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Dietary folate intake and fecundability in two preconception cohorts

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STUDY QUESTION: To what extent is dietary folate intake and total folate intake (dietary and supplemental intakes) associated with fecundability, the per cycle probability of conception?

SUMMARY ANSWER: Preconception dietary folate intake was positively associated with fecundability in a monotonic pattern.

WHAT IS KNOWN ALREADY: Supplemental folic acid has been associated with improved fertility, but little is known about the relation between dietary folate and fecundability.

STUDY DESIGN, SIZE, DURATION: A prospective cohort study including 9559 women trying to conceive without fertility treatment and enrolled in the period 2013-2020.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We used data from two internet-based prospective cohort studies of pregnancy planners from Denmark, where folic acid fortification is not performed (SnartForældre.dk (SF); n = 3755) and North America, where the food supply is fortified with folic acid (Pregnancy Study Online (PRESTO); n = 5804). Women contributed menstrual cycles at risk until they reported conception or experienced a censoring event. We used proportional probabilities regression models to compute fecundability ratios (FRs) and 95% CI, adjusting for potential confounders.

MAIN RESULTS AND THE ROLE OF CHANCE: Compared with a dietary folate intake ≥400 µg/day, the adjusted FRs for women in SF were 0.92 (95% CI: 0.85–0.99) for intake 250–399 µg/day, and 0.80 (95% CI: 0.68–0.94) for intake of <250 µg/day. The corresponding FRs in PRESTO were 0.95 (95% CI: 0.89–1.01) and 0.81 (95% CI: 0.65–1.00). Compared with the highest level of total folate intake (diet folate ≥400 µg/day plus folic acid supplementation), in both cohorts fecundability was lowest among women with the lowest dietary intake <250 µg/day dietary folate and no supplementation (FR: 0.76, 95% CI: 0.59–0.98 [SF] and 0.49, 95% CI: 0.31–0.77 [PRESTO]). Further, total intake dietary folate <250 µg/day plus supplementation was associated with reduced fecundability for SF participants (FR; 0.79, 95% CI: 0.65–0.98) and for PRESTO participants (FR; 0.92, 95% CI: 0.72–1.16).

LIMITATIONS, REASONS FOR CAUTION: It is unknown whether dietary folate and folic acid intake affect fecundability on its own or if there is an interaction with other micronutrients provided in healthy diet. Thus, the observed associations may not reflect dietary folate intake alone, but overall healthy diet.

WIDER IMPLICATIONS OF THE FINDINGS: Recommendations for preconception dietary folate intake and folic acid supplementation are of importance not only to prevent neural tube defects but also to enhance fecundability.

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Key words: dietary folate / folic acid supplementation / fecundability / preconception / pregnancy planners / cohort study

Introduction

Folate is an antioxidant micronutrient primarily present in liver, green leafy vegetables, fruits, whole grains and beans (Delchier *et al.*, 2016). Its synthetic counterpart, folic acid, is used in dietary supplements and for fortification of wheat and other foods. The European Food Safety Authority recommends an average daily intake of 250 µg dietary folate equivalents (DFE) for adult women and 600 µg DFE/day for pregnant women to maintain sufficient serum and red blood cell folate concentration (European Food Safety Authority, 2014). The World Health Organization (WHO) and national authorities recommend 400–800 µg/day folic acid supplementation to prevent neural tube defects (Bibbins-Domingo *et al.*, 2017; Danish National Board of Health, 2017; WHO, 2020). However, the optimal dose of total folate intake and the interplay between dietary and supplemental intake in relation to fertility are less clear.

Folate is essential in DNA synthesis, *S*-adenosylmethionine production and various methylation reactions (Laanpere *et al.*, 2010; Greenberg *et al.*, 2011). Thus, folate is indispensable during periods of rapid cell growth, such as germ cell maturation and fertilization (Ebisch *et al.*, 2007; Laanpere *et al.*, 2010). Furthermore, it has been suggested that folate is beneficial to fertility by lowering oxidative stress (Ruder *et al.*, 2008).

In vitro studies have reported an association between elevated homocysteine concentrations in the follicular fluid and poor oocyte maturity (Szymański and Kazdepka-Ziemińska, 2003; Berker *et al.*, 2009). Supplementation with folic acid increases folate and decreases homocysteine concentrations in the microenvironment of the maturing oocyte (Szymański and Kazdepka-Ziemińska, 2003; Boxmeer *et al.*, 2008). In a randomized trial of 7905 women with no history of infertility, Czeizel *et al*. reported slightly higher conception rates among women taking multivitamins, including 800 µg folic acid, compared with women taking placebo (proportion pregnant 71.3% versus 67.9%, respectively) during a 14-month follow-up period (Czeizel *et al.*, 1996). In a prospective cohort study from 2016, folic acid supplement use (multivitamins or single folic acid tablets) was associated with increased fecundability among 3895 Danish pregnancy planners compared with non-users (Cueto *et al.*, 2016).

A 2015 cohort study of 232 American women undergoing ART found a positive association between total folate intake (from natural sources, fortified foods and supplements), and implantation, clinical pregnancy and live birth rates. However, this association was attenuated when the exposure was exclusively dietary folate, even though it included folic acid from fortified foods (Gaskins *et al.*, 2014).

To our knowledge, no study has examined the role of dietary folate and total folate (dietary and supplemental intakes) on fecundability, the per cycle probability of conception (Weinberg *et al.*, 1989), among couples trying to conceive naturally. We examined the association of dietary folate and total folate intake and fecundability in two similar cohorts of women trying to conceive: SnartForældre.dk (SF) (Mikkelsen *et al.*, 2009; Huybrechts *et al.*, 2010) located in Denmark, where folic acid fortification is not performed; and pregnancy study online (PRESTO) (Wise *et al.*, 2015) located in North America (USA and Canada), where the food supply is fortified with folic acid (Crider *et al.*, 2011).

Materials and methods

Details of the study methods have been described elsewhere (Mikkelsen *et al.*, 2016; Wise *et al.*, 2020). In brief, enrollment is ongoing and began in June 2011 for SF and June 2013 for PRESTO, which was modeled after SF. Participants are primarily recruited via social media and online advertisements. Eligible women were aged 18–49 years (SF) and 21–45 years (PRESTO), in a relationship with a male partner, and trying to conceive without fertility treatment. At study entry, participants in both cohorts completed a comprehensive online questionnaire on demographic, lifestyle, behavioral, medical and reproductive factors. Follow-up questionnaires were completed every 8 weeks for up to 12 months or until reported pregnancy. Ten days after completion of the baseline questionnaire, participants were invited to complete an online food frequency questionnaire (FFQ); the SF_FFQ for SF participants and the National Cancer Institute's Dietary Health Questionnaire II (DHQ II) for PRESTO participants (Subar *et al.*, 2001; Knudsen *et al.*, 2016).

Study population

Of the 8078 eligible women enrolled in SF through September 2020, we excluded women who enrolled before February 2013, when the SF_FFQ was first implemented, yielding a study population of 6856 women. Next, we excluded 768 participants with implausible last menstrual period (LMP) data. To avoid possible reverse causation in which diet was modified because of subfertility, we further excluded 1281 women who were trying to conceive for more than six cycles at study entry. Of the remaining 4807 women, 3861 completed the SF_FFQ (80% completion rate). Finally, we restricted to unique participants (multiple enrollments are possible) and excluded women with implausible total energy intake (<600 or >3800 kcal/day), for a final study population of 3755 women (Fig. 1).

Of the 12 843 eligible women who enrolled in PRESTO before September 2020, we excluded 580 women with missing or implausible LMP data. Of the 12 263 remaining women, we excluded 2744 women who had been trying to conceive more than six menstrual cycles at study entry. Finally, we excluded 3576 women who did not complete the DHQ II (62% completion rate) and 139 women with implausible total energy intake (<600 or >3800 kcal/day), for a final study population of 5804 women.

Assessment of dietary folate intake and folic acid supplementation

Participants both in SF and PRESTO were asked to record their usual intake of foods and drinks in the previous year. The SF_FFQ includes questions related to more than 230 foods and beverages. It was specifically designed for and validated against 4-day food diaries within this study population (Knudsen *et al.*, 2016). The DHQ II includes more than 150 questions on foods and beverages, and a previous version of this instrument (DHQ) was validated using 24 h telephone recalls in a US population. Overall, the de-attenuated correlation coefficients showed moderate reliability of the FFQs (0.49 for folate in SF_FFQ (Knudsen *et al.*, 2016) and 0.69 for dark green vegetables in DHQ (Millen *et al.*, 2006)).

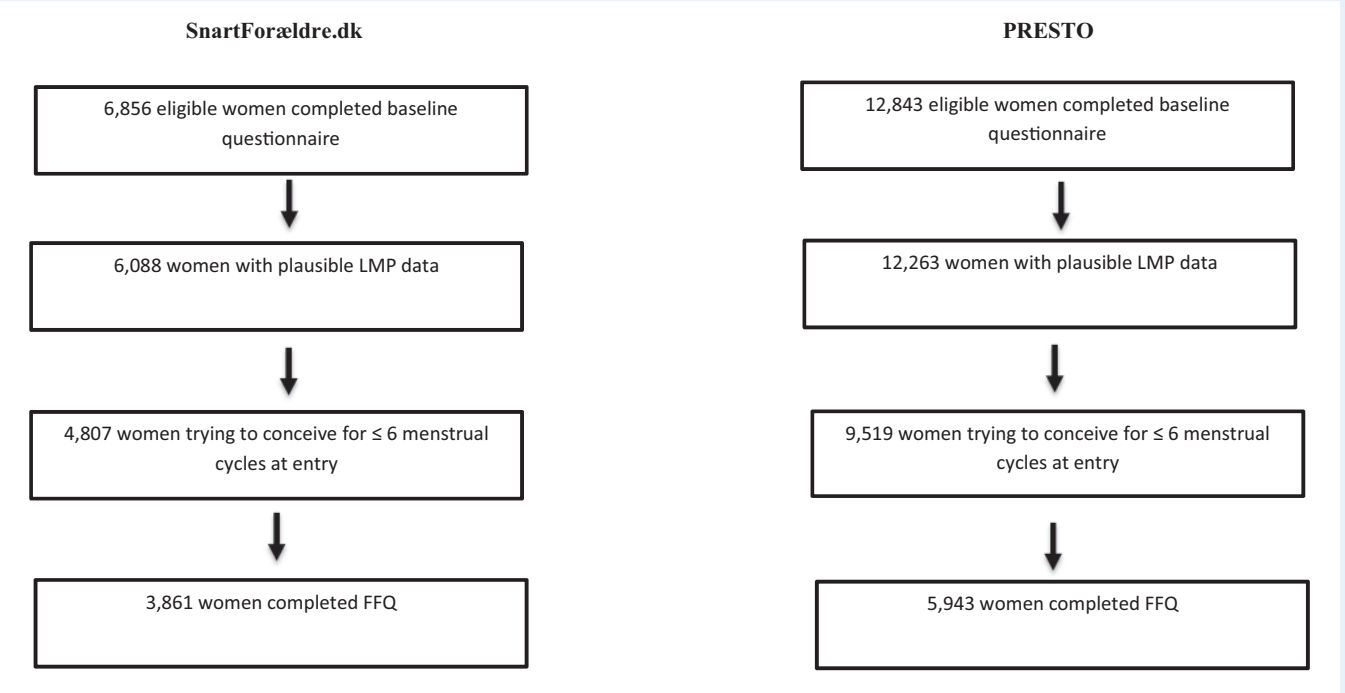

Figure 1. Flowchart of participant exclusions, SnartForældre.dk (February 2013–September 2020) and PRESTO (June 2013–September 2020). FFQ, food frequency questionnaire; LMP, last menstrual period.

To increase reporting accuracy and reduce missing data, the SF_FFQ included help buttons and photos explaining portion sizes, and the DHQ II queried participants to complete all questions. Both FFQs included automated skip patterns to shorten the length of the questionnaires. In both cohorts, we estimated dietary folate intake for each participant based on nutrient compositions of all food items included in the FFQs and servings of folate from individual foods and mixed recipes. In SF, the Danish nutrient database provided data on nutrient compositions and we estimated total dietary intake in μg/day (National Food Institute, 2008). In PRESTO, we used the National Cancer Institute's DIET*CALC software (version 1.5.0) to estimate intake of DFEs in μg/day. DFEs taken into account the difference in bioavailability of natural food folate and folic acid added to foods. It is calculated as the amount of natural food folate plus 1.7 times the amount of added folic acid in foods (Bailey, 1998).

In SF, we asked participants to report use of multivitamin supplements and other single dietary supplements including folic acid. In addition, participants were asked to report the brand of multivitamins revealing if the multivitamins included folic acid. Similarly, in PRESTO, participants reported their use of multivitamins, prenatal vitamins and folic acid as a single line item. For women in both cohorts who reported use of folic acid supplements we assumed that the dosage was at least 400 μg/day, as most marketed multivitamin and prenatal brands contain this dosage. Further, WHO and national Danish guidelines recommend folic acid supplementation of 400 μg/day and the US Preventive Service Task Force recommend 400–800 μg/day to prevent neural tube defects (Bibbins-Domingo *et al.*, 2017; Danish National Board of Health, 2017; WHO, 2020).

Assessment of pregnancy and cycles at risk

On the baseline questionnaire, participants reported the number of menstrual cycles they had tried to conceive, date of LMP, cycle length (number of days) and cycle regularity (yes versus no, according to the question: 'Has your menstrual period been regular in a way that you could usually predict when the next period would start?'). On each follow-up questionnaire, current pregnancy status including intervening pregnancy losses, initiation of fertility treatment and the most recent LMP date were reported. Among PRESTO participants who were lost to follow-up, we searched for additional outcome data by: contacting the participants directly (phone/email); reviewing online baby registries/announcements and linking with birth registries in seven US states. We estimated total menstrual cycles at risk using the following formula: *cycles trying to conceive reported at study entry + [(LMP date from most recent follow-up questionnaire − date of baseline questionnaire)/usual cycle length] + 1* (Cueto, 2016). For women with irregular cycles, we estimated cycle length based on date of LMP at baseline and prospectively reported LMP dates during follow-up.

Assessment of covariates

Baseline characteristics (age, partner age, height, weight, smoking, alcohol and caffeine intake, last method of contraception, sexual transmitted diseases, cycle regularity, timing of intercourse (assessed as 'Do you or your partner do something to time your pregnancy attempt to your fertile window?'), frequency of intercourse and parity) were ascertained identically in the two cohorts with the exception of physical activity, education and race/ethnicity. In SF, total metabolic equivalents (METs) per week were calculated using the International Physical

Activity Questionnaire short-form by summing the MET-hours from walking, moderate and vigorous physical activity (hours/week × 3.3 METs, 4 METs and 8 METs, respectively) (Craig *et al.*, 2003). In PRESTO, total MET-hours per week were calculated by multiplying the average number of hours per week spent participating in various activities by metabolic equivalents estimated from the Compendium of Physical Activities (Ainsworth *et al.*, 2000). Vigorous activities (e.g. bicycling; aerobics) were assigned seven METs, while moderate activities (e.g. walking for transportation; gardening) were assigned 3.5 METs. In SF, education was reported as years of vocational training after basic schooling (none, semi-skilled/basic training, <3 years, 3–4 years, more than 4 years) whereas in PRESTO it was reported as overall years of schooling (less than college/university degree, 4-year college/university graduate, graduate school). We calculated BMI as weight (kg) divided by height squared (m^2). Data on race and ethnicity were not ascertained in SF owing to homogeneity of the population.

Data analysis

Because dietary folate intake in the two cohorts was assessed by different FFQs and because there is food fortification in North America but not in Denmark, we analyzed data separately. We used the nutrient residual method to calculate energy-adjusted dietary folate intake to address potential confounding by total energy intake. Women with higher energy intake on average are more likely to have a higher consumption of dietary folate (Willett *et al.*, 1997). We used the mean total energy intake (SF: 1927 kcal and SF: 1572 kcal) as the constant in the nutrient residual method.

We categorized dietary folate intake (<250, 250–399, ≥400 µg/day) based on the recommended minimum dietary intake of 250 µg DFE per day for adult women and the distribution of dietary folate intake in the two populations (Willett *et al.*, 1997; European Food Safety Authority, 2014). Supplementation was defined as yes (single folic acid and/or multivitamin use) versus no. Further, we created a variable for total folate including dietary folate in µg/day and supplementation yes/no: (i) <250, no supplement, (ii) 250–399, no supplement, (iii) ≥400, no supplement, (iv) <250, yes supplement, (v) 250–399, yes supplement, (vi) ≥400 µg/day, yes supplement).

We described baseline characteristics of the study participants according to the three categories of dietary folate intake. Using life-table methods, we calculated the proportion of women who conceived during follow-up, accounting for censoring events: start of fertility treatment, cessation of pregnancy attempt, withdrawal, loss to follow-up and end of follow-up (12 menstrual cycles).

To examine the association between dietary folate and total folate intake and fecundability, we computed fecundability ratios (FRs) and 95% CI using a proportional probabilities regression model. An FR below one indicates reduced fecundability among the exposed women compared with the unexposed (Weinberg *et al.*, 1989). We used menstrual cycles as the timescale, and each woman contributed discrete menstrual cycles at risk from date of study entry until pregnancy or a censoring event, whichever came first. Thus, we began follow-up at the first observed menstrual cycle after enrollment in the study. To account for left truncation, wherein some participants have been trying to conceive for several cycles (1–6) before enrolling, we used the Anderson-Gill data structure in the regression model (Howards *et al.*, 2007; Schisterman *et al.*, 2013).

In the multivariate regression models, we adjusted for *a priori* defined potential risk factors for subfertility that were associated with dietary folate intake. The covariates considered as potential confounders of the association between dietary folate intake and fecundability are depicted in a directed acyclic graph (Supplementary Fig. S1). The primary model included age in years (cubic splines), partner's age in years (cubic splines), education (<3, 3–4 and ≥4 years after high school), household income (SF: DKK <24 999, 25 000–64 999, 65 000–80 000, >80 000 and PRESTO: US$<50 000, 50 000–99 999, 100 000–149 999 and >150 000), physical activity MET-hours/week (cubic splines), alcohol consumption (0, 1–3, 4–7, 8–13 and ≥14 standard servings/week), parity (parous versus nulliparous), current smoking (yes versus no), folic acid supplement use (yes versus no), timing of intercourse to improve chances of conception (yes versus no) and race/ethnicity (only PRESTO: (i) Hispanic/Latina; (ii) Mixed race, Non-Hispanic; American Indian/Alaskan Native, Non-Hispanic; other Non-Hispanic; (iii) Black, Non-Hispanic; (iv) Asian Non-Hispanic and (v) White, Non-Hispanic) (Model I). We included timing of intercourse as a proxy for intensity of pregnancy attempt as we hypothesize that intentions are associated with healthy diet, and timing of intercourse is associated with fecundability. Intake of sugar-sweetened beverages is positively associated with obesity, and unhealthy dietary practices in general (Luger *et al.*, 2017; Dunford *et al.*, 2021), and inversely associated with dietary folate intake in SF and PRESTO. In a second model, we additionally adjusted for intake of sugar-sweetened beverage as a non-folate containing proxy for unhealthy diet because a potential adverse effect of low dietary folate intake on fecundability could be confounded by unhealthy diet (Model II).

Finally, because dietary folate intake is closely related to a generally healthful diet, e.g. eating vegetables and fruits, we adjusted for diet quality, using the Nutrient Rich Diet Score (NRD15.3) in SF and Healthy Eating Index (HEI-2010) in PRESTO (Model III) (Fulgoni *et al.*, 2009; Guenther *et al.*, 2014). NRD15.3 includes 18 components and has been used to estimate diet quality in European populations (Mertens *et al.*, 2019). HEI is a validated instrument including 12 components that reflects a healthful diet as recommended in federal guidelines in the USA. Both instruments include dietary components that should be promoted or limited. We stratified the primary analysis (Model I) by BMI (≤25 versus >25 kg/m^2) because it is a strong determinant of fertility, and it is associated with diet independent of folate intake.

To diminish the potential for reverse causation, we restricted the study populations to women who had tried to conceive for six cycles or less at study entry. For the same reason, in a sensitivity analysis we further restricted to participants with an attempt time at study entry of two or less cycles.

To evaluate a potential non-linear relation between dietary folate intake and fecundability, we fitted restricted cubic spline regression models adjusted for potential confounders.

The proportion of missing covariate values at baseline ranged from 0.1% (gravidity) to 5.6% (liquor consumption) in SF and from 0.03% (partner age) to 3.3% (household income) in PRESTO. We used multiple imputation (MI) to create 20 imputed data sets for each cohort, using over 100 variables in the MI model (Zhou *et al.*, 2001; Pedersen *et al.*, 2017). Baseline characteristics presented in Table I are based on the first imputed data set. To reduce potential for selection bias owing to differential loss to follow-up, we used MI to impute the outcome

Table I Baseline characteristics by energy-adjusted dietary folate intake (μg/day) in SnartForaeldre.dk (February 2013–September 2020) and PRESTO (June 2013–September 2020) cohorts.

	Energy-adjusted dietary folate intake (μg/day)					
	SnartForældre.dk, N = 3755			PRESTO, N = 5804		
Characteristic	<250	250–399	≥400	<250	250–399	≥400
Number of women (%)	258 (6.9)	2058 (54.8)	1439 (38.3)	172 (3.0)	2330 (40.1)	3302 (56.9)
Age, years (median)	28.0	29.0	29.0	29.0	30.0	30.0
Partner's age, years (median)	30.0	30.0	31.0	30.0	31.0	32.0
Vocational training, >4 years (%)	22.1	42.4	47.3	20.4	37.5	48.3
Household income,[1] (%)	17.4	11.4	9.6	37.2	17.2	14.3
Body mass index, kg/m^2 (median)	24.3	23.1	22.7	30.1	26.5	24.4
Physical activity, MET,[2] h/week (median)	33.0	35.3	43.1	18.1	26.7	32.7
Folic acid and/or multivitamin use, yes (%)	57.0	70.6	76.0	66.3	82.9	85.8
Single folic acid supplement use, yes (%)	19.0	27.6	30.7	11.1	13.5	13.7
Energy intake, kcal/day (median)	1785	1879	1866	1524	1489	1521
Alcohol intake, drinks/week (median)	1.0	1.9	1.6	1.8	2.3	2.0
Current smoking, yes (%)	20.5	10.9	9.1	19.2	5.6	2.7
Caffeine consumption, ≥150 g/day (%)	28.7	44.7	51.4	46.5	55.5	52.9
Sugar-sweetened beverage, ≥3 servings/day (%)	13.2	2.7	0.4	40.1	14.7	7.6
Nutrient Rich Diet Score, median	4.5	5.1	5.4	–	–	–
Healthy Eating Index, median	–	–	–	4.5	6.2	7.0
Irregular cycle, yes (%)	34.1	27.2	26.9	20.9	16.0	13.8
Cycle length, days (median)	29.0	29.0	29.0	28.8	29.0	29.0
Parous, ever had live or still birth, yes (%)	40.7	37.4	28.7	35.5	31.5	29.9
Frequency of intercourse, ≥4 times/week (%)	8.9	11.3	16.1	18.0	14.5	13.8
Timing intercourse to fertile window, yes (%)	74.4	74.2	75.1	70.9	77.0	77.1
Attempt time at study entry, cycles (%)						
0–1 cycles	42.3	51.6	51.4	43.0	52.0	53.8
2–3 cycles	31.4	25.8	26.5	29.7	28.8	28.8
4–6 cycles	26.4	22.6	22.1	27.3	19.2	17.4

[1]SF: DKK < 25 000/month and PRESTO: US$<50 000/year.
[2]MET = total metabolic equivalents.

(pregnant: yes versus no) for participants who did not complete any follow-up questionnaires, and assigned them one cycle of follow-up.

Ethical approval

Participants in both cohorts provided online consent at enrollment. SF is registered at Aarhus University (2016-051-000001, # 431) to comply with Danish law on data protection. The Boston Medical Campus Institutional Review Board approved both studies.

The SAS 9.4 (SAS Institute, Cary, NC, USA) software was used for the statistical analyses.

Results

Based on life-table methods, 74.1% (SF) and 69.5% (PRESTO) of participants conceived within six cycles of attempt. Within 12 cycles of attempt time, the estimates were 87.5% (SF) and 81.4% (PRESTO).

The median age of participants was 29.0 and 30.0 years in SF and PRESTO, respectively. Median energy intake was 1867 kcal/day in SF versus 1508 kcal/day in PRESTO. The median (25th and 75th quartiles) energy-adjusted dietary folate intake in SF was 369 μg/day (313 and 448) and 418 μg/day (354 and 499) in PRESTO. In total, 2316 women (62%) in SF and 2502 (43%) in PRESTO did not reach an intake of 400 μg/day folate solely from dietary sources (Table I). In total, 72% of SF participants and 84% of PRESTO participants reported use of folic acid supplements (folic acid and/or multivitamin) on a daily basis. Among users, 19% of SF participants and 11% of PRESTO participants reported daily use of both a single folic acid supplement and a multivitamin supplement with folic acid. Daily intake of folic acid supplementation varied with parity. Among SF participants, 68% versus 74% of parous and nulliparous women, respectively, reported daily use of folic acid supplements; the corresponding numbers in PRESTO were 79% versus 86%.

Dietary folate intake was positively associated with educational attainment, folic acid supplement use, physical activity, diet quality

Table II Association of energy-adjusted dietary folate intake and fecundability among women in SnartForaeldre.dk and PRESTO.

Dietary folate intake (μg/day)[1]	Pregnancies	Cycles	Unadjusted model FR (95% CI)	Adjusted model I[2] FR (95% CI)	Adjusted model II[3] FR (95% CI)	Adjusted model III[4] FR (95% CI)
SnartForaeldre.dk, N = 3755						
<250	153	958	0.81 (0.69–0.95)	0.80 (0.68–0.94)	0.80 (0.68–0.94)	0.85 (0.78–1.03)
250–399	1414	7491	0.95 (0.88–1.02)	0.92 (0.85–0.99)	0.92 (0.86–1.00)	0.94 (0.87–1.02)
≥400	1008	4993	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
PRESTO, N = 5804						
<250	82	800	0.68 (0.55–0.84)	0.81 (0.65–1.00)	0.82 (0.66–1.02)	0.90 (0.73–1.12)
250–399	1460	9891	0.93 (0.87–0.99)	0.95 (0.89–1.01)	0.96 (0.90–1.02)	0.99 (0.32–1.05)
≥400	2192	13 680	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)

FR, fecundability ratio.
[1]Energy-adjusted dietary folate intake in μg per day.
[2]Model I, adjusted for female age at baseline, partner's age at baseline, vocational training, household income, parity, physical activity, alcohol consumption, current smoking, folic acid supplementation, timing of intercourse and ethnicity (only PRESTO).
[3]Model II, adjusted for intake of sugar-sweetened beverage in addition to Model I adjustments.
[4]Model III, adjusted for Nutrient Rich Diet Score in SF and Healthy Eating Index in PRESTO in addition to Model I adjustments.

(NRD15.3 and HEI) and alcohol intake across both cohorts. In contrast, dietary folate intake was inversely associated with median BMI, intake of sugar-sweetened beverages, current smoking, irregular cycles, parity and household income in both cohorts (Table I).

Compared with dietary folate intake ≥400 μg/day, the adjusted FRs for women in SF were 0.92 (95% CI: 0.85–0.99) for intake 250–399 μg/day, and 0.80 (95% CI: 0.68–0.94) for intake <250 μg/day of (Table II, Model I). The corresponding FRs in PRESTO were similar, 0.95 (95% CI: 0.89–1.01) for intake 250–399 μg/day, and 0.81 (95% CI: 0.65–1.00) for intake <250 μg/day. The estimates were similar after additional adjustment for sugar-sweetened beverage intake (Table II, Model II). After additional adjustment for healthful diet using the NRD15.3 index in SF and HEI-2010 in PRESTO, the association attenuated slightly in SF (FR: 0.85, 95% CI: 0.70–1.03) and more so in PRESTO (0.90, 95% CI: 0.73–1.12) for <250 μg/day versus ≥400 μg/day (Table II, Model I).

The association between <250 μg/day versus ≥400 μg/day dietary folate intake and fecundability was stronger among SF women with BMI <25 (FR: 0.72, 95% CI: 0.58–0.90) versus women with BMI ≥25 (FR: 0.87, 95% CI: 0.68–1.12). In PRESTO, the corresponding association was similar for women with BMI <25 (FR: 0.76, 95% CI: 0.52–1.13) versus BMI ≥25 (FR: 0.83, 95% CI: 0.62–1.04) (Table III).

The association between dietary folate intake (<250 versus ≥400 μg/day) and fecundability remained virtually unchanged when restricted to participants who had tried to conceive for ≤2 menstrual cycles at study entry in SF (adjusted FR: 0.73, 95% CI: 0.59–0.89) and PRESTO (adjusted FR: 0.77, 95% CI: 0.60–0.98).

The cubic splines for SF and PRESTO illustrate a monotonic positive association between folate intake and fecundability (Fig. 2A and B).

In the analyses of total folate intake, we used the highest level of intake (diet folate ≥400 μg/day plus folic acid supplement) as the reference category, and observed in both cohorts that fecundability was lowest among women with the lowest dietary folate intake (<250 μg/day dietary folate intake and no supplementation) (FR: 0.76, 95% CI: 0.59–0.98 [SF] and 0.49, 95% CI: 0.31–0.77 [PRESTO]) (Table IV, Model I). Dietary intake of 250–399 μg/day and no folic acid supplementation was likewise associated with reduced fecundability (FR: 0.88, 95% CI: 0.79–0.98 [SF] and 0.79, 95% CI: 0.69–0.90 [PRESTO]). Among SF participants, the FR for dietary intake <250 μg/day plus folic supplementation was 0.79, 95% CI: 0.65–0.98 and the corresponding FR in PRESTO was 0.92, 95% CI: 0.72–1.16 (Table IV, Model I).

Discussion

Overall, we observed a monotonic association between preconception dietary folate intake and fecundability. Total folate intake, i.e. a dietary intake below 250 μg/day and no folic acid supplementation was associated with a reduced fecundability of 24% in SF and 51% in PRESTO. Relative to no supplementation, in both cohorts fecundability was higher among women with low dietary folate intake (<250 μg/day) who supplemented with 400 μg/day folic acid, however, supplementation did not appear to compensate completely for the low dietary intake.

Our findings agree with those from two randomized trials and two cohort studies of reproductive-aged women, all indicating a positive association of preconceptional folic acid supplementation and fertility (Czeizel *et al.*, 1996; Westphal *et al.*, 2006; Chavarro *et al.*, 2008; Cueto *et al.*, 2016). However, only one of these studies assessed fecundability and none examined dietary folate, although total folate intake includes natural folate from foods as well as synthetic folate from fortified foods and supplements.

In a study of 232 women undergoing ART, higher dietary folate intake including supplementation was associated with higher rates of

Table III Fecundability and energy-adjusted dietary folate intake stratified by BMI in SnartForaeldre.dk (N = 3755) and PRESTO (N = 5804) participants.

	Dietary folate intake[1]	Pregnancies	Cycles	Unadjusted model	Adjusted model[2]	Pregnancies	Cycles	Unadjusted model	Adjusted model[2]
				FR (95% CI)	FR (95% CI)			FR (95% CI)	FR (95% CI)
BMI				**SnartForaeldre.dk**				**PRESTO**	
<25	<250	83	579	0.75 (0.61–0.93)	0.72 (0.58–0.90)	25	197	0.74 (0.51–1.09)	0.76 (0.52–1.13)
	250–399	984	5006	0.97 (0.89–1.06)	0.91 (0.84–1.00)	652	3907	0.92 (0.85–1.00)	0.94 (0.87–1.03)
	≥400	722	3559	1.00 (Ref.)	1.00 (Ref.)	1286	6961	1.00 (Ref.)	1.00 (Ref.)
≥25	<250	70	379	0.88 (0.69–1.12)	0.87 (0.68–1.12)	57	603	0.73 (0.57–0.94)	0.83 (0.64–1.07)
	250–399	430	2485	0.90 (0.79–1.03)	0.90 (0.79–1.03)	808	5984	0.99 (0.90–1.08)	0.98 (0.89–1.07)
	≥400	286	1434	1.00 (Ref.)	1.00 (Ref.)	906	6719	1.00 (Ref.)	1.00 (Ref.)

FR, fecundability ratio.
[1]Energy-adjusted dietary folate intake in μg per day.
[2]Adjusted for female age at baseline, partner's age at baseline, vocational training, household income, parity, physical activity, alcohol consumption, current smoking, folic acid supplementation, timing of intercourse and ethnicity (only PRESTO).

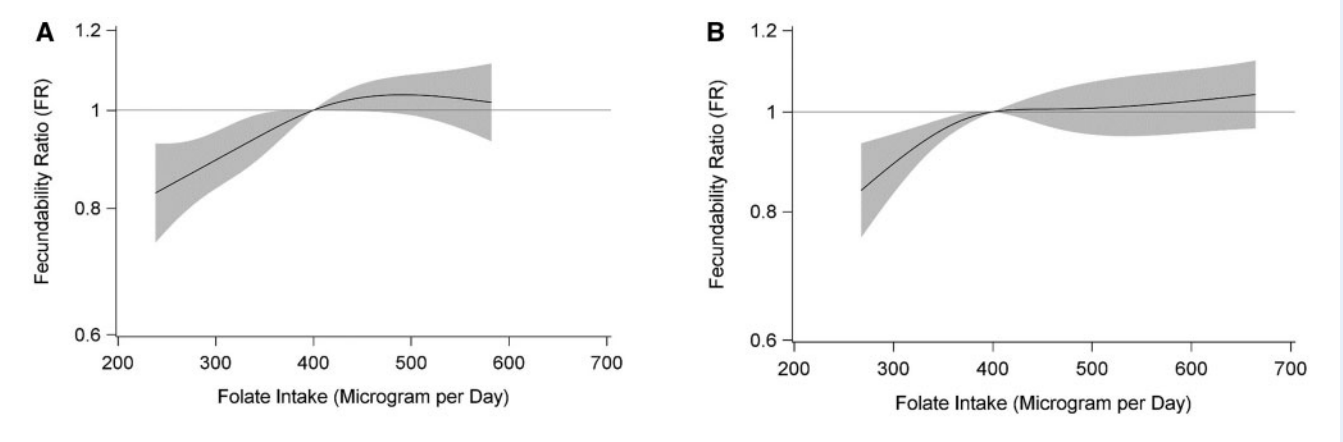

Figure 2 Dietary folate intake and fecundability among participants in SnartForaeldre.dk and PRESTO, fitted by restricted cubic splines. (**A**) SnartForaeldre.dk, (**B**) PRESTO. The lines indicate fecundability ratio and the shaded area indicates the 95% CI. The reference level is dietary folate intake 400 μg/day and the curves are adjusted for age, partner's age, vocational training, household income, parity, physical activity, alcohol consumption, current smoking, timing of intercourse and race/ethnicity (PRESTO).

implantation, clinical pregnancy and live birth (Gaskins *et al.*, 2014). Among these women, 78% took at least 400 μg/day folic acid and 19% took at least 1000 μg/day. There was a positive linear relation between live birth rates and folic acid supplements up to 1200 μg/day and likewise for DFEs up to 3200 μg/day. Further, a study of 259 healthy women of reproductive age found that diets high in synthetic folate were associated with increased levels of progesterone and lower risk of sporadic anovulation (Gaskins *et al.*, 2012). Although these studies did not evaluate fecundability, the results are consistent with our findings.

In both cohorts, dietary intake was estimated from all individual foods and mixed recipes. Over- and underreporting is a potential problem when FFQs are used to obtain dietary intake (Willett *et al.*, 1997; Subar *et al.*, 2003). However, for example, mean folate and energy intake in SF seems plausible compared with data reported by women aged 25–34 years in the Danish background population (non-energy-adjusted folate: 387 μg/day versus 340 and energy: 1927 kcal/day versus 2101 kcal/day) (National Food Institute, 2015). Similarly, mean folate and energy and intake reported by women in PRESTO was consistent with intake reported in NHANES 2017–2018 for women aged 30–39 years (non-energy-adjusted folate (FDE): 433 versus 450 μg/day and energy: 1572 kcal/day versus 1885 kcal/day) (US Department of Agriculture Research Service, 2020).

Danish participants appeared to have lower absolute intake of dietary folate compared with PRESTO participants (energy-adjusted median of 369 versus 418 μg/day). This difference is most likely

Table IV Association of total folate intake (energy-adjusted dietary folate and supplementation) and fecundability among women in SnartForaeldre.dk and PRESTO.

Total folate intake (dietary and supplement)[1]			Unadjusted model	Adjusted model I[2]	Adjusted model II[3]	Adjusted model III[4]
	Pregnancies	Cycles	FR (95% CI)	FR (95% CI)	FR (95% CI)	FR (95% CI)
SnartForaeldre.dk, N = 3753						
<250, no supplement	59	397	0.76 (0.60–0.98)	0.76 (0.59–0.98)	0.77 (0.60–1.00)	0.82 (0.63–1.08)
250–399, no supplement	412	2207	0.90 (0.81–1.00)	0.88 (0.79–0.98)	0.89 (0.80–1.00)	0.90 (0.80–1.02)
≥400, no supplement	229	1236	0.90 (0.79–1.02)	0.96 (0.84–1.09)	0.96 (0.84–1.09)	0.96 (0.84–1.10)
<250, yes supplement	94	561	0.80 (0.66–0.98)	0.79 (0.65–0.98)	0.80 (0.65–0.99)	0.85 (0.68–1.07)
250–399, yes supplement	1002	5284	0.93 (0.86–1.01)	0.92 (0.84–1.00)	0.92 (0.85–1.01)	0.94 (0.86–1.03)
≥400, yes supplement	779	3757	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
PRESTO, N = 5804						
<250, no supplement	19	298	0.40 (0.26–0.62)	0.49 (0.31–0.77)	0.50 (0.32–0.79)	0.56 (0.36–0.88)
250–399, no supplement	214	1805	0.75 (0.66–0.86)	0.79 (0.69–0.90)	0.80 (0.78–0.99)	0.83 (0.72–0.95)
≥400, no supplement	280	2152	0.82 (0.73–0.92)	0.88 (0.79–0.99)	0.88 (0.78–0.99)	0.90 (0.80–1.01)
<250, yes supplement	63	502	0.83 (0.65–1.04)	0.92 (0.72–1.16)	0.96 (0.76–1.22)	1.05 (0.82–1.34)
250–399, yes supplement	1246	8086	0.93 (0.88–1.00)	0.96 (0.90–1.02)	0.97 (0.91–1.04)	1.00 (0.93–1.07)
≥400, yes supplement	1912	11 528	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)

FR, fecundability ratio.
[1]Energy-adjusted dietary folate intake in micrograms per day and daily supplementation yes/no.
[2]Model I, adjusted for female age at baseline, partner's age at baseline, vocational training, household income, parity, physical activity, alcohol consumption, current smoking, timing of intercourse, and ethnicity (only PRESTO).
[4]Model II, adjusted for intake of sugar-sweetened beverage in addition to Model I adjustments.
[5]Model III, adjusted for Nutrient Rich Diet Score in SF and Healthy Eating Index in PRESTO in addition to Model I adjustments.

attributable to folic acid fortification of foods in North America, but differences in the FFQ instruments preclude a one-to-one comparison. In addition, we did not ask about cooking methods. Steaming food involves little loss of folate, in contrast to boiling (Delchier *et al.*, 2016). Differences in absolute dietary folate intake across cohorts is unlikely to alter the relative ranking of intake of participants within cohorts (Tabacchi *et al.*, 2016).

Misclassification may have occurred in the assessment of folic acid supplementation, as the participants reported if they took a supplement on daily basis, but not the dosage of folic acid or how many pills they took per day. We assumed that supplement users consumed at least 400 µg/day, equal to the dosage in most marketed multivitamin and single folic acid brands.

Although some misclassification of dietary folate intake and folic acid supplementation is likely, because both were reported at baseline, before pregnancy is recognized, the misclassification is likely non-differential, which would tend to bias the extreme exposure categories (<250 µg/day) toward the null. Because participants are invited to complete the FFQ only 10 days after baseline, we similarly expect any bias related to the exclusion of participants who did not complete the FFQ (SF: 23% and PRESTO: 37%) to have little impact on the study results (Greenland, 1977).

In both cohorts, unhealthy diet is likely the most important confounder of the association between dietary folate and fecundability, thus in an effort to address this, we used two different strategies. First, we adjusted for intake of sugar-sweetened beverages as a proxy for unhealthy diet. We used this proxy as it is associated with poor diet quality (Dunford, 2017) and of importance it does not contain folate (natural or from fortification). This adjustment did not change the estimates considerably. Second, we adjusted for NRD15.3 and HEI, both well-known instruments to assess diet quality. Unfortunately, we were not able to remove major sources of folate included in these instruments as folate is present in almost all foods, and the specific contributors may vary considerably across individuals. Further, if we remove foods with a high content of folate, we will also remove foods rich in other micronutrients, which may affect fecundability on their own or jointly with folate (Crider *et al.*, 2012). The association between folate intake and fecundability was attenuated after adjustment for NRD15.3, which may partly be caused by over-adjustment. In conclusion, although we adjusted for these and other potential confounders, such as smoking, education and income, we cannot exclude residual and/or unmeasured confounding from socioeconomic factors and poor diet.

The molecular mechanisms relating low total folate intake to reduced fecundability are complex and not fully understood. It is unknown whether intakes of dietary folate or folic acid supplements are associated with changes in DNA methylation on its own or if there is an interaction with other micronutrients (Crider *et al.*, 2012). Adequate folate intake is required to maintain normal one-carbon metabolism and epigenetic processes (Crider *et al.*, 2012). It has been suggested that insufficient folate status disrupts DNA methylation and increases homocysteine levels, which in turn may interrupt normal oocyte and follicular maturation, fertilization and embryo growth (Laanpere *et al.*, 2010).

Considering the optimal folate intake in relation to fecundability, both the source (natural versus synthetic) and the amount should be taken into account. Naturally occurring folate and synthetic folic acid vary in biochemical expression as folic acid is more oxidized, monoglutamated and stable than natural folate (Laanpere *et al.*, 2010). However, both forms of folate are converted into 5-methyl tetrahydrofolate during transit through the intestinal mucosa indicating that the amount of folate maybe more important than form of intake. The recommended intake of 250 μg DFE/day for adult women to maintain sufficient serum and red blood cell folate concentration seems too low for women trying to conceive (European Food Safety Authority, 2014). In most studies reporting a positive effect of folate on fertility, the total folate exposure was well above 400 μg/day (Gaskins and Chavarro, 2018). Our findings suggest that supplementation of 400 μg/day folic acid may not be sufficient to compensate for low dietary folate intake (<250 μg/day) in relation to fecundability, regardless of whether fortification is provided. Unfortunately, our data are not detailed enough to assess whether low dietary intake may be substituted with folic acid supplementation of, e.g. 800 μg/day or more. Further, because other B vitamins are of importance for the folate-requiring metabolic processes related to fertility, folic acid supplementation alone may not make up for low dietary folate intake (Bailey and Gregory, 1999).

In summary, preconception dietary folate intake was associated with fecundability in a monotonic pattern. A dietary folate intake below 250 μg/day, appears to be detrimental to fecundability if not supplemented with folic acid even in a population where the food supply is fortified with folic acid.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data used for this article cannot be shared publicly due to Danish data protection regulations. Anonymized data will be shared on reasonable request to the corresponding author.

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Authors' roles

E.M.M., H.T.C., E.E.H., L.A.W., H.T.S., E.T. and K.J.R. designed the study. E.M.M. and H.T.C. wrote the first and successive drafts of the paper. B.H.J., B.R.J. and A.H.R. carried out the statistical analysis. All authors contributed to the interpretation of results, reviewed and approved the final manuscript.

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Conflict of interest

None declared.

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