outcome also may have occurred, but it is unlikely to be related to vitamin supplement use. Therefore, any misclassification errors in assessing supplement use and pregnancy status should bias our results toward the null.

Previously identified predictors of FA or MV use indicate various demographic, lifestyle, and behavioral differences between users and non-users, *e.g.*, users are less likely to smoke and to

consume alcohol.[30,31] Such health behaviors also may be related to fecundability and thus may confound the estimates. In the proportional probabilities regression model, we adjusted for several lifestyle factors, but some residual confounding remains possible, because of roughly categorized variables in the questionnaires. Dietary factors may be a source of unmeasured confounding. However, the bioavailability of FA in supplements is generally higher than that of dietary folate,[32-34] as synthetic FA is more stable and absorbable.[35] Thus, it seems unlikely that a dietary factor would meaningfully confound the effect of FA supplementation on fecundability.

The study population comprised self-selected volunteers enrolled via the internet. Because all our comparisons were made within the population of our study participants, and because women who volunteered for the study did so before the occurrence of the outcome (pregnancy), the internal validity of the study should not be affected by differences between study participants and the general population.[36]

In a randomized trial of 7 905 women enrolled in the Hungarian Family Planning Program, Czeizel *et al.*[19] reported higher conception rates (64.6%) among women taking MV supplements including 800 μg FA than among women taking placebo-like trace elements (62.4%) [OR = 1.10, 95% CI: 1.00 to 1.21] during a 14 month follow-up period. In a trial of 30 women, who had tried unsuccessfully to conceive for 6-36 months, Westphal *et al.*[15] also demonstrated increased pregnancy rates among women taking a MV supplement including 400 μg FA for 3 months, compared with women taking placebo. In our study, the associations between FA supplementation and fecundability were stronger among women with irregular menstrual cycles and among women with both short [<27 days] and long [≥30 days] cycle length. This suggests that the biological effect of FA on fecundability may be mediated in part by menstrual cycle hormones. FA supplementation could influence fecundability by several different mechanisms, such as alterations in DNA

biosynthesis, multiple methylation reactions, and accumulation of homocysteine. However, our data did not include biological specimens to investigate the biological mechanisms of our findings.

Although the exact duration of preconceptional FA supplementation was not assessed in this study, we found no evidence of increased fecundability among women using FA supplements for 1 year or more compared with those using FA for less than 1 year. A previous study showed an increase in plasma folate levels after three weeks among folate-depleted women receiving 300 μg of dietary folate per day,[37] suggesting that folate deficiency is remedied quite quickly after supplementation. Another study found a significant increase in red-cell folate concentrations after three months of supplementation with 400 μg FA per day.[32] Thus, it seems plausible that the biological effect of FA supplementation on fecundability may occur within a short time after the onset of supplementation, emphasizing the importance of daily FA supplementation and indicating that longer preconceptional supplement use may not contribute to increased fecundability. As data on the exact dose of FA ingested was not collected in this study, an in depth evaluation of a potential dose-response relation between FA supplementation and fecundability was not possible.

CONCLUSION

Our findings suggest that preconceptional FA supplementation was associated with increased fecundability and this association appeared to be stronger among women with irregular cycles and among women with either short or long cycle length. Longer duration of FA supplementation (one year or more) did not increase fecundability.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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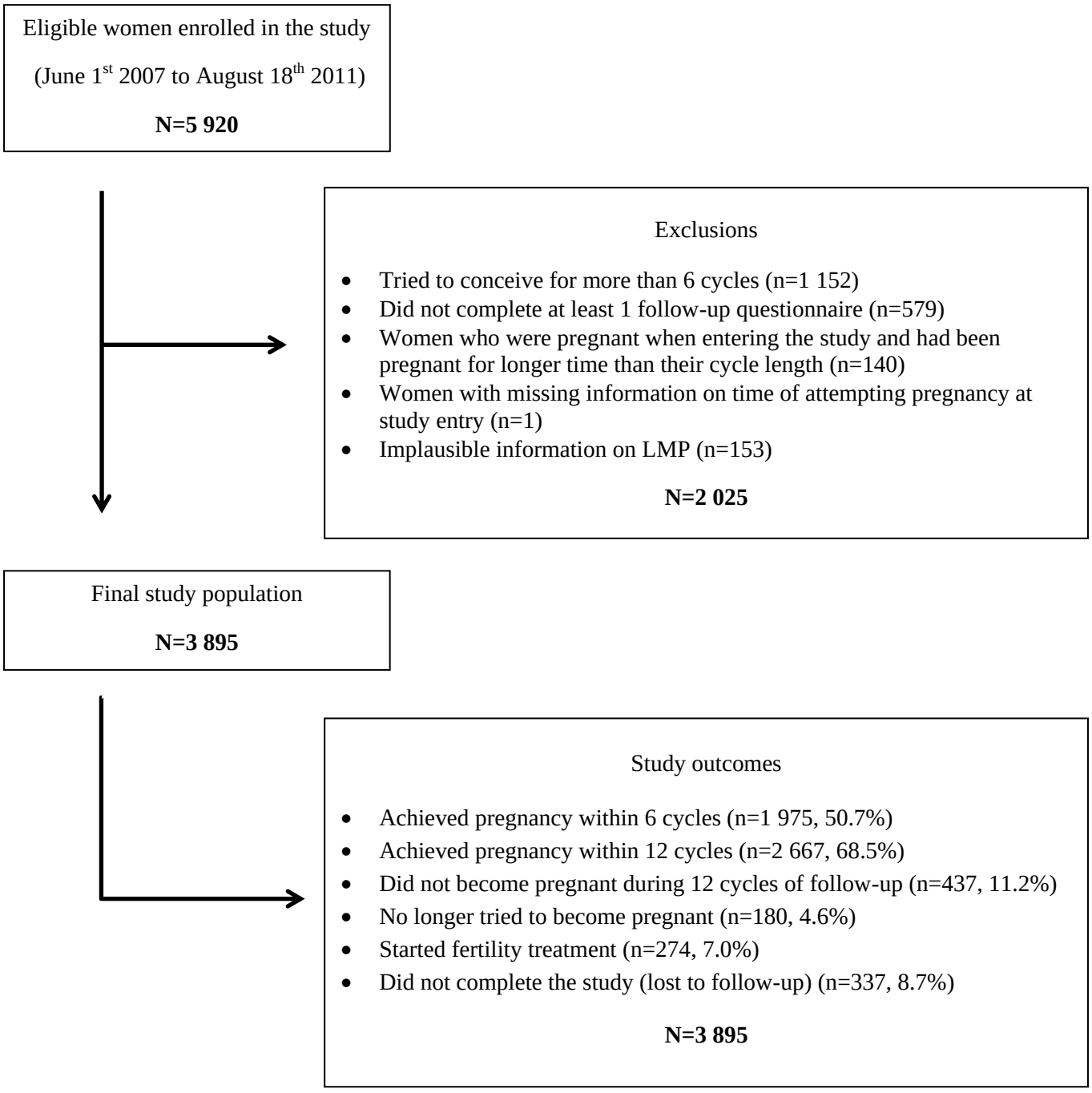
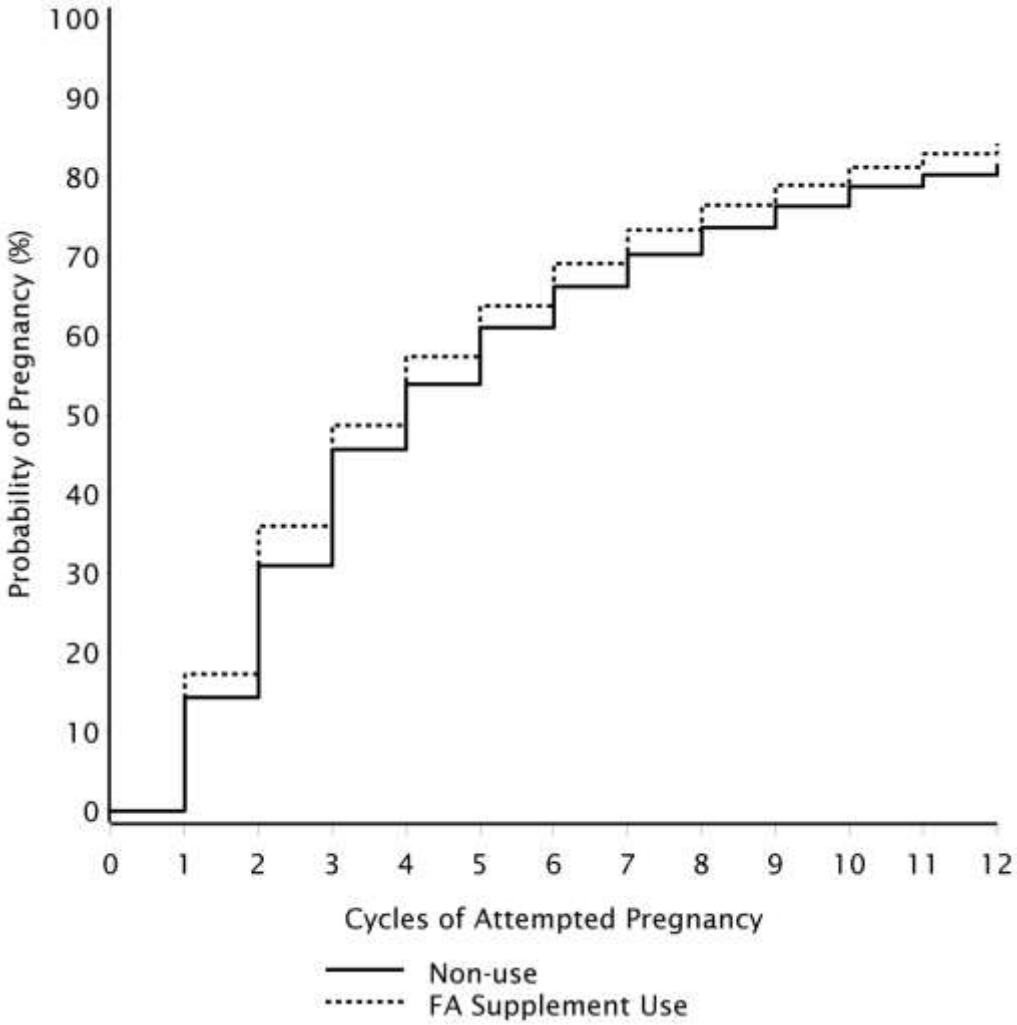
FIGURE LEGENDS

Figure 1. Flow chart and study outcomes

Figure 2. Kaplan-Meier, pregnancy probability curves by FA supplement use (N=3 895)


Eligible women enrolled in the study
(June 1st 2007 to August 18th 2011)
N=5 920
Exclusions
Tried to conceive for more than 6 cycles (n=1 152)
Did not complete at least 1 follow-up questionnaire (n=579)
Women who were pregnant when entering the study and had been pregnant for longer time than their cycle length (n=140)
Women with missing information on time of attempting pregnancy at study entry (n=1)
Implausible information on LMP (n=153)
N=2 025
Final study population
N=3 895
Study outcomes
Achieved pregnancy within 6 cycles (n=1 975, 50.7%)
Achieved pregnancy within 12 cycles (n=2 667, 68.5%)
Did not become pregnant during 12 cycles of follow-up (n=437, 11.2%)
No longer tried to become pregnant (n=180, 4.6%)
Started fertility treatment (n=274, 7.0%)
Did not complete the study (lost to follow-up) (n=337, 8.7%)
N=3 895



The curves are adjusted for age at baseline, vocational training, cycle regularity, parity, previous spontaneous abortion, pap smear, physical activity level, pack-years of smoking, alcohol intake, timing and frequency of intercourse, BMI, and last method of contraception used.

Table 1. Baseline characteristics of 3 895 women by supplement use at baseline

	Vitamin supplement use				
	FA supplementation		Subgroups of FA supplementation		
Characteristic	Non-use[1]	FA supplementation[2]	FA and MV	MV exclusive	FA exclusive
No. of women, n (%)	1 335 (34.3)	2 560 (65.7)	1 419 (36.4)	824 (21.2)	317 (8.1)
Age, years (mean)	28.0	28.6	28.8	28.5	27.8
Partner's age, years (mean)	30.6	30.9	31.0	30.8	30.4
Level of education (%)					
Short (none, semi-skilled,<3 years)	50.8	37.2	34.2	41.6	38.8
Medium (3-4 years)	29.8	37.0	37.0	36.4	38.5
Long (>4 years)	19.4	25.9	28.8	22.0	22.7
Body mass index (mean)	24.7	23.9	23.8	23.9	24.0
Physical activity, MET hrs/wk (median)	21.0	21.0	22.8	21.0	19.3
Pack-years of ever smoking (mean)	2.6	1.9	1.8	2.0	1.8
Alcohol intake, drinks/wk (mean)	2.8	2.4	2.3	2.6	2.8
Pap smear[3], ≥1time last three years (%)	74.8	83.6	84.6	83.4	79.5
Last method of contraception, (%)					
Oral contraceptives	62.8	60.9	60.5	62.0	59.1
Barrier methods	25.3	28.8	29.5	27.8	27.8
Parous, ever had live birth (%)	32.7	33.2	33.9	33.9	28.1
Previous spontanous abortion, yes (%)	8.6	10.4	11.4	9.6	7.9
Doing something to time intercourse, yes (%)	36.1	51.4	55.0	44.9	52.1
Frequency of intercourse, ≥4 times/wk (%)	19.3	19.5	19.2	18.9	22.4
Attempt time before study entry, (%)					
0-1 cycles	54.5	53.1	50.7	56.1	55.5
2-3 cycles	25.4	26.3	26.8	26.8	22.4
4-6 cycles	20.1	20.7	22.5	17.1	22.1
Irregular cycles, yes (%)	23.1	24.5	23.3	25.4	27.4
Cycle length, days (mean)	30.5	30.7	30.5	30.9	30.9

FA = folic acid; MV = multivitamin; MET = total metabolic equivalents

[1] Non-use includes no supplement use 1 273 (32.7%) and use of other single vitamin/mineral supplements than folic acid 62 (1.6%).

[2] FA supplementation includes use of both FA and MV, use of MV exclusively, and use of FA exclusively.

[3] Attending the national screening program for cervical cancer (pap smear)

Table 2. Fecundability by supplement use among 3 895 women

	Pregnancies	Cycles	Unadjusted Model		Adjusted Model[2]	
			FR	95% CI	FR	95% CI
Vitamin supplement use						
Non-use[1]	732	4 997	1.00	Ref.	1.00	Ref.
FA supplementation	1 935	11 341	1.22	1.13 to 1.32	1.15	1.06 to 1.25
FA and MV	1 069	6 340	1.21	1.11 to 1.33	1.12	1.03 to 1.23
MV exclusive	602	3 404	1.24	1.12 to 1.37	1.20	1.08 to 1.32
FA exclusive	264	1 597	1.20	1.05 to1.37	1.15	1.00 to 1.31

FR = fecundability ratio; CI = confidence interval; FA = folic acid; MV = multivitamin

[1] Non-use includes no supplement use and use of other single vitamin/mineral supplements than folic acid.

[2] Models for FA supplementation, FA and MV use, MV exclusive, and FA exclusive are adjusted for age at baseline, vocational training, parity, previous spontaneous abortion, pap smear, physical activity level, pack-years of smoking, current smoking status, alcohol intake, timing and frequency of intercourse, BMI, and last method of contraception used.

Table 3. Fecundability by FA supplement use stratified by selected factors (N = 3 895)

	Pregnancies	Cycles	Unadjusted Model		Adjusted Model[2]	
			FR	95% CI	FR	95% CI
Age 18-30 y						
Non-use[1]	531	3 640	1	Ref.	1	Ref.
FA supplement use	1 380	7 903	1.24	1.13 to 1.36	1.18	1.07 to 1.30
Age 31-40 y						
Non-use	201	1 357	1	Ref.	1	Ref.
FA supplement use	555	3 438	1.17	1.00 to 1.36	1.09	0.93 to 1.27
Nulliparous						
Non-use	452	3 503	1	Ref.	1	Ref.
FA supplement use	1 232	8 119	1.23	1.11 to 1.36	1.16	1.05 to 1.29
Parous						
Non-use	280	1 494	1	Ref.	1	Ref.
FA supplement use	703	3 222	1.22	1.07 to 1.39	1.14	1.00 to 1.30
Irregular periods						
Non-use	141	1 130	1	Ref.	1	Ref.
FA supplement use	435	2 634	1.39	1.16 to 1.66	1.35	1.12 to 1.65
Regular periods						
Non-use	591	3 867	1	Ref.	1	Ref.
FA supplement use	1 500	8 707	1.18	1.08 to 1.29	1.11	1.01 to 1.22
Cycle length, short (<27 d)						
Non-use	89	704	1	Ref.	1	Ref.
FA supplement use	227	1 317	1.47	1.17 to 1.85	1.36	0.95 to 1.95
Cycle length, medium (27-29 d)						
Non-use	430	2 785	1	Ref.	1	Ref.
FA supplement use	1 090	6 599	1.14	1.02 to 1.26	1.10	0.98 to 1.22
Cycle length, long (≥30 d)						
Non-use	302	2 212	1	Ref.	1	Ref.
FA supplement use	845	4 742	1.35	1.19 to 1.52	1.24	1.10 to 1.41

FR = fecundability ratio; CI = confidence interval; FA = folic acid; MV = multivitamin

[1] Non-use includes no supplement use and use of other single vitamin/mineral supplements than folic acid.

[2] Models for the stratified analysis are adjusted for vocational training, previous spontaneous abortion, pap smear, physical activity level, pack-years of smoking, current smoking status, alcohol intake and BMI. Further, each model includes adjustment for the other covariates stratified for, with the exception of cycle regularity and cycle length.

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