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The effect of continued folic acid supplementation beyond the first trimester on unmetabolised folic acid in late gestation

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B.Sc in Nutritional Sciences with Co-op Option
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A thesis submitted in fulfilment of the Doctor of Philosophy Degree in Sciences

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Table of Contents

Abstract

Folic acid (FA) is the synthetic form of the vitamin folate. Pregnant women are advised to take FA supplements before conception and during the first three months of pregnancy to reduce their chances of having a neural tube defect (NTDs) affected pregnancy, severe birth such as spina bifida and anencephaly. However, NTDs occur in the first month of pregnancy, and after that, there is no known benefit of continuing FA. Current recommendations do not advise stopping FA, so most women continue to take FA throughout pregnancy. Concerns have been raised that excess FA, especially in later pregnancy, may lead to adverse maternal and child health outcomes. These include large for gestational age, gestational diabetes, autism spectrum disorders, and childhood allergy. Australian Health authorities could simply change recommendations to advise women to women to stop FA once pregnancy is confirmed. Policymakers warn that changing recommendations will confuse women and undermine three decades of successful public health efforts encouraging women to take FA to reduce NTDs. All harms are based on observational evidence, which is insufficient to change policy. Only a high-quality RCT can supply definitive proof. Circulating unmetabolised folic acid (UMFA) in serum is used as a biomarker of excess FA intake and has been associated with an increased risk of adverse outcomes in some studies. Before commencing a large RCT, we first wanted to determine whether removing FA from prenatal supplements after the first trimester reduces the amount of UMFA at 36 weeks gestation.

We conducted a two-arm, parallel, double-blind RCT to address this question. Women with a singleton pregnancy 12-16 weeks (n=103) gestation were randomly assigned to a multi-micronutrient supplement without (intervention) or with 800 µg folic acid/day (control, current standard practice) from enrolment until 36 weeks gestation. We chose 800 µg/d because it is Australia's most commonly used FA dose. Of the 103 women randomised, 90 finished the study. The primary outcome was the difference in maternal serum UMFA concentration between the groups at 36 weeks. Only 12% of women had UMFA above the limit of quantification (LOQ); thus, we could not compare UMFA concentration. However, fewer women in the no folic acid (intervention) compared to the 800 µg folic acid/day (control) group (72% [n=33/46] vs 98% [n=43/44]; p = 0.001) had UMFA above the LOD. Removing FA from multi-micronutrient prenatal supplements after 12 weeks gestation reduced the number of women with detectable UMFA at 36 weeks gestation. However, differences in UMFA concentration between treatment groups were not quantifiable. Whether continued FA use beyond the first trimester increases the risk of adverse maternal and child

health outcomes remains unclear. This can only be answered with adequately powered RCTs to determine if removing folic acid from supplements after the first trimester alters the rate of outcomes. Only after this trial is complete can an informed decision be made about the risks of continued FA supplementation after the first trimester.

Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications Arising from This Thesis

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Sulistyoningrum DC, Sullivan TR, Skubisz M, Palmer DJ, Wood S, Marten SF, Trim PJ, Makrides M, Green TJ, Best KP. Detection of maternal serum unmetabolised folic acid following multivitamin and mineral supplementation with or without folic acid after 12 weeks' gestation: a randomised controlled trial.
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Other Related Publications during Candidature (Listed as Appendices)

Best KP, Green TJ, **Sulistyoningrum DC**, Sullivan TR, Aufreiter S, Prescott SL, Makrides M, Skubisz M, O'Connor DL, Palmer DJ. Maternal Late-Pregnancy Serum Unmetabolized Folic Acid Concentrations Are Not Associated with Infant Allergic Disease: A Prospective Cohort Study. J Nutr. 2021 Jun 1;151(6):1553-1560. doi: 10.1093/jn/nxab040. PMID: 33851208. (Appendix 1)

Samson KLI, Loh SP, Lee SS, **Sulistyoningrum DC**, Khor GL, Mohd Shariff ZB, Ismai IZ, Makrides M, Hutcheon JA, Roche ML, Green TJ, Karakochuk CD. The Inclusion of Folic Acid in Weekly Iron-Folic Acid Supplements Confers no Additional Benefit on Anemia Reduction in Nonpregnant Women: A Randomised Controlled Trial in Malaysia. J Nutr. 2021 Aug 7;151(8):2264-2270. doi: 10.1093/jn/nxab115. PMID: 33978167. (Appendix 2)

Samson KLI, Loh SP, Lee SS, **Sulistyoningrum DC**, Khor GL, Shariff ZBM, Ismai IZ, Yelland LN, Leemaqz S, Makrides M, Hutcheon JA, Roche ML, Karakochuk CD, Green TJ. Weekly iron-folic acid supplements containing 2.8 mg folic acid are associated with a lower risk of neural tube defects than the current practice of 0.4 mg: a randomised controlled trial in Malaysia. BMJ Glob Health. 2020 Dec;5(12):e003897. doi: 10.1136/bmjgh-2020-003897. PMID: 33272946; PMCID: PMC7716666. (Appendix 3)

Samson KLI, Loh SP, Khor GL, Mohd Shariff Z, Yelland LN, Leemaqz S, Makrides M, Hutcheon JA, **Sulistyoningrum DC**, Yu JJ, Roche ML, De-Regil LM, Green TJ, Karakochuk CD. Effect of once weekly folic acid supplementation on erythrocyte folate concentrations in women to determine potential to prevent neural tube defects: a randomised controlled dose-finding trial in Malaysia. BMJ Open. 2020 Feb 5;10(2):e034598. doi: 10.1136/bmjopen-2019-034598. PMID: 32029499; PMCID: PMC7044827. (Appendix 4)

Probst Y, **Sulistyoningrum DC**, Netting MJ, Gould JF, Wood S, Makrides M, Best KP, Green TJ. Estimated Choline Intakes and Dietary Sources of Choline in Pregnant Australian Women. Nutrients. 2022; 14(18):3819. https://doi.org/10.3390/nu14183819. (Appendix 5)

Conference Presentations Arising from This Thesis

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Abstract Tittle: Randomised controlled trial protocol for evaluating the effect of folic acid supplementation beyond the first trimester on maternal unmetabolised folic acid (UMFA) in late gestation

Dian C Sulistyoningrum, Timothy J Green, Debra J Palmer, Thomas R Sullivan, Simon Wood, Maria Makrides, Monika Skubisz, Karen P Best

Awarded Postgraduate Oral Presentation Award (Appendix 6)

South Australia Health Medical Research Institute Research Showcase Oral Presentation 2021

Perinatal Society of Australia and New Zealand Poster Presentation 2022

Abstract Tittle: Detection of Serum Unmetabolised Folic Acid at 36 Weeks' Gestation in Pregnant Women Taking Multi-Vitamin and Mineral Supplements With or Without 800 µg Folic Acid: A Randomised Controlled Trial

Sulistyoningrum DC[1,2], Sullivan TR[1,4], Skubisz M[1,2], Palmer DJ[3,4], Wood S5[,6], Snel MF[7], Trim PJ[7], Makrides M[1,2], Green TJ[1,2], Best KP[1,2]

International Congress of Nutrition Poster Presentation 2022

Abstract Tittle: Detection of serum unmetabolised folic acid at 36 weeks' gestation in pregnant women taking multi-vitamin and mineral supplements with or without 800 µg folic acid

Dian C Sulistyoningrum[1,2], Thomas R Sullivan[1,3], Monika Skubisz[1,2], Debra J Palmer[4,5], Simon Wood[6,7], Marten F Snel[2,8], Paul J Trim[2,8], Maria Makrides[1,2], Timothy J Green[1,2], Karen P Best[1,2]

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Abbreviations

ASD	:	autism spectrum disorder
BMI	:	body mass index
CDC	:	Centers for Disease Control and Prevention
DHF	:	dihydrofolate
DHFR	:	dihydrofolate reductase
FPB	:	folate binding protein
GDM	:	gestational diabetes mellitus
Ig-E	:	immunoglobulin E
MBA	:	microbiological assay
MTHF	:	methyl tetrahydrofolate
NHMRC	:	National Health and Medical Research Institute
LC-MS/MS	:	liquid chromatography-tandem mass spectrometry
LGA	:	large-for-gestational-age
LOD	:	limit of detection
LOQ	:	limit of quantification
NSAID	:	non-steroid anti-inflammatory drug
NTD	:	neural tube defect
PABA	:	para-aminobenzoic acid
PCFT	:	proton-coupled folate transporter
RANZCOG	:	Royal Australia and New Zealand College of Obstetricians and Gynaecologists
RBC	:	red blood cell
RCT	:	randomised clinical trial
RFC	:	reduced folate carrier
SAM	:	s-adenosyl methionine
SAH	:	s-adenosyl homocysteine
SPE	:	solid-phase extraction
THF	:	tetrahydrofolate
UMFA	:	unmetabolised folic acid
WHO	:	World Health Organisation

Chapter 1:

Introduction

Neural tube defects (NTDs) are caused by the failure of the neural tube to close properly, which it normally does around 28 days post-conception (1). The neural tube becomes the brain and spinal cord. There are several types of NTDs, the most common are anencephaly and spina bifida (2). Anencephaly occurs when the neural tube that becomes the brain fails to close, and the baby dies at birth or soon after. Spina bifida occurs when the lower neural tube fails to close which becomes the spinal cord that leads to nerve damage and varying degree of paralysis and other disabilities.

Globally, it is estimated that 300,000 babies are born with NTDs each year (3). The incidence of NTDs varies geographically; for example, rates in Northern China were estimated at 200 per 10,000 births between 2004 to 2005, whereas in Spain, the rates were estimated at 1.3 per 10,000 births each year between 2003 to 2012 (3). In Australia, the incidence annually between 2006-2008 was estimated at 8.8 per 10,000 births (3).

The exact aetiology of NTDs is believed to involve the interplay between genetic and environmental (modifiable) factors. Because the neural tube closes early in pregnancy, modifiable factors around the time of conception and early pregnancy are important. Over 50 years ago, it was suspected that a lack of folate might increase the risk of NTDs (4). However, it was not until the 1990s when high quality randomised controlled trials (RCTs) were published, showing that taking folic acid prior to and during early pregnancy reduced the risk, and that folic acid could prevent NTDs (5, 6).

In response to this finding, health authorities around the world issued recommendations for women planning pregnancy to take folic acid. In Australia, The Royal Australia New Zealand College of Obstetricians and Gynaecologists (RANZCOG) recommends that “folic acid be taken for a minimum of one month before conception and the first 12 weeks of pregnancy” (7). The recommended dose of folic acid is at least 400 µg per day to aid prevention of NTDs. These public health messages were only partially effective

because the neural tube closes early in pregnancy before many women know they are pregnant and many pregnancies are unplanned (8, 9). In response, many governments mandated the addition of folic acid to a food staple such as wheat flour. The advantage of fortification is that it is passive and does not require behaviour change. In Australia, the government mandated the addition of folic acid to bread flour (10). This fortification has increased the intake of folic acid and has been associated with a temporal decline in the incidence of NTDs in women of childbearing age (11).

In Australia, many women take folic acid as part of prenatal multivitamin mineral supplements throughout pregnancy (12). While the benefit of taking folic acid prior to and during early pregnancy is not in questioned, there are no proven benefits beyond the first trimester. The common practice of continuing folic acid supplementation beyond the first trimester (13) is worrying due to increasing evidence from observational studies that exposure to excess folic acid in late pregnancy may be associated with adverse maternal and child health outcomes, such as gestational diabetes mellitus, large-for-gestational age, autism spectrum disorders, allergic disease (14, 15). Allergic disease is of particular interest because Australia has among the highest rates of allergy in the world.

In Australia, the market-leading supplement provides 800 µg per day (11). Together with a current practice of continuing folic acid supplementation beyond the first trimester and at a dose higher than what is recommended, women may need to discontinue folic acid supplementation after the first trimester. However, currently all studies suggesting that late-term folic acid causes harm are observational and cannot infer causation. We need better evidence from RCTs before we risk compromising decades of public health policy to encourage women to take folic acid supplements to prevent NTDs. Unmetabolised folic acid (UMFA) is a biomarker of excess folic acid. In acute dosing studies in non-pregnant individuals, UMFA rises rapidly after folic acid ingestion and falls over the following hours

(16, 17). The greater the dose of folic acid, the higher the UMFA concentration and the longer it is detected in serum. The effect of chronic folic acid administration on UMFA is less clear. UMFA has been detected in maternal blood samples in several population studies (18-21) and in one RCT in a country without mandatory fortification (22). However, there are no published RCTs investigating the effect of higher doses of prenatal folic acid containing supplements (i.e. the 800 µg/d used in Australia), on top of folic acid from mandatory fortification on UMFA concentration.

Although the importance of taking folic acid supplements in early pregnancy to reduce NTDs is not in doubt, supplementation beyond this time is questionable. In this thesis, I aim to investigate the effect of removing folic acid from prenatal supplements *after* 12 weeks gestation compared with the common practice of continuing folic acid supplementation of 800 µg/day throughout pregnancy on maternal serum UMFA at 36 weeks gestation.

In what follows, I provide a literature review, the published trial protocol and the main study results, followed by concluding remarks including strengths, limitations and future directions.

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Chapter 2:

Literature Review

In the following literature review, I will briefly cover the discovery of folate, folate metabolism, and the functions of folate. The chapter begins with a review of folate recommendations and folate biomarkers, including UMFA a potential biomarker of folic acid excess. A discussion of folic acid and NTD prevention will follow with a review of public health policies to prevent NTDs, including folic acid supplementation recommendations and food fortification. The next section will then review other potential benefits and harms of folic acid during pregnancy for the health of the mother and child. Afterwards, I will outline the rationale for the current study.

2.1 Folate

The term folate refers to any of a group of water-soluble B vitamin compounds with nutritional properties and a chemical structure similar to folic acid. In the 1930s, Lucy Wills identified a new "haematopoietic factor" found in Brewer's Yeast that cured macrocytic anaemia in pregnant women and named it Will's Factor (1). Folate was subsequently isolated from spinach leaves in 1941 and synthesised in its pure crystalline form in 1943 (2). The name was later changed to folate, the term *folic* is derived from the Latin *folium* meaning leaf, due to its abundance in green leafy vegetables (3).

Folates are found naturally in many foods with green leafy vegetables and beef liver being particularly good sources (3). Folic acid is the synthetic form of folate and is typically used in dietary supplements and added to fortify foods due to its stability and high bioavailability (3). Folate plays an important role in a number of important biochemical pathways, most notably those involved in one-carbon metabolism, purine and pyrimidine, and amino acid synthesis (4). Folate deficiency in its most severe form leads to megaloblastic anaemia, which is characterised by a low haemoglobin, and low red blood cell count, as well as the presence of large red blood cell precursors called megaloblasts in the bone marrow (3).

2.1.1. Forms of folate

All folates consist of para-aminobenzoic acid (PABA) molecule linked at one end to a pteridine ring by a methylene bridge and at the other to glutamic acid by a peptide linkage. Folic acid, which occurs rarely in nature, is the most oxidised form of folate, whereas 5-methyl tetrahydrofolate (5-MTHF) is the most reduced form of folate, and the form of folate is normally found in greatest abundance in circulation (3).

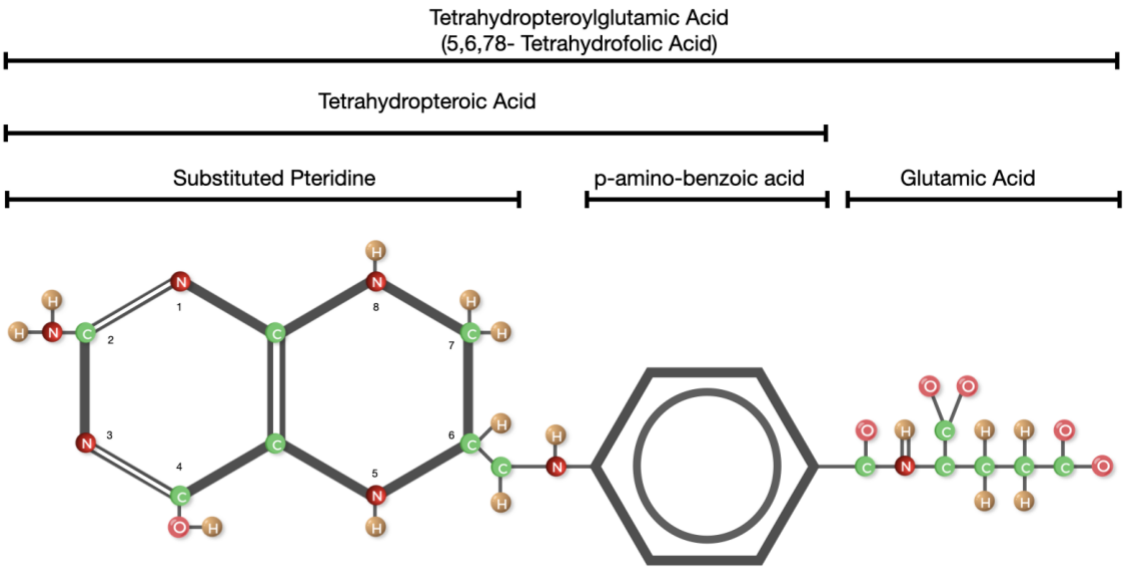
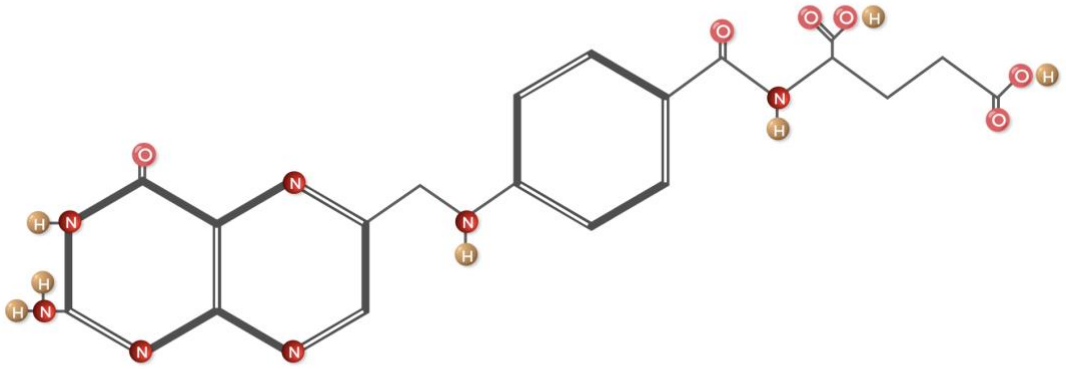
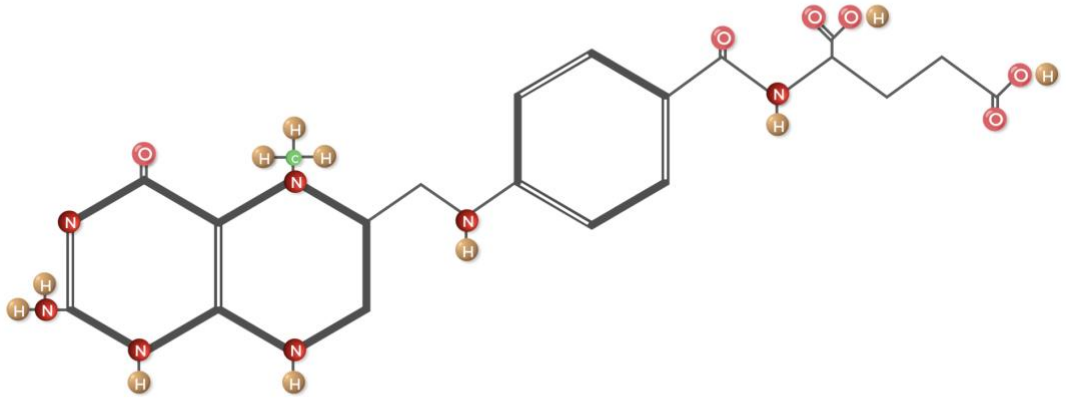


Figure 1A. Chemical structure of tetrahydrofolate



Folic Acid
($C_{19}H_{19}N_7O_6$)



5-Methyltetrahydrofolate
($C_{20}H_{25}N_7O_6$)

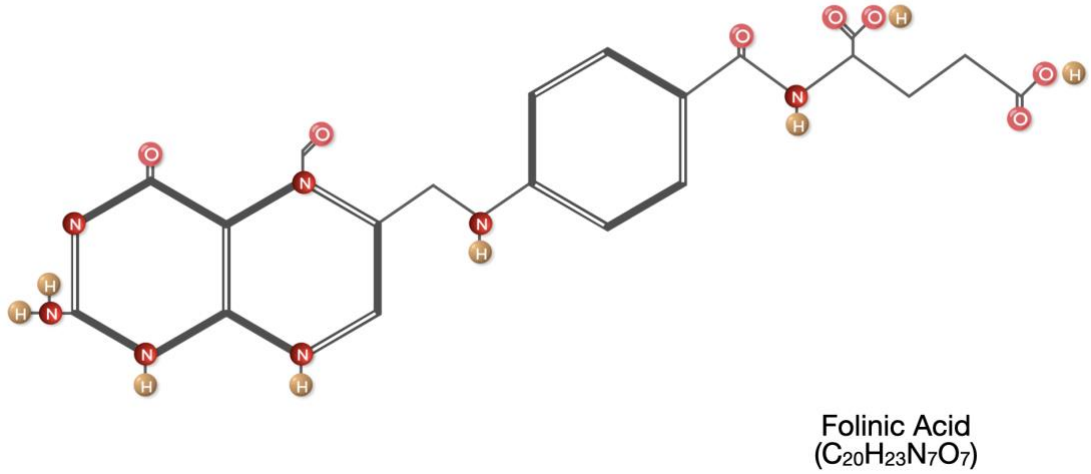


Figure 1B. Structures of Folic acid, 5-Methyltetrahydrofolate, Folinic acid (Adapted from (3))

Naturally occurring folates exist in varying degrees of reduction with one-carbon units attached at the N^5 nitrogen atom of the pteridine ring and/or the N^{10} nitrogen atom of the p-aminobenzoyl group. Most naturally occurring folates contain an additional one to six glutamate molecules joined in a peptide linkage to the γ-carboxyl of glutamate.

2.1.2 Intestinal Absorption, Transport, Storage, and Excretion of Folate

Naturally occurring dietary folates usually have an additional 2-8 glutamate residues attached in a peptide linkage. These additional glutamates must be cleaved before absorption, by mucosal folyl γ-glutamyl carboxypeptidase (EC 3.4.22.12) (commonly known as folate conjugase), an enzyme found in the proximal intestinal brush border (3).

Once cleaved, the deconjugated folates are absorbed by active uptake across the brush border of the enterocyte and facilitated by two transporters; and the reduced folate carrier (RFC). The proton-coupled folate transporter has a greater affinity for folic acid than reduced folates (3). In contrast, the reduced folate carrier has a greater affinity for reduced folates than

folic acid. Folates can also be absorbed into the enterocyte through passive diffusion, which accounts for 20-30% of absorption when the folate intake is high (3).

Folates mainly circulate in portal circulation as 5-methyl tetrahydrofolate (5-MTHF) bound to albumin (low-affinity protein binder) or a high-affinity Folate Binding Protein (4). Folic acid circulates freely in plasma. Folate storage is primarily in the liver and prior to storage, absorbed monoglutamylated folates are converted to polyglutamylated folates, a reaction catalysed by the enzyme, folylpolyglutamate synthase (EC 6.3.2.12). The addition of glutamates to monoglutmayl folate allows the cell to maintain folate within the cell at concentrations greater than those of extracellular fluids (3). In order to export from the cell polyglutamylated folates must be converted to the monoglutamylated form of folate, a reaction catalysed by conjugase.

Folate is mainly excreted in the urine after being converted to acetamidobenzoylglutamate, which is formed through the oxidative cleavage of the folate molecule at the C9-C10 bond (3).

2.1.2.1 Folic acid metabolism

Folic acid must be reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) in order to be metabolically active, reactions catalysed by dihydrofolate reductase (see Figure 2 – Folic acid metabolism). At a low intake, folic acid is reduced to THF, which mainly occurs in the enterocyte and liver (3).

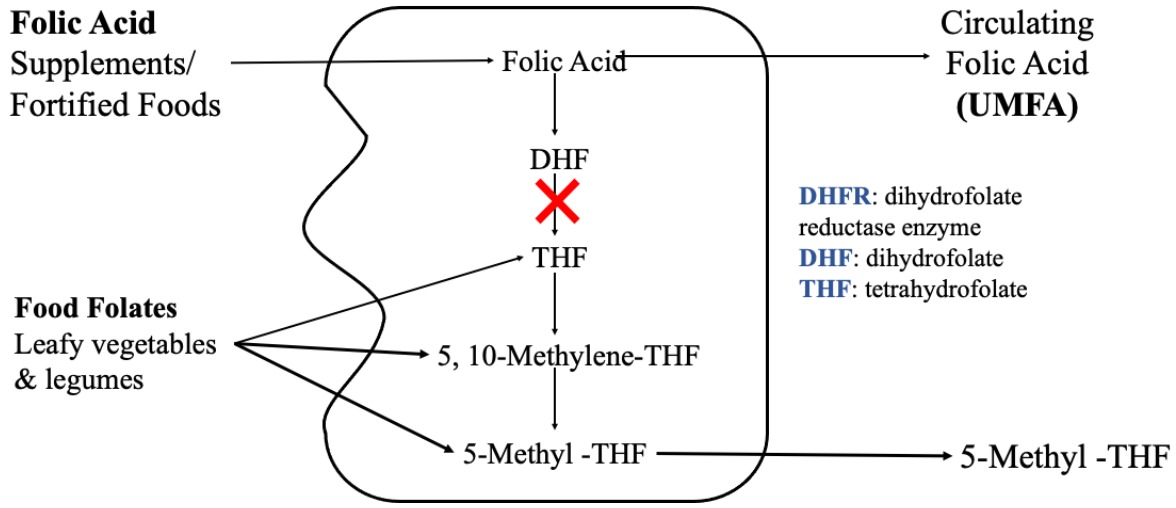


Figure 2. Folic acid metabolism

THF is further reduced to 5-MTHF which is released into circulation. At higher intakes of folic acid, DHFR becomes saturated and folic acid passes unaltered into circulation (see Figure 2).

Circulating folic acid is often referred to as unmetabolised folic acid (UMFA). The amount of folic acid ingested resulting in UMFA is not known and affected by several factors, including genetics, the amount of folic acid consumed, and the frequency of consumption during the day. Kelly et al (1997) reported the appearance of UMFA in serum following the acute consumption of folic acid-fortified food (either as isotonic saline, milk, or white bread) after > 200 µg of intake (5). A pharmacokinetic study in 20 healthy male volunteers reported that following ingestion of 800 µg of folic acid, UMFA concentrations peaked around 2.5 hours after ingestion which immediately started disappearing and completely disappeared after 12 hours (6). The effects of consuming this dose chronically on UMFA concentrations are not known.

2.1.3. Metabolic functions of folate

Folate in its coenzyme forms is involved in shuttling (oxidation and reduction) one-carbon units (CH_3) in purine and thymidine synthesis and amino acid metabolism. Folate is indirectly involved in DNA methylation by providing a methyl group to homocysteine making methionine which in turn is converted to S-adenosylmethionine (SAM), the universal methyl donor (3) (**see Figure 3**).

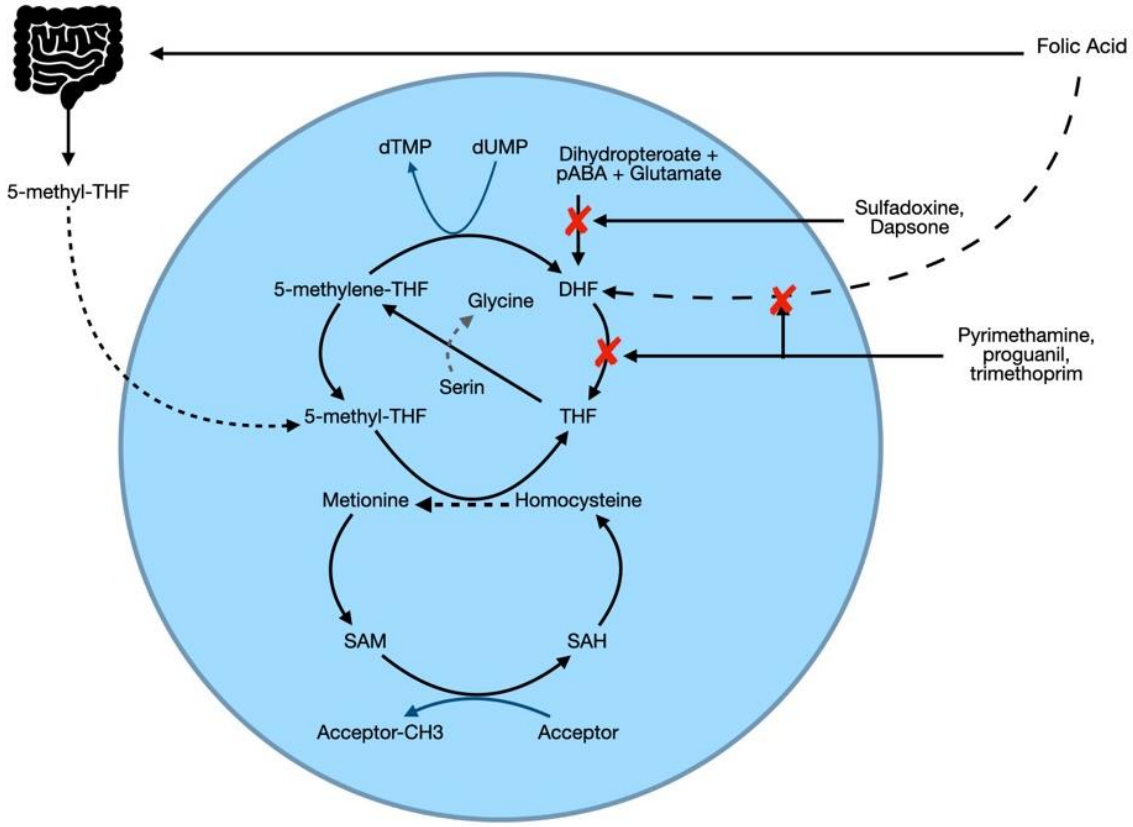


Figure 3. Metabolic functions of folate (Adapted from (7)). THF, tetrahydrofolate; DHF, dihydrofolate; SAM, s-adenosyl methionine; SAH, s-adenosyl homocysteine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; pABA, para-aminobenzoic acid.

The mechanism by which UMFA could be harmful is unclear. Concerning increased allergy, it has been suggested folic acid's role in one-carbon metabolism is a mediator. As described in Figure 3, circulating UMFA from excess folic acid could lead to excess SAM, the major methyl (CH3) donor, that could increase DNA methylation. However, currently

there is no sufficient in vivo study to support this hypothesis. A previous *in vivo* study of in utero supplementation of methyl donors in mice was retracted. The study reported that DNA methylation altered the expression of *Runx3* (Runt-related transcription factor 3) gene, which is involved in immune cell regulation (8). The study showed that maternal diet high in methyl donors decreased transcriptional activity of *Runx3* in lung tissues of the offspring, which then contributed to the severity of allergic airway disease. Further mechanistic study is necessary to elucidate the mechanism by which excess maternal folic acid exposure in late gestation modifies gene expressions in the offspring.

There have been a couple of reports in humans that showed DNA methylation alteration following folic acid supplementation. In a randomised clinical trial (RCT) - folic acid supplementation in second and third trimester (FASSTT) trial reported that folic acid supplementation at 400 µg in the second and third trimester alters the DNA methylation of genes involved in neurodevelopment (9, 10).

2.1.4. Dietary Intake Recommendation for Folate

Folate intake recommendations were set in 2006 by the National Health and Medical Research Council of Australia, and are published as Nutrient Reference Values (NRVs) for Australia and New Zealand (11). These folate intake recommendations were adopted from the 1997 United States Institute of Medicine Dietary Reference Intakes (12). Folate intake recommendations were established jointly by Canada and The US and are expressed as Dietary Folate Equivalents (DFEs) which is used to reflect the higher bioavailability of folic acid from supplements and fortified foods than naturally occurring folate. Naturally occurring folates in food are assigned a value of 1 µg DFE and folic acid from fortified foods and supplements 1.7 µg DFE (12).

Folate intakes vary by life stage and age. Like most other nutrients, the NRVs for folate include 3 values; Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), and an Upper Limit (UL). The EAR is the folate intake that is estimated to meet the requirement of half the healthy individuals in a group. The RDA is the average daily dietary intake level that is sufficient to meet the folate requirement of nearly all (97 to 98 percent) healthy individuals in a group because dietary advice is being given to individuals for whom we do not know their requirement the RDA is the most appropriate values to use. The RDA for folate in non-pregnant women over 14 years is 400 µg/d DFE. Due to the high demand for folate, the RDA is increased in pregnancy and lactation, 600 and 500 µg/d DFE, respectively (11). The NRVs for folate do not include the additional folic acid recommended for women capable of becoming pregnant who are advised to consume an additional 400 µg per day of folic acid, either as a supplement or from folic acid-fortified foods.

An upper limit (UL) is defined as the highest level of daily intake that is likely to pose no adverse health effects in most human individuals. The UL for folate is set at 1000 µg, and only applies to folate as folic acid not naturally folic acid. Both folate and B12 deficiency lead to megaloblastic anaemia. The UL was set due to concerns in B12 deficiency, folic acid might correct the anaemia, but allow the neurological damage caused by B12 deficiency to go undiagnosed (11).

2.1.5 Folate deficiency

Folate deficiency may arise from insufficient intake, increased requirement during pregnancy, malabsorption due to excessive alcohol intake and smoking, and due to certain drugs interfering with folate metabolism such as nonsteroid anti-inflammatory drugs (NSAIDs) and anticonvulsant (phenytoin). Folate deficiency impedes the rapid cell division

in the bone marrow causing the RBCs to be immature and enlarged. Because the RBCs are too large, they impede the cells to exit the bone marrow to enter the bloodstream and deliver oxygen. The clinical consequence of folate deficiency is megaloblastic anaemia, which is when the red blood cells (RBCs) became enlarged.

2.1.6 Folate biomarkers

2.1.6.1 Serum and red blood cell (RBC) folate

The most commonly used biomarkers for folate status are serum folate or RBC folate. Serum folate concentrations reflect current folate status and are affected by recent folate intake. Serum folate rises after ingestion of folate and falls after a few hours. Thus, it should ideally be measured in a fasted state. Meanwhile, the red blood cell folate (RBC) concentration reflects a long-term folate status and is not affected by recent folate intake. Serum folate concentration is not recommended as a biomarker for NTD prevention. However, a cut-off of less than 6.8 nmol/L is often used to define severe deficiency as below this level the risk of megaloblastic anaemia rises markedly (12). Similarly, the cut-off for RBC folate concentration for severe folate deficiency is <305 nmol/L (12). Red blood cell folate concentration has been used at the population level to define NTD risk, with concentrations greater than 905 nmol/L associated with a low risk of NTDs (13). This cut-off is based on the microbiological assay in which folic acid is used as the calibrator. Recently the US CDC changed the calibrator to 5-MTHF resulting in a change in the cut-off to 748 nmol/L (14).

2.1.6.2 Unmetabolised folic acid (UMFA)

While serum or plasma and RBC folate concentrations are more well-established folate biomarkers, unmetabolised folic acid (UMFA) has been gaining more attention in the

past two decades. As previously reviewed, UMFA reflects the excess folic acid mostly from fortified foods and supplements that cannot be metabolised because the enzyme has reached saturation.

Unlike serum or plasma and RBC folate measurements, methods to measure UMFA vary from one laboratory to another, and there is not one gold standard method. While liquid-chromatography tandem mass spectrophotometry (LC-MS/MS) is the most reliable and common method used in most laboratories, the components of the analysis such as the range of standard curve, the limit of detection (LOD), and the limit of quantification (LOQ) have not been standardised. Therefore, in interpreting UMFA concentrations, one must consider these factors.

Recent evidence has highlighted concern surrounding chronic exposure of folic acid on health as the presence of UMFA has been detected in pregnant women in Germany (15), Australia (16), and Canada (17), as well as in all other population groups in the US (18). It remains unclear whether it is the presence of UMFA or the amount of UMFA that is of concern. It also still remains unclear what the clinical relevance of circulating UMFA on human health is. There are currently no clinical cut-offs of UMFA concentration related to human health.

2.1.7 Folate and neural tube defects

Folic acid taken prior to and during early pregnancy reduces the risk of a neural tube defect (NTD) affected pregnancy. The possibility that folate or folic acid might reduce NTDs was first raised by Hibbard in 1964 (19). Since 1965, a series of observational and quasi-experimental studies suggested that a lack of folate was associated with increased NTD risk and that giving folic acid periconceptionally during pregnancy could reduce NTDs (20-22).

Definitive proof that folic acid reduces NTDs risk came in 1991 from a double-blind 2x2 factorial RCT conducted at 33 centres in seven countries by the British Medical Research Council (23). Women (n=1817) with a prior history of an NTD-affected pregnancy were randomly assigned to one of four treatment groups: folic acid alone (4 mg), other multivitamins but not folic acid, both folic acid and multivitamins, and neither. Women in the trial were asked to consume the supplement once daily from the time of randomisation until 12 weeks of pregnancy. Data were obtained from 1195 women in which the outcome of the pregnancy was known. Of the women who received folic acid with or without other vitamins there were 6/593 giving a recurrence rate of 1%. In the groups not receiving folic acid, there were 21/602 affected pregnancies, with a recurrence rate of 3.4%. Receiving folic acid reduced the recurrence of NTDs by almost 80%. Unsurprisingly, the study was halted early when the interim analysis showed such a large benefit of folic acid.

That folic acid could prevent the first occurrence of an NTD in pregnancy was demonstrated by Czeizel and Dudas in 1992 (24). Women (n=4682) were randomised to either a supplement containing multivitamins with 800 µg folic acid and trace elements or a supplement containing trace elements only. There were no cases of NTDs in the group who received folic acid, but there are 6 in the group who received only the trace elements ($p = 0.02$). Although the supplement contained vitamins other than folic acid based on the findings from the British Medical Research Council study, it was concluded that folic acid was likely responsible for the reduction in NTDs.

In 1999, a sizable volume of research has investigated public health intervention in China (25). Originally designed to be an RCT, it was turned into a public health intervention study due to ethical concerns. The study intervention was 400 µg/d folic acid from the premarital examination until the end of the first trimester of pregnancy in two areas of China, one in the North with high background rate of NTDs (50 to 60 per 10000 births per year) and

another in the South with a low background rate (1 per 10000 births per year). Women in both regions who took folic acid during the periconceptional period had lower rates of NTD affected pregnancies. The magnitude of the reduction in NTDs was higher in the Northern region than the Southern region, 79% and 41%, respectively. The study confirmed that 400 µg per day folic acid could lower the risk of NTDs.

2.1.8 Other potential benefits of folate during pregnancy

It has been suggested that folic acid during pregnancy may have additional health benefits for mothers and children. Most studies include a reduction in the risk of pre-eclampsia, cleft palate and lip, heart defects and prematurity. Preeclampsia is a condition of high blood pressure in pregnancy that occurs in ~3% of pregnancies in Australia (26). Treatment for preeclampsia is delivery of the fetus (26). A large prospective cohort study found an association between folate supplementation or multivitamins containing folic acid and a reduction in pre-eclampsia in high-risk women (OR 0.17, 95% CI: 0.03, 0.95) (27). However, a meta-analysis of observational studies (28) and randomised studies found no differences in outcomes with folic acid or multivitamin containing folic acid. Moreover, a randomised clinical trial of 2464 pregnant women in five countries: Argentina, Australia, Canada, Jamaica and the UK has shown that supplementation with 4.0 mg per day of folic acid compared with the placebo after the first trimester did not prevent pre-eclampsia among high-risk women (RR 1.10, 95% CI: 0.90,1.34) (29).

In addition to preeclampsia, there are several reports, mostly observational and small trials, suggesting that folate may be associated with a reduced risk of other adverse pregnancy outcomes. However, a 2015 Cochrane review concluded that there was insufficient data to evaluate the effects of supplementation with folic acid on outcomes such as cleft lip and

palate, congenital cardiovascular anomalies, other congenital anomalies, and miscarriages (30).

2.1.9 Potential harms of higher exposure to folate and folic acid during pregnancy

More than 80% of Australian women report taking a multivitamin and mineral supplement throughout pregnancy, often containing large amounts of folic acid (31). The market leader of prenatal multivitamin supplements in Australia promotes their product as "vitamins and minerals to support you throughout the different stages of pregnancy: before, during and after." This product contains double the amount of folic acid 800 µg per day recommended in early pregnancy for the prevention of neural tube defects. The extensive promotion of supplements to pregnant women by the supplement industry disregards current recommendations from the NHMRC and the RANZCOG. The marketing of such products is not supported by evidence of improvement in maternal or infant outcomes and pregnant women may be vulnerable to unsubstantiated messages about giving their baby the best start in life, regardless of cost or potential harm. In addition to supplementation, Australian women receive 150 to 200 µg per day of folic acid from food containing fortified bread flour (32).

Recent reports of the potential harm of excess folic acid intake during late gestation appear. A study from India published in 2008 reported that higher maternal RBC folate at 28 weeks of pregnancy predicted greater adiposity (fat mass and body fat percentage) and higher insulin resistance in the offspring at 6 years of age (33). Similarly, a more recent meta-analysis that pooled three studies found a significant increase in gestational diabetes mellitus (GDM) risk with the highest versus lower category of maternal RBC folate (34). The dose-response analysis revealed that for every 200 ng/mL increase of RBC folate, the risk of GDM

increased by 8% (34). The meta-analysis found no significant increased risk of GDM associated with the highest level of folic acid supplement intake and plasma folate.

More recent studies reported that the use of folic acid supplementation in prenatal multivitamin and mineral supplements was positively and significantly associated with birthweight (35). Babies born above the 90th percentile of gestational age are classified LGA. Consequently, LGA imposes maternal complications such as prolonged labour, perineal tears, and post-partum haemorrhage (36). Furthermore, LGA babies have higher risk of hypoglycaemia, respiratory distress, and fetal death (36), with long-term consequences of increased risk of adverse health outcomes such as obesity, diabetes and cardiovascular disease (37, 38). The mechanism by which maternal folic acid affects fetal development and birthweight is not fully understood. However, epigenetics programming through DNA methylation may be the underlying mechanism. Furthermore, mothers with GDM are more likely to give birth to babies with higher birthweight or large-for-gestational age babies (39, 40).

While the potential harms of folate during pregnancy reviewed in the previous paragraph pertains to RBC folate, concerns about the potential detrimental effects of folate or folic acid during pregnancy have been stemmed from the accumulation of unmetabolised folic acid (UMFA). This is particularly of concern in the population of women from countries with mandatory folic acid fortification in addition to women taking folic acid supplement periconceptionally, although current evidence is inconclusive.

As reviewed by Wiens and DeSoto (41), some recent studies have reported the slight protective effect of folic acid supplementation against autism spectrum disorder (ASD) (42, 43), but other studies have reported that folic acid supplementation increased ASD risk (44). However, these studies have limitations in their relation to this thesis. While the protective effect of folic acid supplementation against ASD was primarily associated with its use during

early gestation, the potential harm with ASD was associated with cord blood UMFA as opposed to maternal UMFA in late gestation. Similar to its association with ASD, excess folic acid intake due to folic acid supplementation during pregnancy against a background of folic acid fortification has raised concern for an increased risk of allergic disease in the offspring.

2.1.9.1 Exposure to folate or folic acid during pregnancy and allergy diseases in children

For the relevance of this thesis, it is important to highlight the association between folate and folic acid intake during pregnancy and allergic disease in the offspring. The prevalence of allergy diseases has increased in the past two decades. Australia, in particular, has among the highest prevalence of allergic disorders in high-income countries (45). Findings from observational studies examining the association between folate or folic acid exposure during pregnancy and allergy risk have been equivocal. This is mostly due to the heterogenous study design including differences in timing and method of estimating folate or folic acid exposure. Methods varied among studies from dietary folate intake, folic acid supplement use to folate/folic acid biomarkers (See **Appendix 6** and **Appendix 7**, pg. 135 to 146). Studies reporting associations between maternal UMFA concentration and allergic disease have not been available until recently (16, 46). Similarly, allergic disease outcomes in offspring varied from asthma and wheezing, rhino conjunctivitis/hay fever, atopic dermatitis/eczema, food allergy, to sensitisation.

A review from 2017 highlighted the potential contributing role of folate/folic acid supplementation during pregnancy in the development of allergic diseases in the offspring (47). However, evidences from epidemiological studies have been conflicting. An observational study from my research group examined associations between maternal serum UMFA and folate concentration and allergic disease in 1-year-old children (**Appendix 1**)

(16). Best et al reported the maternal serum folate and UMFA concentrations between 36-40 weeks of pregnancy were not associated with physician-diagnosed eczema, IgE-mediated food allergy, and skin prick test in offspring at 1 year of age (16).

This work contrasts a study of 1,394 children from the Boston Birth Cohort who participated in the follow-up study of the Children's Health Status USA. McGowan et al reported that maternal total folate concentrations at delivery between mothers whose children later developed food allergy versus those whose children did not develop a food allergy at 2.4 years of age (on average) were significantly different 30.2 vs. 35.3 nmol/L (46). In addition, maternal total folate concentrations in the third quartile (30.4 – 44.8 nmol/L) versus the first (6.64 – 19.7 nmol/L) were associated with lower odds of developing food allergy at (on average) 2.4 years of age.

Findings from various countries on folate and folic acid intake or status during pregnancy from observational studies and clinical trials are summarised in **Appendix 6** and **Appendix 7** (**Appendices** pg. 135 to 146). There are currently no RCTs that have investigated folate and folic acid intake or status including UMFA concentration in late gestation and how it affects adverse health outcomes in mothers and their offspring.

In summary, it is becoming increasingly clear that the benefits of folic acid supplementation are not proven beyond the first month post-conception, and indeed potential harms exist. We have a responsibility to assess the benefits and risks of folic acid containing prenatal supplements designed for use beyond early pregnancy.

2.2 Public health policies related to folate and folic acid in pregnancy.

2.2.1 Folic acid supplementation

Strong evidence showed that folic acid supplementation during periconceptional period is an essential public health policy to prevent NTDs. The Royal Australia and New

Zealand College of Obstetricians and Gynaecologists (RANZCOG) recommend that folic acid should be taken at 400 µg per day one month before pregnancy until 12 weeks of pregnancy to aid with NTDs prevention despite compliance to folic acid supplement intake has been reported to be challenging. Watson et al reported that less than 50% of women in Victoria and New South Wales, Australia took periconceptional folate supplements (48). Supplement use is highly correlated with socioeconomic and educational status (49). A study reported that education campaigns encouraging women to increase their supplement use have not been effective among high-risk populations (50).

2.2.2. Folic acid fortification

Folic acid supplementation alone is an ineffective public health measure for NTD prevention. The main reason is that many pregnancies are unplanned while folic acid is needed at least one month before conception. A recent report in Australia showed that one in four pregnancies is planned (51). Therefore, food fortification is another public health measure in addition to folic acid supplementation to aid in NTDs prevention. Folic acid fortification to reduce NTD risk has been one of the most successful public health initiatives in the past 85 years as reviewed by Crider et al 2011 (52). Now, at least 80 countries have imposed mandatory folic acid fortification of staple foods, including Australia, Canada, and the USA (53). The USA mandated folic acid fortification since 1998 and Australia started in 2009. More recently, the UK has started to mandate folic acid fortification as of September 2021 (54).

Folic acid fortification has implicated folate intake, status, and NTD risk. In Australia, the introduction of mandatory folic acid fortification has increased folate intake by 145 µg per day (142% increased) (32). This consequently resulted in around 14% decrease in NTD incidence in all women with the highest reduction among Aboriginal women (~75%) and

teenagers (55%) (32). In the USA, since the introduction of folic acid fortification in 1998, there has been a decline in 20% of anencephaly (55). Globally, a systematic review of 4078 studies reported that mandatory folic acid fortification results in a reduction of spina bifida prevalence regardless of the type of birth cohort (56).

2.4 Gap of knowledge

While it is clear that folate is essential during the periconceptional period, the use of folic acid during this period is also warranted with a specific recommended dose for NTDs prevention. However, it remains unclear whether a higher dose of folic acid with a longer period of exposure beyond the first 12 weeks of pregnancy has additional benefits or imposes any harm. Recent observational evidence on the adverse effect of excess intake of folic acid, elevated folate status (serum and RBC folate) and/or UMFA is inconclusive as discussed in Section 2.1.9. Similarly, the biological mechanism by which all three indicators of excess folic acid remains unclear. Even though it is postulated that it is through one carbon metabolism pathway including DNA methylation, the evidence is still lacking (57) and warrants further investigation. Furthermore, many women continue to take folic acid supplements beyond the recommended time period. Therefore, stronger evidence is warranted to investigate the effect of folic acid beyond the first trimester of pregnancy.

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Chapter 3:

Clinical Trial: Folic Acid Study Protocol

Statement of Authorship

Title of Paper	Study protocol for a randomised controlled trial evaluating the effect of folic acid supplementation beyond the first trimester on maternal plasma unmetabolised folic acid in late gestation
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Principal Author

Name of Principal Author (Candidate)	Dian C Sulistyoningrum		
Contribution to the Paper	Advised on the methodological aspect of the trial including programming data collection management system, blood collection, biobanking of samples, and laboratory analysis. Drafted the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/10/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tim Green		
Contribution to the Paper	Supervised the student and conceived the trial and proposed the trial design, designed the prenatal supplement and had it manufactured, drafted and reviewed the manuscript		
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Contribution to the Paper	Conceived the trial, proposed the trial design, designed the prenatal supplement, reviewed and edited the manuscript		
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Contribution to the Paper	Supervised the student, conceived the trial, proposed the trial design and managed, the trial, drafted and reviewed the manuscript		
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BMJ Open

Study protocol for a randomised controlled trial evaluating the effect of folic acid supplementation beyond the first trimester on maternal plasma unmetabolised folic acid in late gestation

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ABSTRACT

Introduction Taking folic acid containing supplements prior to and during early pregnancy reduces the risk of neural tube defects. Neural tube defects occur prior to 28 days postconception, after which, there is no proven benefit of continuing to take folic acid. However, many women continue to take folic acid containing supplements throughout the pregnancy. At higher intakes, folic acid is not converted to its active form and accumulates in circulation as unmetabolised folic acid (UMFA). Recently, concerns have been raised about possible links between late gestation folic acid supplementation and childhood allergy, metabolic disease and autism spectrum disorders. We aim to determine if removing folic acid from prenatal micronutrient supplements after 12 weeks gestation reduces circulating levels of maternal UMFA at 36 weeks gestation.

Methods and analysis This is a parallel-design, double-blinded randomised controlled trial. Women ≥12 and <16 weeks' gestation with a singleton pregnancy and able to give informed consent are eligible to participate. Women (n=100; 50 per group) will be randomised to receive either a micronutrient supplement containing 0.8 mg of folic acid or a micronutrient supplement without folic acid daily from enrolment until delivery. The primary outcome is plasma UMFA concentration at 36 weeks gestation. Secondary outcomes include red blood cell folate and total plasma folate concentration. We will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression with adjustment for baseline UMFA levels and gestational age at trial entry. The treatment effect will be described as a mean difference with 95% CI.

Ethics and dissemination Ethical approval has been granted from the Women's and Children's Health Network Research Ethics Committee (HREC/19/WCHN/018). The results of this trial will be presented at scientific conferences and published in peer-reviewed journals.

Trial registration number ACTRN12619001511123.

Strengths and limitations of this study

- We will determine if discontinuing folic acid supplementation after 12 weeks of gestation results in lower levels of unmetabolised folic acid.
- Unmetabolised folic acid is a biomarker of excess folic, and has been associated with a number of adverse pregnanacy outocomes.
- This study is not powered to determine the effect of continuing folic acid supplements after the first trimester on clinical outcomes.
- The study findings will be generalisable to countries which like Australia have mandatory folic acid fortification.
- This research will inform the need for larger trials to determine if folic acid beyond the first trimester leads to adverse maternal and infant health outcomes.

INTRODUCTION

Evidence from randomised controlled trials[1 2] and a large public health intervention[3] showed that taking folic acid containing supplements prior to and during early pregnancy reduces the incidence of neural tube defects (NTD). Based on these findings, public health agencies around the world issued recommendations advising women to take folic acid supplements prior to conception and during early pregnancy.[4] For example, in Australia, the government recommends that women trying to become pregnant take a folic acid supplement of 0.5 mg/day 12 weeks prior to conceiving and for the first 12 weeks of pregnancy.[5]

The neural tube closes in the first month of pregnancy, beyond this time there is no proven benefit of taking folic acid.[6] However,

many women continue to take folic acid as part of a prenatal vitamin and mineral supplement throughout pregnancy.[7] In Australia, for example, a randomised controlled trial of pregnant women showed that more than 80% of women were taking a prenatal supplement containing folic acid at some time during their pregnancy,[8] with the market leading supplement containing 0.8 mg of folic acid. Furthermore, almost 80 countries, including Australia, have mandated the addition of folic acid to food staples, typically wheat flour, to reduce NTDs in unplanned pregnancies.[9] As such, the combination of food fortification along with prenatal supplement use may expose women and their fetus to excessive amounts of folic acid.

There is emerging evidence that higher intakes of folic acid in pregnancy may have negative health effects on the offspring including autism spectrum disorders[10–12] and insulin resistance.[13] An increased risk of childhood allergic disease is chief among these concerns with several studies reporting an inverse association with folic acid[14–24] However, results are inconsistent and some studies report no relationship between folic acid intake and allergy outcomes in offspring[25–27] or a reduction in risk of allergic disease.[28] These studies vary greatly in regard to the timing and measurement of exposure and only one study differentiated between maternal total plasma folate and maternal plasma unmetabolised folic acid (UMFA).[29]

Folic acid is the synthetic form of the vitamin folate that is used in supplements and fortified foods because of its high bioavailability and stability compared with naturally occurring folate in food.[30] Once consumed, folic acid must be converted into an active form, 5-methyltetrahydrofolate.[31] At higher intakes folic acid is not converted to its active form and accumulates in plasma as UMFA.[32] Circulating UMFA has been proposed as a biomarker of excess folic acid intake.[33] Without proven benefit and with the suggestion of harm, the amount of folic acid in prenatal supplements may need to be reduced after the first trimester. We aim to determine if removing folic acid from prenatal multivitamin supplements after the first trimester (12 weeks gestation) reduces the accumulation of maternal UMFA measured at 36 weeks gestation.

Hypotheses

Removing folic acid from prenatal supplements after 12 weeks of gestation will limit the accumulation of UMFA in maternal plasma at 36 weeks of gestation.

METHODS AND ANALYSIS

Trial design

A multicentre two-arm parallel design, double-blinded randomised controlled trial.

Participating centres

The sponsoring institution and Trial Coordinating Centre is the South Australian Health and Medical Research Insititute (SAHMRI) based at the Women's and Children's Hospital (WCH). We will also seek approval to conduct the trial at Flinders Medical Centre (FMC), Adelaide, South Australia.

Study population

Participants are pregnant women with a singleton pregnancy enrolled between ≥12 and <16 weeks gestation. Enrolment commenced on 18 December 2019 and recruitment is ongoing. Data collection will continue through to May 2021.

Eligibility criteria

Inclusion criteria

To be eligible for participation women must be:

1. Carrying a singleton pregnancy ≥12 and <16 weeks gestation.
2. Currently taking a folic acid containing supplement and planning to continue this throughout pregnancy.
3. Able to give informed consent.

Exclusion criteria

Women will be ineligible for trial participation if they meet any the following criteria:

1. Carrying a fetus with a confirmed or suspected fetal abnormality.
2. Unwilling to cease current folic acid containing supplement/s.
3. Past history of an NTD affected pregnancy.
4. Currently taking medication known to interfere with folate metabolism (eg, methotrexate, sulphasalazine, anticonvulsants, antimalarials or barbiturates).
5. Known haemolytic anaemia or haemoglobinopathy.
6. Known to carry the TT variant of the methylene tetrahydrofolate reductase gene (MTHFR C77T) polymorphism.
7. Intolerance or allergy to prenatal vitamin and mineral supplements.

Study treatments

Participating women will be randomised to receive either a micronutrient supplement in tablet form, containing 0.8 mg folic acid (the dose in the most commonly used supplement in Australia or an identical micronutrient supplement containing no folic acid. The composition of micronutrients within the intervention and control supplements are formulated to approximate leading brands of prenatal micronutrient supplements available in Australia (table 1). Intervention and control supplements are identical in size, shape, colour and packaging and only differ in the removal of folic acid from the intervention supplement. Women will be asked to consume one supplement per day from enrolment (≥12 and <16 weeks of gestation) until delivery.

Manufacture of study supplements

Intevention and control supplements are manufactured in a licensed facility in accordance with the Code of Good Manufacturing Practice (GMP) for Medicinal Products

Table 1 Ingredients of supplements for intervention and control groups

Ingredients	Intervention	Control	Unit
Folic acid	0	0.8	mg
Calcium	250	250	mg
Iron	27	27	mg
Thiamine	1.4	1.4	mg
Riboflavin	1.4	1.4	mg
Niacinamide	18	18	mg
Vitamin B_6	1.9	1.9	mg
Vitamin B_{12}	2.6	2.6	µg
Pantothenic acid	6	6	mg
Biotin	30	30	mg
Vitamin C	85	85	mg
Vitamin E	13.5	13.5	IU
Magnesium	50	50	mg
Zinc	7.5	7.5	mg
Manganese	2.0	2.0	mg
Iodine	0.22	0.22	mg
Copper	1	1	mg
Selenium	30	30	µg
Vitamin D_3	10	10	µg
b-carotene	2500	2500	IU

and have been donated to the trial by Factors Group of Companies, Coquitlam, British Columbia, Canada. The supplements are packaged and labelled in accordance with GMP including an individual product identifier, batch number, expiry date and the statement 'for clinical trial use only'. The pharmacist or the investigator's designee maintains accurate records of the dispensing of study product. Unused study supplements will be destroyed in compliance with applicable regulations.

Monitoring adherence to study treatment

Research personnel will maintain regular contact with participating women to monitor and encourage supplement adherence and study compliance and answer any questions as they arise. At each contact, women will be asked if they have missed any supplements in the last week and if so, how many have been missed. Women will be asked to return unused supplements at the final study visit (36 weeks' gestation) and the proportion of supplements returned will serve as the primary measure of compliance. A woman will be classified as compliant if she takes greater than 80% of her study supplements. At this visit women will be issued with enough supplements to last until the delivery of their baby.

Outcome measures

The primary outcome is maternal plasma UMFA concentration at 36 weeks gestation.

Secondary outcomes

- Maternal plasma total and red blood cell folate levels at 36 weeks' gestation.
- Gestational age at birth, birth weight, birth length, birth head circumference.

Safety outcomes

- Neonatal complications requiring admission to the neonatal unit.
- Pregnancy complications requiring hospital admission.
- Serious adverse events defined as: maternal or fetal (>20 weeks) deaths, fetal loss (<20 weeks), maternal or neonatal admissions to intensive care and major congenital anomalies.

Participant timeline

Women will be randomised and asked to cease their current prenatal supplements immediately and for the duration of the study. At enrolment, following informed consent and prior to commencement of the study treatment, research personnel will collect baseline clinical and demographic data including: contact details, self-reported ethnicity, gravida, parity, age, supplement and prescription drug use, weight, height, highest level of education, occupation and smoking status. Maternal dietary intakes of folate and other one-carbon nutrients during early and late pregnancy will be collected with the use of an 80-item semiquantitative Food-Frequency Questionnaire—Dietary Questionnaire for Epidemiological Studies (V.3.2).[34] A 10 mL venous blood sample will be collected by venepuncture to assess UMFA, folate status and full blood count. The time the woman last ate and drank as well as the time her last supplement was taken will be recorded. Research personnel will contact the participant 1 week following the enrolment visit and then monthly to ensure adherence and record adverse events (figure 1). At 36 weeks' gestation participating women will attend a clinic appointment for collection of venous blood sample for UMFA and folate analysis and full blood count. Participants will be asked to return unused supplements which will be counted as a measure of compliance. The Food-Frequency Questionnaire will be repeated and women will be given enough supplements to last the remainder of their pregnancy. Following delivery, research personnel will extract details of pregnancy, labour and birth from the woman and her baby's medical records. Blood samples will be analysed for UMFA according to established methods.[35] Plasma folate (nmol/L) and erythrocyte folate (nmol/L) concentrations will be determined using the folate microbiological assay harmonised by the Centers for Disease Control and Prevention.[36]

Sample size

A sample size of 90 women (45 per group) will provide >90% power to detect a standardised difference in mean UMFA concentration at 36 weeks gestation between

	Screening	Enrolment	Allocation	+1 week		Post-allocation				
TIMEPOINT		$-t_1$	0	t_1	t_{1a}*	t_2	t_3	t_4	t_5	t_6
ENROLMENT:	<12w	≥12 to <16w		13 to17w	16w	20w	24w	28w	32w	36w
	Clinic	Clinic	Clinic	Phone	SMS	SMS	SMS	SMS	Phone	Clinic
Eligibility screen	X	X								
Consent to Contact	X									
Informed consent		X								
Randomisation			X							
Demographic data		X								
Allocation			X							
INTERVENTION:			←	—	—	—	—	—	→	
ASSESSMENTS:										
Maternal Folate status		X								X
Maternal UMFA status		X								X
Food Frequency Questionairre		X								X
Adverse events					X	X		X		X
Serious Adverse events										X
Compliance – maternal report					X	X	X	X	X	X
Compliance – Supplement count										X

Figure 1 Folic acid trial schedule. *This timepoint will be completed only for women enrolled <14 weeks gestation. SMS, short message service; UMFA, unmetabolised folic acid.

groups of 0.60 (two-tailed alpha =0.05, correlation between UMFA concentrations at baseline and 36 weeks of gestation=0.60).[29] Calculations were performed based on a standardised mean difference (mean difference divided by SD of outcome at 36 weeks gestation) due to considerable variability in the literature in the reported SD for UMFA concentration in pregnancy.[16 29] A standardised mean difference of 0.60 represents a medium effect size and would demonstrate biologically excessive folic acid consumption. To allow for 10% lost to follow-up, we will randomise 50 women per group.

Recruitment

Pregnant women will be recruited through a combination of flyers,posters, a digital media campaign and through in-person recruitment at antenatal clinics. Women who meet eligibility criteria and agree to participate are invited to attend an enrolment appointment at our research clinics at the WCH or FMC, Adelaide between 12 and 16 weeks gestation.

Randomisation procedures

Participants will be randomised using a secure web-based randomisation service. Allocation will follow a computer-generated randomisation schedule using balanced variable block sizes, prepared by an independent statistician who is not involved with trial participants or data analysis. A unique four-digit study identification number and a coloured coded study pack are assigned to each participant. Stratification will be by gestational age at trial entry 12 to ≤14 weeks or >14 to 16 weeks gestation.

Blinding

The independent unblinded statistician (not involved in any other way in the trial) allocated two colours to the intervention group and two colours to the control group. Supplements were subsequently packaged and labelled with a colour by two unblinded staff members who have no other involvement in the trial. Research personnel, participants and their family, care providers, outcome assessors and data analysts remain blinded to colour allocation and therefore randomisation group.

The intervention and control supplements are identical in size, shape, colour, packaging and labelling and uniquely identified by the coloured product identification label (yellow, pink, blue or green) only. The randomisation code for an individual participant may be unblinded by the independent statistician in the event of an emergency.

Data collection and trial management

Data are collected by trained research personnel and entered directly into an electronic case report form with password protection and defined user-level access Research Electronic Data Capture (REDCap). A record of all women approached, screened for eligibility and

consented will be recorded.[37] Once consented and randomised, REDCap has been designed to automatically calculate study milestones for each participant. This information is readily available for clinical trial staff to enable scheduling of appointments and sample collection. Summary reports including screening data, enrolment, appointment attendance, sample collection, serious adverse events and study completion are generated from REDCap and reviewed at monthly trial steering committee meetings. Electronic data are stored on secure servers at South Australia Health and Medical Research Institute and released only to persons authorised to receive those data.

Data and safety monitoring

We do not anticipate any serious adverse events related to participation in this trial. Regardless, an independent (blinded) clinician will review all serious adverse events and determine whether there is any likelihood that involvement in the trial could have contributed to the event. Determinations of causality will be made from medical records retrieved for this purpose. All serious adverse events will be captured and reported to the Human Research Ethics Committee.

Statistical analysis

Statistical analyses will be performed on an intention-to-treat basis according to a pre-specified statistical analysis plan. For the primary outcome, we will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression, with adjustment for baseline UMFA and the stratification variable gestational age at trial entry (12 to ≤14 weeks or >14 weeks). The treatment effect will be described as a mean difference with 95% CI. Secondary outcomes will be analysed using linear and logistic regression models for continuous and binary outcomes, respectively, again with adjustment for gestational age at trial entry. Safety outcomes will be compared between groups using Fisher exact tests. In all analyses, a two-sided $p<0.05$ will be taken to indicate statistical significance.

Ethics and dissemination

Human Research Ethics Approval

This protocol, the informed consent and participant information document and all participant communication have been approved by the Women's and Children's Health Network Research Ethics Committee (HREC) (HREC/19/WCHN/018) and Governance (SSA/19/WCHN/080). Governance approval has also been obtained from FMC. Any subsequent modifications will be reviewed and approved by the HREC and governance of each study site. The study will be conducted in compliance with the current approved version of the protocol. Any change to the protocol document or informed consent form that affects the scientific intent, study design, patient safety or may affect a participant's willingness to continue participation in the study will be considered a major amendment. All such amendments will be submitted to the HREC for approval. Any other changes to the protocol (such as administrative changes to dates and study personnel) will be considered minor amendments and will be notified to the HREC as appropriate.

Confidentiality

Participant confidentiality is strictly held in trust by the participating investigators, research staff and their agents. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Regulatory authorities may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records for the women and/or infants in this study subject to individuals having obtained approval/clearance through State/National Governments and HREC as required by local laws. Clinical information will not be released without written permission of the parent, except as necessary for monitoring by HREC or regulatory agencies.

Patient and public involvement

The study was supported by a consumer advisory group which provided input to the protocol. A Consumer representative from our SAHMRI Women and Kids Consumer Advisory Group partnered with us for the design of the study, informational material to support the intervention, and the burden of the intervention from the participant's perspective. We will meet with the consumer representative for this trial and the full Consumer Advisory group on a regular basis for the duration of the study. At the end of the study, the consumer advisory group will be given the opportunity to comment on the findings and contribute to the dissemination plan.

Dissemination plan

Study findings will be submitted for peer-reviewed publication and for presentation at appropriate local and international conferences. In addition, study findings will be disseminated to participants through a one-page lay summary. Results will be made available to the wider community through social media avenues and the SAHMRI website.

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Contributors KPB, TG, MM, DP and MS conceived the trial and proposed the trial design; TS advised on sample size calculations, trial design and analysis; SW and TG designed the prenatal supplement and had it manufactured; DS advised on analytical methodology; DS, TG and KPB drafted the protocol, all authors contributed to refinement of the study and approved the final manuscript.

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Disclaimer The funder/s have no role in the study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication and have no authority over any of these activities.

Competing interests MM reports that she has a financial relationship outside the submitted work with Trajan Nutrition as a member of the board. SW is a consultant for the Factors Group of Companies. DS, TG, DP, TS MS and KPB have nothing to disclose.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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Chapter 4:

Clinical Trial: Folic Acid Study Outcome

Statement of Authorship

Title of Paper	Detection of maternal serum unmetabolized folic acid following multivitamin and mineral supplementation with or without folic acid after 12 weeks' gestation: a randomized controlled trial.
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Contribution to the Paper	Contributed to the trial proposal and original trial design. Advised on the methodological aspect of blood collection, sample biobanking and laboratory analysis. Programmed the study data management system, conducted analysis of blood samples for serum and blood cell folate, participated in data collection, and performed extraction of samples for UMFA analysis. Interpreted the data and drafted the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Manuscript

Detection of maternal serum unmetabolized folic acid following multivitamin and mineral supplementation with or without folic acid after 12 weeks gestation: a randomized controlled trial

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Abstract: (1) Background: Folic acid (FA) supplements are recommended to be taken periconceptionally until 12 weeks gestation, but many women continue taking them throughout pregnancy, raising concern for excess exposure to FA. Unmetabolized folic acid (UMFA) is proposed to be the biomarker of excess FA. To determine if removing FA from prenatal multivitamin and mineral supplements after 12 weeks gestation reduces concentrations of serum UMFA at 36 weeks gestation. (2) Methods: A double-blind, parallel-group, randomized controlled trial. Women with a singleton pregnancy 12-16 weeks gestation were randomly assigned to a multi-micronutrient supplement containing no FA (intervention) or 800 µg FA/d (control) until 36 weeks. Maternal serum UMFA, folate, and red blood cell (RBC) folate were analyzed. (3) Results: UMFA was detected in 86% of the 103 randomized women. However, only 12% (n=11/90) had UMFA above the limit of quantification. Compared to the control group (folic acid), fewer women in the intervention group (no folic acid) had detectable UMFA (72% [n=33/46] vs. 98% [n=43/44]; p = 0.001) and lower maternal serum and RBC folate concentrations (median 23.2 vs. 49.3 nmol/L, 1335 vs. 1914 nmol/L, p< 0.,001). (4) Conclusions: Removing FA from prenatal supplements reduced the number of women with detectable UMFA at 36 weeks gestation; however, differences in UMFA concentration between treatment groups were not quantifiable.

Keywords: prenatal supplements; pregnancy vitamins; supplementation guidelines; antenatal; prenatal nutrition; unmetabolized folic acid; excess folic acid.

1. Introduction

Neural tube defects (NTDs) are serious birth defects caused by the failure of the neural tube to close properly, usually occurring around 28 days post-conception (1). Folic acid taken before conception and during early pregnancy is proven to reduce a woman's risk of having an NTD-affected pregnancy (2-4); therefore, health authorities in most countries recommend

women take a folic acid-containing supplement before conception (5, 6). In Australia, women are advised to take a supplement containing 500 µg of folic acid per day for a minimum of one month before trying to conceive and for the first three months of pregnancy (6). Although there is no conclusive evidence for any overall benefit of folic acid supplementation beyond 12 weeks gestation (31 trials involving 17,771 women) (7), many women continue to take folic acid supplements throughout their whole pregnancy, often at amounts up to 800 µg/day or higher (8). Because NTDs occur in the first month of pregnancy and many pregnancies are unplanned, more than 80 countries, including Australia, Canada, and the USA, have mandated folic acid fortification of staple foods with folic acid, further increasing folic acid exposure of pregnant women (9).

The common practice of continuing folic acid supplementation beyond the first trimester, especially in countries with folic acid fortification, is worrying due to increasing reports suggesting exposure to excess folic acid in late pregnancy may be associated with adverse child health outcomes, including an increased risk of allergic disease (10-12). Although findings from observational studies have been inconsistent, evidence from randomized controlled trials (RCTs) is lacking. The suggestion of risk necessitates further exploration of excess folic acid intake during pregnancy.

Folic acid is the synthetic form of folate not found naturally in food. Because of its high bioavailability and stability, it is the form of folate used in supplements and to fortify food (13). When consumed, folic acid is reduced and methylated to 5-methyltetrahydrofolate (5-MTHF) in the enterocyte or liver. At higher intakes, the enzymes required to convert folic acid to 5-MTHF are saturated, and the excess folic acid circulates in its unmetabolized form (UMFA) (14). UMFA has been proposed as a potential biomarker of excessive folic acid intake (14). Concerns have been raised over whether high concentrations of circulating UMFA may adversely affect the developing fetus (15). In acute dosing studies in nonpregnant individuals,

UMFA rises rapidly after folic acid ingestion and falls over the following hours (14, 16). The greater the dose of folic acid, the higher the UMFA concentration and the longer it is detected in serum. The effect of chronic folic acid administration on UMFA is less clear.

UMFA has been detected in maternal blood samples in several population studies (17-20) and one RCT in a country without mandatory fortification (21). However, there are no published RCTs investigating the effect of commonly used higher dose prenatal folic acid containing supplements combined with background intake from mandatory fortification on UMFA concentration.

The importance of taking folic acid supplements in early pregnancy to reduce NTDs is not in doubt. However, supplementation beyond this time is in question. We aimed to investigate the effect of removing folic acid from prenatal supplements *after* 12 weeks gestation compared with the common practice of continuing folic acid supplementation of 800 µg/day throughout pregnancy on maternal serum UMFA concentration at 36 weeks gestation.

2. Materials and Methods

2.1 Trial design and oversight

This trial was a multicenter, double-blind, placebo-controlled, parallel-group RCT. The trial protocol, published previously (22) was developed by the authors and approved by the Women's and Children's Health Network Research Ethics Committee – HREC/19/WCHN/018 and Flinders Medical Centre – SSA/20/SAC/61. The trial was conducted according to the 2007 National Statement on Ethical Conduct in Human Research and the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and prospectively registered with the Australia New Zealand Clinical Trials Registry - ACTRN12619001511123.

2.2 Study participants and setting

Pregnant women living in South Australia were recruited to the trial between December 2019 and November 2020. Women with a singleton pregnancy between 12^{+0} and 16^{+0} weeks gestation, who were taking a folic acid-containing supplement and planning to continue it throughout pregnancy, were eligible to participate. Women were excluded if they; were carrying a fetus with a confirmed or suspected fetal abnormality, had a prior history of an NTD-affected pregnancy, or were taking medications that interfere with folate metabolism. Women were recruited in person at their first antenatal clinic appointment or remotely through a Trial Recruitment Company (TrialFacts Australia, Melbourne, Victoria). They utilize an online digital marketing campaign and an electronic pre-screening survey.

2.3 Randomization, blinding and masking

After written informed consent was obtained, women were randomized by research personnel using a secure web-based randomization service and stratified by gestational age at trial entry 12^{+0} to $\leq 14^{+0}$ weeks or $>14^{+0}$ to 16^{+0} weeks gestation. Allocation followed a computer-generated randomization schedule using randomly permuted blocks of sizes 4 and 6 (1:1 ratio), prepared by an independent statistician who was not involved with trial participants or data analysis. A unique and uninformative four-digit study identification number (Study ID) was assigned to each participant together with one of four colors for their group assignment (blue, pink, yellow, green). The intervention and control supplements were identical in size, shape, color, packaging, and labeling and identified by a colored label only. Participants, researchers, and laboratory personnel remained unaware of the group assignments until the data analysis was complete.

2.4 Trial interventions

Women in the intervention group received multivitamin and mineral supplements without folic acid. Women in the control group were assigned the same formulation with the addition of 800 µg of folic acid/d (S1 Table). Following randomization, women were provided with two bottles, each containing 125 caplets, and advised to cease any folic acid-containing supplements for the duration of the trial. The assigned study supplements were taken once daily, orally from trial entry (12 weeks to 16 weeks gestation) until the day before the clinic visit and blood draw at 36 weeks gestation. Intervention and control supplements (PreNuro®) were formulated to provide daily multivitamin and mineral levels for prenatal supplementation. They were manufactured in a licensed facility following the Code of Good Manufacturing Practice of Medicinal Products (23) by The Factors Group of Nutritional Companies Inc (Burnaby, British Columbia, Canada). The company had no other role in the trial.

2.5 Data collection

Baseline characteristics were collected at enrollment and included gestational age, maternal age, height and weight, race, education, pre-pregnancy and current supplement use, annual household income, parity and alcohol intake, and smoking in the three months leading up to pregnancy. Women were asked to complete an electronic 80-item food frequency questionnaire (FFQ) (The Dietary Questionnaire for Epidemiological Studies v3.2, Cancer Council, Victoria) at enrollment (baseline) and 34 weeks gestation to estimate folate intakes from foods. Adherence to the trial regimen and the occurrence of any adverse events were assessed by monthly electronic surveys sent by short message survey or phone call by study staff. Women returned for an in-person visit at 36 weeks gestation so that the number of unused caplets could be recorded and a venous blood sample could be obtained by trained research personnel. Women were asked to refrain from taking their study supplement and consuming foods high in folic acid on the day of sample collection. Birth data, including gestational age,

birth weight, length, and head circumference, were extracted from maternal and infant medical records or by parental report.

2.6 36 Weeks sample collection

A 10 ml non-fasting venous blood sample was collected into two evacuated containers containing no anticoagulant and ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer®). The EDTA vacutainer was inverted 10 times, and an aliquot was placed in a cryovial, diluted 1 in 11 with 1% ascorbic acid, and incubated for 30 minutes at 37ºC. The serum vacutainer was left to clot at room temperature for at least 30 minutes. Vacutainers were centrifuged at 1500xg for 15 minutes at 4ºC, and serum and plasma were aliquoted into cryovials and stored at $^{-}$80ºC until analyzed.

2.7 Blood analysis

A Complete Blood Count was performed using an automated hematology analyzer by SA Pathology (Adelaide, Australia). Serum UMFA was measured using the method of Hannisdal et al. (24). Briefly, 10 µL (100 ng/mL) of d^4 folic acid (F680302-0.5G, Novachem, Australia) was added to 100 µL of participant serum. Samples were deproteinized by adding 500 µL methanol, incubated at -20°C for 1 hour, and centrifuged for 15 minutes (12,000xg). The supernatant was collected and placed in a 96-well plate, dried under nitrogen at 65°C, and reconstituted with 100 µL 0.1% formic acid. Fetal bovine serum was used as a blank to generate standard curve and quality control (QC) samples. Known concentrations of folic acid were spiked into fetal bovine serum to give the final concentrations of 0.566 limit of quantification (LOQ), 1.13, 2.27, 5.66, 11.3, 22.7, 45.3, 227, and 566 nmol/L for the standard curve and 1.42, 2.83, 17.04 and 113.12 nmol/L for the QC samples. Standards and QC samples were extracted

using the same method as described above alongside the patient samples. Any standards or QCs outside +/- 15% accuracy were excluded.

Liquid chromatographic separation of a 5 µL injection volume was achieved using an Acquity UPLC system (Waters Corporation, USA) fitted with a BEH Premier C18 (2.1 x 100 mm, 1.8 µm) chromatographic column maintained at 45°C. A linear gradient from 99% mobile phase A (0.1% formic acid) to 99% mobile phase B (acetonitrile 0.1% formic acid) over 2.5 minutes, followed by a 1 min hold at 99% B and a 1.5-minute re-equilibration at 99% A. Mass analysis was performed using a 5500 Triple Quadrupole (Sciex, Canada) in Multiple Reaction Monitoring mode, transitions monitored are listed in S3 Table. Data integration and analysis were performed using Analyst 1.6.2 software (Sciex, Canada).

Whole blood and serum folate concentrations were determined using the microbiological method, using standardized kits from the US Centres for Disease Control and Prevention (US CDC; Atlanta, GA) (25-29). This method is based on the method of O'Broin and Kelleher (26), uses 96 well microplates, 5-methyl tetrahydrofolate (Merck Eprova) as a calibrator, and chloramphenicol-resistant Lactobacillus rhamnosus (ATCC 27773TM) as the test organism. High- and low-quality controls (QC) provided by the US CDC, whole blood and plasma folate, were run in quadruplets on every plate. RBC folate was calculated by subtracting plasma from whole blood folate and correcting for hematocrit.

As per instructions (27): if all QC results were within mean (2 SD) limits, the assay was accepted; if more than one of the QC results were outside of the mean (2 SD) limits or any of the QC results were outside of the mean (3 SD) limits, then the assay was rejected. Results from assay runs that passed QC were used when the quadruplets were below 15%. If the coefficient of variation (CV) of the quadruplets was above 15%, the largest outlier was removed. The results were recorded if the CV of the remaining triplicates was below 10%; otherwise, the sample measurement was repeated.

At the population level, WHO recommends RBC folate concentrations be >906 nmol/L in women of reproductive age to prevent NTDs. This RBC folate value was generated using folic acid as the calibrator (30, 31). We used a newer method recommended by the US CDC that uses 5-methyl tetrahydrofolate as the calibrator. Since 5-methyl tetrahydrofolate gives lower RBC folate concentrations than folic acid, we used a cutoff of >748 nmol/L to define the optimal RBC folate concentration for NTD risk reduction (31-33).

2.8 Outcome measures

The primary outcome was the concentration of UMFA in maternal serum at 36 weeks gestation. Secondary outcomes included maternal serum and RBC folate concentrations at 36 weeks gestation and birth outcomes, including gestation age at birth, birth weight, birth length, and birth head circumference.

2.9 Changes to outcomes and trial design

We adapted some aspects of our methodology due to the COVID-19 pandemic. As per the recently released CONSERVE statement (34), we have described our original methods (22) and our adaptations as follows (35). When the trial commenced in December 2019, women were recruited from antenatal clinics, and a baseline blood sample was collected at enrollment. In March 2020, due to COVID-19 restrictions in South Australia, in-person enrollment was suspended, and we could no longer collect the baseline blood sample. Eighteen women were recruited before in-person enrollment was suspended. Screening methods were modified to include online screening coupled with a digital marketing campaign and e-consent using Research Electronic Data Capture (REDCap, Vanderbilt University). REDCap is a secure web application for building and managing online surveys and databases. Enrollment and all study visits up to 36 weeks gestation were conducted via telephone, and supplements were couriered

to participants. Birth data could no longer be extracted from medical records and were obtained by maternal report. Maternal and infant characteristics at birth, such as gestational age at birth, birth weight, birth length, and head circumference, were collected to establish a comparison between treatment groups, as this study was not powered to evaluate clinical outcomes. We would caution about drawing conclusions due to the small sample size and lack of control for multiple testing.

2.10 Sample size and statistics

A target sample of 90 women (45 per group) was chosen to provide >90% power to detect a standardized difference in mean UMFA concentration at 36 weeks gestation between groups of 0.60 (two-tailed alpha = 0.05, correlation between UMFA concentrations at baseline and 36 weeks gestation = 0.60) (21). Calculations were performed based on a standardized mean difference (mean difference divided by SD of the outcome at 36 weeks gestation) due to considerable variability in the literature in the reported SD for UMFA concentration in pregnancy (10, 21).

All analyses were undertaken on an intention-to-treat basis according to a pre-specified statistical analysis plan (Supporting information). The trial was originally designed with serum UMFA concentration at 36 weeks (primary outcome) defined as a continuous outcome, with mean concentrations to be compared between groups using linear regression. However, a blinded review of 36-week UMFA concentrations unexpectedly revealed a high proportion of samples (88%) where the measurement was below the limit of quantification (LOQ, 0.566 nmol/L). We followed US Food and Drug Administration recommendations that values below the LOQ be reported as below the LOQ (36). Consequently, the primary outcome definition was changed to UMFA concentration at 36 weeks, dichotomized into above or below the limit of detection (LOD); this change was made before the statistical analysis plan was finalized and

the treatment groups unblinded. The LOD was determined as a peak height of *ca* 4 times the background noise. Its concentration was not calculated because it is not a true linear relationship to the standard curve at the LOD. The proportion of women with a UMFA concentration above the LOD was compared between groups using logistic regression. Others have quantified UMFA concentrations below the LOQ utilizing the ratio of the peaks folic acid: d^4 folic acid and treated values below the LOD as LOD//√2 for analysis (37). To assess the sensitivity of results to this approach, in a posthoc analysis, we used the Wilcoxon rank-sum test to compare UMFA values between groups when including values below the LOQ and treating values below the LOD as the lowest possible concentration.

Secondary outcomes were analyzed using linear regression models, with log transformations applied where appropriate to satisfy model assumptions better. All analyses were adjusted for gestational age at trial entry since this was used to stratify the randomization, with analyses of birth anthropometrics adjusted for infant sex. Statistical calculations were performed using Stata v16 (College Station, TX: StataCorp LP).

3. Results

3.1. Trial participants

A total of 103 women were randomized, 51 to the no folic acid group (intervention) and 52 to the 800 µg folic acid/d (control) group. After withdrawal of consent (n = 7), loss to follow-up (n = 4), unable to attend the clinic visit (n = 1), and preterm birth before 36 weeks gestation (n = 1), primary outcome data were available for 90/103 (87%) of women (Figure 1). The average age of women entering the trial was 31 years, and more than 80% of the participants were Caucasian. Most women (87%) had completed secondary education, and 55% had an annual household income higher than AUD$105,000. Among women assessed at baseline (n =18), median UMFA was 1.2 (IQR 0.3, 2.0; maximum 34.2) nmol/L with no values

below the LOD and 4 below the LOQ. Overall mean total folate intake (±SD) was 585 ± 264 µg/day dietary folate equivalent (DFE) at baseline and 559 ± 253 µg/day DFE at 36 weeks. Folic acid added to food, and natural folate intake was 179 ± 120 µg/day, 285 ± 108 µg/day at enrollment and 166 ± 116 µg/day, and 282 ± 104 at 36 weeks and did not differ markedly between the groups **(**Table 1**)**. Adherence to the trial supplements was similar between the intervention and control groups, with 85% of women who returned bottles (74%) consuming >80% of supplements to 36 weeks gestation, comparable with results from compliance questioning at study visits.

Figure 1. Participant flowchart

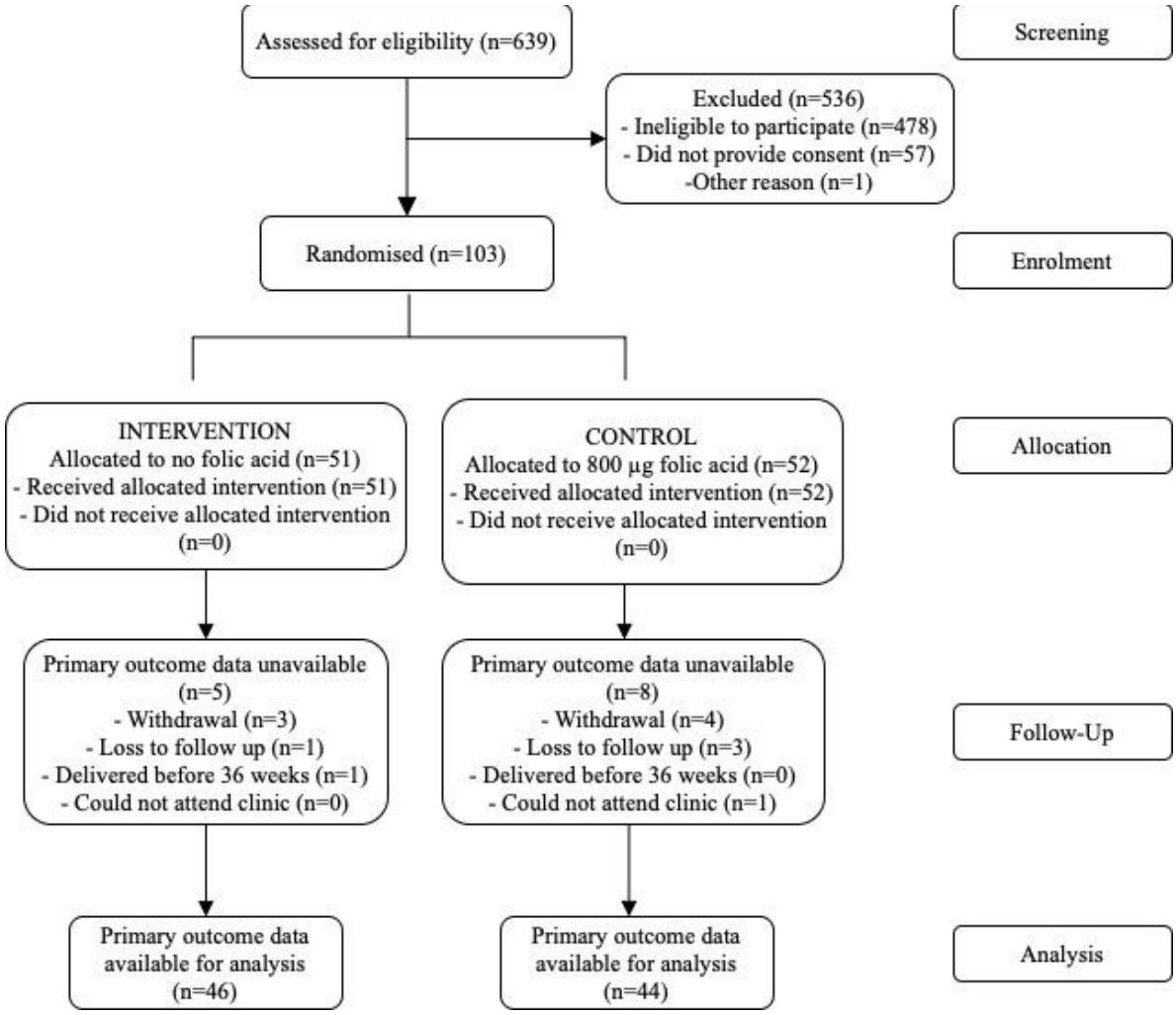


Table 1. Maternal baseline characteristics and folate intake at 36 weeks gestation[1]

Characteristic	Intervention No folic acid (n = 51)	Control 800 µg folic acid/d (n = 52)
Age, y	30.7 ± 5.2	31.4 ± 4.4
Gestational age at trial entry		
12 to < 14 wk	32 (63)	32 (62)
≥14 to 16 wk	19 (37)	20 (38)
Maternal BMI at enrollment (n=94)	25.2 ± 5.0	27.0 ± 6.2
Ethnicity		
European	41 (80)	44 (85)
Other	10 (20)	8 (15)
Completed secondary education	46 (90)	44 (85)
Annual household income		
AUD$70,000 or less	9 (18)	9 (17)
AUD$70,001 - $105,000	12 (24)	7 (13)
AUD$105,001 - $205,000	23 (45)	26 (50)
>AUD$205,000	5 (10)	7 (13)
Prefer not to disclose	2 (4)	3 (6)
Parity		
0	27 (53)	20 (38)
Smoked tobacco in 3 mo before pregnancy	5 (10)	5 (10)
Consumed alcohol in 3 mo before pregnancy	34 (67)	41 (79)
Folic acid supplement intake at enrollment, µg/d		
250-<500	2 (4)	1 (2)
500-<750	24 (47)	28 (56)
>750	25 (49)	21 (42)
Serum unmetabolized folic acid nmol/L (n=18)[3]	1.6 (0.2, 4.3)	0.9 (0.2, 1.8)
Folate intake at baseline, µg/d (n=88)		
Total dietary folate[2]	644 ± 298	528 ± 214
Folic acid from fortified food	204 ± 127	155 ± 110
Natural food folate	303 ± 124	268 ± 88
Folate intake at 34 weeks, µg/d (n=84)		
Total dietary folate[2]	581 ± 269	538 ± 238
Folic acid from fortified food	179 ± 131	152 ± 100
Natural food folate	282 ± 105	281 ± 103

[1]Values are mean ± SD, *n* (%), median (25^{th},75^{th})

[2]As µg Dietary Folate Equivalents = 1.7 x µg folic acid from fortified food + µg natural food folate

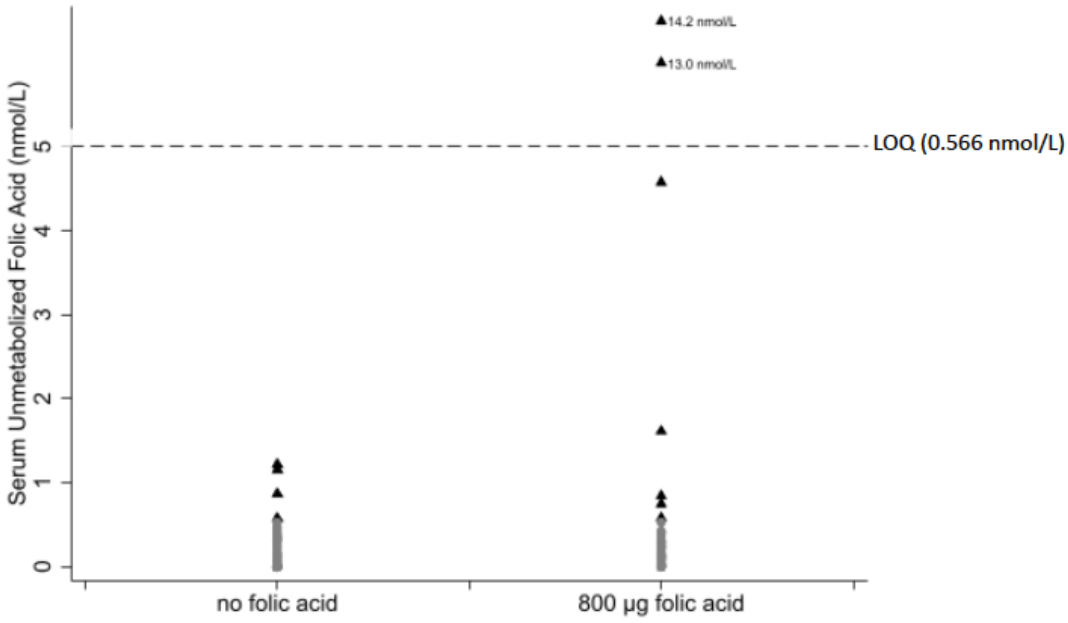
[3]Blood was collected from 18 women at baseline before Covid-19 restrictions prevented in-person baseline blood sample collection (see Section 2.9).

3.2. Outcomes

3.2.1. Unmetabolized folic acid (UMFA)

Differences between the groups in UMFA concentrations could not be reliably determined because only 12% (n=11/90) of the sample were above the LOQ (0.566 nmol/L) (4/46 in the no folic acid group and 7/44 in the 800 µg folic acid/d group). However, the proportion of women with UMFA above the LOD at 36 weeks gestation was significantly lower in the no folic acid compared to the 800 µg folic group/d; 72% (n=33/46) vs. 98% (n=43/44), p=0.001. Similar results were observed in a supplementary analysis treating values between the LOQ and LOD as observed and values below the LOD as the lowest possible concentration (Wilcoxon rank-sum p-value = 0.003; probability of a larger UMFA value in the intervention arm = 0.33 (vs. null hypothesis value of 0.50)).

Figure 2. Serum unmetabolized folic acid in women at 36 weeks gestation



3.2.2. Serum and red blood cell folate

Maternal serum folate concentrations were lower in the no folic acid group compared to the 800 µg folic acid/d group; median 23.2 nmol/L vs. 49.3 nmol/L, the ratio of geometric means was 0.56 (95% confidence interval (CI), 0.46 to 0.68 nmol/L), p<0.001 (Table 2). Similarly, median RBC folate concentrations were significantly lower in the no folic acid group than the 800 µg folic acid/d group; 1340 nmol/L vs. 1910 nmol/L, the ratio of geometric means was 0.69 (95% CI, 0.61 to 0.77), p<0.001 (Table 2). Serum and RBC folate concentrations were within normal clinical range for folate deficiency in both the intervention and control groups, >6.8 nmol/L for serum folate and >305 nmol/L for RBC folate concentrations.

Table 2. Blood folate concentrations at 36 weeks and neonatal outcome by treatment group

Outcome	No folic acid[1]	800 µg folic acid/d[1]	Treatment effect[2] (95% CI)	P-value
Maternal serum folate, nmol/L (n=90)	23.2 (18.0, 28.4)	49.3 (32.7, 57.7)	0.56 (0.46, 0.68)[3]	< 0.001
Maternal red blood cell folate, nmol/L (n=90)	1340 (1150, 1510)	1910 (1530, 2300)	0.69 (0.61, 0.77)[3]	< 0.001
Gestational age at birth, wks (86)	39.3 ± 1.7	39.0 ± 1.3	0.3 (-0.4, 1.0)[4]	0.36
Birth weight, g (n=90)	3331 ± 519	3383 ± 473	-44 (-255, 166)[4]	0.68
Birth length, cm (n=78)	49.3 ± 2.8	49.9 ± 2.7	-0.5 (-1.7, 0.8)[4]	0.46
Birth head circumference, cm (n=68)	34.1 ± 2.1	35.0 ± 1.3	-0.9 (-1.8, -0.1)[4]	0.04

[1] Values are median (IQR) or mean ± SD
[2] Adjusted for gestational age at trial entry for all outcomes and additionally for infant sex for birth anthropometric outcomes
[3] Treatment effect expressed as a ratio of geometric means (95% CI)
[4] Treatment effect expressed as a mean difference (95% CI)

3.2.3. Birth outcomes

There were no significant differences between the groups in birth outcomes, including gestational age, birth weight, and length, except for head circumference, which was lower in the no folic acid group compared to the 800 µg folic acid/d group (mean difference: -0.9cm; 95% CI, -1.8, -0.1, P=0.04) (Table 2).

3.2.4. Safety and adverse events

Adverse events were comparable between the groups, with nausea the most common symptom overall at 1 week post-randomization (27%) and at 20 weeks gestation (29%). One infant in each group was admitted to the Neonatal Intensive Care Unit, which was classified as a Serious Adverse Event. All serious adverse events were reviewed and categorized as unlikely related to the trial product or trial protocol.

4. Discussion

We examined the effect of removing folic acid from prenatal supplements after 12 weeks gestation on maternal UMFA in late pregnancy in a country with mandatory folic acid fortification. UMFA was detected in a smaller proportion of women randomized to the no folic acid supplement than the supplement containing 800 µg folic acid/d. Though UMFA was detected in the majority of women in our study (86%), most samples were below the LOQ (88% < LOQ 0.566 nmol/L), which meant we could not reliably report differences between groups in mean UMFA concentration.

Our results are similar to the findings of the only other published RCT investigating the effect of prenatal folic acid supplementation on UMFA concentration. Pentieva et al. reported that women randomized to folic acid supplements were more likely to have detectable plasma UMFA at 36 weeks gestation than women randomized to placebo (42% vs. 16%) (21) but also found no significant difference in the mean concentration of UMFA between groups (0.13 ± SD 0.49 vs. 0.44 ± SD 0.80, interaction p-value = 0.38) (21). The dose of folic acid used by Pentieva et al. (21) was lower than that found in common prenatal multivitamin and mineral supplements in Australia and many other countries, which range from 500 µg to 1,000 µg/day (20, 38). Furthermore, the Pentieva et al. study was conducted in Northern Ireland, which at the time only had voluntary folic acid fortification (21). We had expected to detect higher UMFA concentrations in our study as women in our control group were receiving double the amount of folic acid and were also exposed to a background of folic acid as a result of

mandatory fortification in Australia. The prevalence of detectable UMFA in our population (86%) is similar to reports from observational studies in pregnant women in Australia (93%, > 0.03 to 244.7 nmol/L) (17), USA (81%, 0.23 to 1.47 nmol/L) (18) and Canada (97%, 0.00 to 0.91 nmol/L) (20). However, UMFA concentration differs substantially between studies and appears to be influenced by recent folic acid intakes, which may explain the variability. Pfeiffer et al. reported detectable levels of UMFA in nearly all National Health and Nutrition Examination Survey (NHANES) participants (>95%, range >0.3 to 397 nmol/L) (39). NHANES is a representative sample of the US population and includes men, women, and children. Although 38% of NHANES survey participants were fasting for >8 hours, Pfeiffer et al. reported that the detection of UMFA was evident regardless of fasting status; yet concentrations differed significantly by the length of fasting (39).

We asked participants to avoid taking their study supplement on the day of their blood collection because we were interested in the chronic effect of folic acid supplementation on UMFA, not the acute effect, as this is well established (14, 16, 40). Zheng et al. reported that following a single dose of 800 µg folic acid in 20 healthy male subjects, UMFA increased, peaking at around 2.5 hours in plasma but returned to undetectable levels within 12 hours (40). Although we could detect UMFA in most participants, even those receiving no folic acid from study supplements (our intervention group), we had expected that chronic dosing of folic acid from early pregnancy would result in a greater accumulation of UMFA. However, this was not the case, and we could not quantify UMFA even in women who received 800 µg/d FA.

Comparison of our results with other studies of UMFA is difficult due to differences in measurement methods. We only quantified down to the LOQ, which means we did not report values below the lowest value in the standard curve for UMFA. Other researchers quantify down to the LOD, which, if the term is being applied correctly, means that they are extrapolating below their lowest standard.

The serum and RBC folate differences were as expected and consistent with other prenatal folic acid supplementation trials (21, 30, 41). At 36 weeks gestation, median serum folate was ~26 nmol/L lower, and median RBC folate was 600 nmol/L lower in the group receiving no folic acid versus 800 µg folic acid/d. Importantly all women remained above serum and RBC folate concentrations indicative of deficiency, >6.8 nmol/L and >305 nmol/L, respectively (42).

Our study has many strengths, including a low attrition rate and a high rate of supplement adherence. We asked women to refrain from taking their study supplement for 24 hours prior to their blood sample collection to reduce the variation in UMFA caused by recent high-dose folic acid exposure. A limitation of our study is the absence of a baseline maternal blood sample at enrollment due to COVID-19 restrictions, which meant we could not examine changes in UMFA over time.

In conclusion, our trial showed that removing folic acid from prenatal multivitamin and mineral supplements reduced the number of women with detectable UMFA at 36 weeks gestation; however, differences in UMFA concentration between treatment groups were not quantifiable. The high within-subject variation when measured under standardized conditions and the lack of evidence regarding what concentration of UMFA is normal suggests that UMFA may not be the best biomarker to determine chronic excess folic acid exposure. There is no question that folic acid supplementation is essential before and in early pregnancy, but investigating excess intake, especially in countries with mandatory fortification, is warranted. High-quality randomized trials powered with clinical endpoints are needed to resolve concerns regarding the potential adverse effects of excess folic acid in late pregnancy on children's health.

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Chapter 5:

Discussion and Future Directions

I aimed to determine the effect of removing folic acid from multivitamin and mineral supplements after the first trimester until 36 weeks of gestation on the levels of unmetabolised folic acid (UMFA) at 36 weeks' gestation. In my RCT, the proportion of women with detectable UMFA was significantly higher in the 800 µg folic acid group than in the control group (0 µg folic acid). However, a mean difference in UMFA concentrations could not be determined because 88% of participants had UMFA concentrations below the level of detection (LOD, 0.55 nmol/L). Although the benefit of folic acid is clear for preventing neural tube defects (NTDs), it is important to note that the timing and the dose of folic acid are crucial. Recommendations from health agencies such as the WHO and RANZCOG are for women to take between 400 to 500 µg of folic acid per day to be taken one month before conception up until 12 weeks of gestation (1, 2). However, no guidelines advise women to discontinue folic acid after the first trimester. As such, most women continue taking folic acid throughout pregnancy (3, 4). It remains unclear whether the continued use of folic acid-containing supplements beyond the recommended time and dosage has benefits or harms to maternal and childhood health outcomes. Some observational studies have reported possible adverse outcomes related to excess folic acid exposure and/or elevated folate status, but other studies as reviewed in Chapter 2 have not reproduced these findings. The question remains whether there is an accumulation of UMFA, purported to be the best biomarker of chronic exposure to folic acid, in late gestation and whether the accumulation of UMFA would have any implications on maternal and child health. This thesis provides the first evidence from an RCT showing the effect of continued use of folic acid-containing multivitamin and mineral supplements on UMFA concentrations in late gestation in a country with mandatory folic acid fortification.

My RCT showed that UMFA might not be the best biomarker to measure chronic exposure to folic acid in late gestation. As described earlier in Chapter 2, the rise of UMFA is

resulted from the saturation of the dihydrofolate reductase that converts folic acid to tetrahydrofolate. An earlier study reported that folic acid starts to appear in circulation after ingesting fortified foods with folic acid above 200 µg (5). Specifically, a pharmacokinetics study in 20 healthy men showed that at 800 µg of folic acid taken orally, folic acid peaks after 2.5 hours of ingestion, gradually disappears after that, and completely disappears at 12 hours (6). In the RCT in this thesis, participants were instructed not to take the supplement in the morning of the blood draw at 36 weeks of gestation. Because the interest is in chronic exposure to folic acid, the instruction is given to ensure that there is no residual folic acid coming from recent intake, either from foods or supplements. Currently, no studies report the effect of chronic folic acid exposure at 800 µg or more of folic acid per day on UMFA. I showed regardless of treatment groups (0 µg vs. 800 µg of folic acid per day), UMFA was detected in both groups, 72% vs. 98%, respectively. Despite the mean difference in UMFA, concentrations could not be quantified because the overall concentrations were low.

Several studies have reported the presence of UMFA in late gestation in women living in countries with and without mandatory folic acid fortification. All of these studies are observational, except for one reporting an RCT of 400 µg folic acid in Irish women (7). Pentieva et al. reported a significant difference in the proportion of women with detectable UMFA between 400 µg folic acid vs. 0 µg, which is 42% vs. 16%. Similarly, in my RCT, I reported differences in detectable UMFA, but the proportion of women with detectable UMFA was approximately double than that in Pentieva et al. It is possible that the doubling of the dose of 800 µg of folic acid may contribute to this. In Pentieva et al., only 16% of women receiving no folic acid had detectable UMFA compared to the women in the RCT of this thesis which is 72%. The difference could be attributed to the background of dietary folic acid intake. While Australia has mandatory fortification, the UK, including Ireland, did not

mandate folic acid fortification when the study took place. As of September 2021, the UK now mandates folic acid fortification.

An earlier report from my research group of Western Australian women reported a similar proportion of women (93%) with detectable UMFA in late gestation (8). However, the study was observational. A controlled feeding study in pregnant women in the US where the women consumed 750 µg folic acid per day reported 81% of women had detectable UMFA (9).

Although the proportion of women with detectable UMFA is quite similar between populations, the ranges of UMFA concentration differ quite significantly in different populations. In Chapter 4, it is reported that the range of UMFA was from undetectable to 14.2 nmol/L. Meanwhile, Pentieva et al reported the highest detectable UMFA concentration was 2.25 nmol/L (7). West et al reported the range of serum UMFA concentrations from 0.1 to 0.65 ng/mL, which is 0.23 to 1.47 nmol/L (9). The highest detectable UMFA in late pregnancy was reported in the cohort of Western Australian women where the range of UMFA concentrations was from 0.03 to 244.7 nmol/L (8).

Several reasons might be in play to explain the differences in the range of detectable UMFA concentrations in these studies. One of the most significant ones is the method used to measure UMFA concentrations. Even though liquid chromatography-tandem mass spectrometry (LC-MS/MS) is commonly used, serum sample preparation and extraction, standards, and determination for limit of detection (LOD) and limit of quantification (LOQ) differ significantly between laboratories. LC-MS/MS methods by Pfeiffer et al and Hannisdal et al are the two most common LC-MS/MS methods to quantify UMFA concentrations. The sample preparation and setting of LOD and LOQ differ significantly. Hannisdal et al. method only requires 60 µL of serum that can be used to measure UMFA while Pfeiffer et al method requires 275 µL to measure three folate forms including UMFA. When serum samples are

limited, Hannisdal method would be more feasible to perform (10). However, Hannisdal's is a less sensitive method to Pfeiffer's (11), where Hannisdal's LOD and LOQ are 0.27 nmol/L and 0.53 nmol/L, Pfeiffer's are 0.07 nmol/L and 0.14 nmol/L, respectively.

While it would be easy to assume that the UMFA method being used in this thesis is not a sensitive method, this is not the case. In establishing the methods, the LC/MS experts overseeing and performing the method validation for UMFA follow the US FDA's Bioanalytical Method Validation Guidance for Industry (12). The manual provides specific guidelines on how to set the limit of quantification (LOQ) as such it should be based on visual inspection and a signal to noise ratio (SNR) between 10-20. For the method used in this thesis, it is set at 14 and as such the LOQ is 0.566 nmol/L. As for the LOD a SNR of 3:1 to 10:1 should be used, and 3 is used in this thesis.

There is currently no standardised consensus in how to set the LOD and LOQ for UMFA measurement using LC/MS. Equally so, there is also currently no consensus in how to interpret the data if the value is below the LOD or LOQ. A couple approaches have been used by researchers which include: (i) Replacing any unquantifiable UMFA values with zero (7) and (ii) Using the LOD and dividing it with 2 or with $\sqrt{2}$ as referred to by more recent publication by Patti et al. (13). In this thesis, a more conservative approach has been used rather than performing data extrapolation as the standard curve below the LOD and LOQ is not linear.

At present, it remains unclear whether the presence of UMFA or higher concentrations of UMFA is more relevant. Currently, there is no clinical cut-offs of UMFA concentrations for certain clinical outcome both in mothers and the offspring. For this reason, Hannisdal's may be a fairly sensitive approach if the presence of UMFA is in contrast to the more pertinent concentration. Another differing factor of the two methods is in the sample preparation. The Pfeiffer's method requires a lengthier preparation using solid phase

extractions (SPE), which was also explored in this thesis where a volume 2.7x of serum sample compared to the current method was used, but lower folic acid was detected. Hannisdal's, on the other hand, does not require SPE procedures.

Unlike the microbiological assay that has been standardised internationally to measure serum and red blood cell (RBC) folate, the LC-MS/MS has not been standardised between laboratories. Considerations for standardisation may include: sample preparation steps, determination of LOD and LOQ, and range of concentrations and manufacturer for standards. Following which, comparisons between laboratories should be performed. It also remains important to determine whether it is the cut-off of UMFA concentration or presence of UMFA that has clinical relevance.

Furthermore, this thesis reported that RBC folate concentrations were significantly higher between the 800 µg folic acid group compared to the no folic acid group, raising concern the potential harmful effect of elevated RBC folate concentration independent of UMFA. There is currently no upper level cut off for RBC folate levels. However, government and public health agencies recommend the Upper Tolerable Level of folic acid is 1000 µg per day. The main concern relating to elevated RBC folate due to high dose of folic acid intake is in its relations with masking vitamin B12 deficiency (masking megaloblastic anemia). This is of a particular concern among women of reproductive age. Other evidence in regard to elevated RBC folate have primarily been among other population groups: males or elderly. These potential adverse health outcomes include neurological diseases (14) and abdominal aortic calcification (15). Within the scope of this thesis, further research is necessary whether these adverse health outcomes may also be implicated in pregnant women exposed to high dose of folic acid during pregnancy.

Finally, this thesis provides the first contemporary evidence of current practice of folic acid supplementation during pregnancy in a country with mandatory folic acid

fortification. Future research should address whether the continued use of folic acid-containing multi-micronutrient prenatal supplement leads to adverse health outcomes in mothers and the offspring. Additionally, it is important if UMFA is the biomarker of interest for chronic exposure of excess folic acid and whether or not elevated UMFA, as also mentioned by Maruvada et al (16), affects any biological pathways related to health outcomes in mothers and offspring.

Concluding remarks

Folic acid fortification has been proven to be one of the most effective public health initiatives for lowering the prevalence of congenital impairments, notably neural tube defects. However, over the years, the practice has changed from what is recommended to raising concern about chronic exposure of excess folic acid during pregnancy on maternal health and the children's health. This thesis showed that at 800 µg of folic acid per day, it did not significantly increase UMFA concentrations in late gestation. There is a possibility that UMFA may not be the best biomarker for it, or if the practice of chronic excess folic acid alone may be potentially harmful via another mechanism that is not UMFA accumulation. It is also important to standardise the methods used to measure UMFA concentrations from blood processing until quantification, such as serum and RBC folate concentrations.

Furthermore, although UMFA may not be the mediating factor by which chronic excess folic acid exposure during pregnancy affects maternal health and the children's health, we must not disregard the mounting observational evidence showing the association. An adequately powered randomised clinical trial with clinical outcome would be an important next step to provide stronger evidence to inform about the effect of continued folic acid exposure during pregnancy on adverse health outcomes both in the mothers and offspring.

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Appendices

Appendix 1

Best KP, Green TJ, **Sulistyoningrum DC**, Sullivan TR, Aufreiter S, Prescott SL, Makrides M, Skubisz M, O'Connor DL, Palmer DJ. Maternal Late-Pregnancy Serum Unmetabolized Folic Acid Concentrations Are Not Associated with Infant Allergic Disease: A Prospective Cohort Study. J Nutr. 2021 Jun 1;151(6):1553-1560. doi: 10.1093/jn/nxab040. PMID: 33851208.

My contribution in the paper is in sample management for the analysis of serum folate and UMFA. I performed all of the experiment to quantify serum folate concentration using microbiological assay. I also analysed the data for serum folate concentration.

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See corresponding commentary on page 1367.

Maternal Late-Pregnancy Serum Unmetabolized Folic Acid Concentrations Are Not Associated with Infant Allergic Disease: A Prospective Cohort Study

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ABSTRACT

Background: The increase in childhood allergic disease in recent decades has coincided with increased folic acid intakes during pregnancy. Circulating unmetabolized folic acid (UMFA) has been proposed as a biomarker of excessive folic acid intake.

Objective: We aimed to determine if late-pregnancy serum UMFA and total folate concentrations were associated with allergic disease risk in the offspring at 1 y of age in a population at high risk of allergy.

Methods: The cohort consisted of 561 mother–infant pairs from Western Australia. To be eligible the infant had to have a first-degree relative (mother, father, or sibling) with a history of medically diagnosed allergic disease. Maternal venous blood was collected between 36 and 40 wk of gestation. Serum UMFA was measured by LC–tandem MS. Serum total folate was determined using a microbiological method with chloramphenicol-resistant *Lactobacillus rhamnosus* as the test organism, and was collected between 36 and 40 wk of gestation. UMFA concentrations were measured by tandem MS using stable isotope dilution; folate concentrations were determined using the microbiological method with standardized kits. Infant allergic disease outcomes of medically diagnosed eczema, steroid-treated eczema, atopic eczema, IgE-mediated food allergy, allergen sensitization, and medically diagnosed wheeze were assessed at 1 y of age.

Results: Median (IQR) concentrations for UMFA and serum folate were 1.6 (0.6–4.7) and 53.2 (32.6–74.5) nmol/L, respectively. Of the infants, 34.6% had medically diagnosed eczema, 26.4% allergen sensitization, and 14.9% had an IgE-mediated food allergy. In both adjusted and unadjusted models there was little evidence of association between UMFA or serum folate and any of the infant allergy outcomes.

Conclusions: In this cohort of children at high risk of allergic disease there was no association between maternal UMFA or serum folate concentrations measured in late pregnancy and allergic disease outcomes at 1 y of age. *J Nutr* 2021;151:1553–1560.

Keywords: unmetabolized folic acid, allergic disease, atopic dermatitis, eczema, food allergy, folate, folic acid, infant, pregnancy

Introduction

The prevalence of early-childhood atopic diseases, predominantly atopic dermatitis (eczema) and food allergy, has increased over recent decades. There is emerging evidence that immune function at birth is predictive of whether a child will develop allergic disease (1–4). Therefore, the in utero period may be critical in determining immune development trajectories towards an allergic phenotype (1, 5) and maternal diet during pregnancy could be a potential modifiable early-life influence of allergic disease development.

This increase in early-life allergic diseases has coincided with an increase in folic acid intake during the perinatal period. Women are advised to take folic acid containing supplements (6, 7) prior to and during early pregnancy to reduce the

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incidence of neural tube defects (NTDs) (8–10). In addition, to further reduce NTDs, >80 countries worldwide have mandated the addition of folic acid to food staples such as flour (11). NTDs occur in the first month of pregnancy, yet many women continue to take folic acid–containing supplements throughout pregnancy with no known benefit.

Folic acid is a synthetic form of folate; due to its high bioavailability, stability, and low cost, it is used in supplements and for food fortification. Once consumed, folic acid is normally converted to tetrahydrofolate, by the enzyme dihydrofolate reductase (DHFR), before being further converted to 5-methyltetrahydrofolate. Higher intakes of folic acid can saturate the capacity of DHFR, leading to the presence of unmetabolized folic acid (UMFA) in the circulation (12–15). Circulating UMFA has been detected in pregnant women and in cord blood (16–19). There is speculation that UMFA in the circulation is a biomarker of excessive folic acid intake and may be causing harm through epigenetic changes to fetal gene expression, with subsequent increased disease risk (20, 21).

Animal models have shown that pregnant mice fed diets high in folic acid exhibit altered expression of immune genes through changes in DNA methylation in the offspring. Such changes have been associated with enhanced severity of allergic airway disease (22). During pregnancy, the human fetus begins to develop and adapt its immune system to maternal diet, lifestyle, and environmental exposures (23–25). The measurement of these in utero exposures, especially in the latter stages of pregnancy, of known epigenetic modifiable factors like folic acid is critical.

Several observational cohort studies have reported inconsistent associations between higher prenatal folic acid or folate intakes and risk of allergic disease in the offspring (26, 27–39); however, many rely on dietary assessment or recall of supplement use to measure exposure. Of the studies that examined biomarkers to determine exposure, only one differentiated between specific forms of folate (34). This nested case-control study from the United States, reported that UMFA concentrations in cord blood were associated with an increased risk of food allergy, but not food allergen sensitization; however, other allergic disease outcomes were not reported (34).

Australia is an ideal setting to examine associations between folic acid in pregnancy and allergic disease outcomes in the offspring. It has among the highest prevalence of allergic disorders in the developed world in addition to high prenatal folic acid exposures from food fortification and high rates of prenatal folic acid supplementation (40, 41). To date, no studies have examined the association between maternal late gestation UMFA and infant allergic disease. We aimed to determine if maternal serum UMFA or folate concentrations in late pregnancy predicted infant allergic disease outcomes at 1 y of age in a pregnancy cohort of women carrying a fetus at high hereditary risk of allergic disease (history of allergic disease in at least 1 immediate family member).

Methods

Study population

The data presented here come from mother–infant pairs from a prospective cohort study in Perth, Western Australia. The study was designed to explore whether maternal diet, lifestyle, and environmental factors influence offspring susceptibility to allergic disease. Pregnant women >18 y of age whose unborn infant had a first-degree relative (mother, father, or sibling) with a history of medically diagnosed allergic disease (asthma, allergic rhinitis, eczema, and/or food allergy) were recruited from local participating maternity antenatal clinics and classes between November 2011 and December 2016. Women were >36 wk of gestation at enrollment, with a singleton pregnancy, nonsmokers (while pregnant), and healthy with no known complications (including immunodeficiency, pre-eclampsia, and major congenital anomalies). The original cohort study was approved by the Princess Margaret Hospital Human Research Ethics Committee (1942EP), and all participants provided written informed consent. The maternal blood analysis for this study was also approved by the Women's and Children's Health Network Human Research Ethics Committee in 2019 (HREC/19/WCHN/21).

The investigators in the original cohort aimed to have ~600 mother–infant pairs with infant allergy outcomes at 1 y of age. This was based on previous cohorts that have examined associations between maternal diet in pregnancy and allergic disease outcomes in infants with a high hereditary risk of allergic disease (42, 32).

Maternal data and blood collection

Maternal baseline data were collected between 36 and 40 wk of gestation, including history of allergic disease, education, ethnicity, parity, and pet ownership (cat, dog, or both). Maternal nonfasting blood samples were collected from the cubital vein into a serum clot activator tube (Vacuette, Z Serum Clot activator; Greiner Bio-One GmbH). The blood samples were allowed to clot and blood was centrifuged at 500 × *g* for 30 min at room temperature; serum was placed into 1–2-mL tube aliquots and stored at −80°C until analysis.

Infant allergic disease assessment and definitions

At 1 y of age the participating infants were assessed at the Princess Margaret Hospital in Perth, Australia. A parent was asked if the infant ever had eczema that was diagnosed by a medical doctor (medically diagnosed eczema) during the first year of life, and if the eczema skin lesions were responsive to topical steroid treatment prescribed by a medical doctor (steroid-treated eczema). The parent was also asked if the infant had any wheeze symptoms that had been diagnosed by a medical doctor (medically diagnosed wheeze). IgE-mediated food allergy was based on history of immediate IgE-mediated symptoms (within 60 min of food ingestion), including angioedema, urticaria, cough, wheeze, stridor, vomiting, diarrhea, cardiovascular symptoms, and allergen sensitization to the same food detected by positive skin-prick test (SPT) at the 1-y-of-age visit. SPT was conducted to detect allergen sensitization to common Australian food and environmental allergens including cow milk, hen's egg, peanut, cashew nut, wheat, rye grass, house dust mite, and cat (Hollister-Stier Laboratories), as well as histamine as a positive control. A response was considered positive if the mean of the horizontal and perpendicular wheal diameter was ≥3 mm in size than the mean wheal of the negative control site at 15 min. Sensitization was defined as a positive SPT result to at least 1 of the allergens assessed. Each infant's SPT and clinical allergy assessment results were confirmed by the research physician. Infant birth details (including delivery mode, gestational weight, gestational age, and infant sex) were also collected.

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Address correspondence to KPB (e-mail: karen.best@sahmri.com).
Abbreviations used: DHFR, dihydrofolate reductase; NTD, neural tube defect; SPT, skin-prick test; UMFA, unmetabolized folic acid.

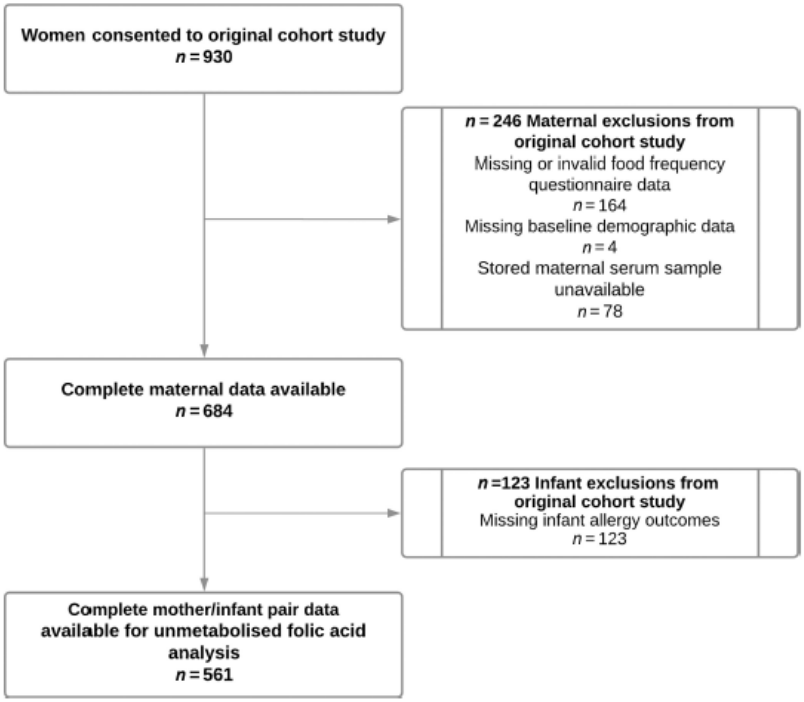

FIGURE 1 Participant flow of the original cohort study and selection process for the data included in the maternal serum unmetabolized folic acid and folate analysis.

Serum UMFA

Between August 2019 and July 2020 maternal serum samples were analyzed for UMFA by stable isotope dilution–tandem MS following the methods of Pfeiffer et al. (43) using spectrometrically verified standards of folic acid and an internal standard of 13C5-folic acid (Merck). Briefly, samples and standard spiked with folic acid-[13C5] in 1% ammonium formate, 0.5% ascorbic acid buffer (pH 3.2) were loaded onto phenyl cartridges (1 mL; Phenomenex) previously conditioned with 2 mL each of methanol, acetonitrile, and 1% ammonium formate buffer (pH 3.2) and were allowed to equilibrate for 1 min. The loaded cartridges were washed sequentially with 3 mL 0.05% ammonium formate buffer (pH = 3.4, 0.25% ascorbic acid), and folic acid was eluted from the columns using an elution buffer (0.5 mL) of 49% water, 40% methanol, 10% acetonitrile, 1% concentrated acetic acid, and 0.5% ascorbic acid. Eluted samples were stored at −80°C until analysis by tandem MS at the Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto, Canada.

Eluted solutions were separated chromatographically on a Kinetex PFP (50 × 3.0 mm, 2.6-μm particle size) column (Phenomenex). The mass-to-charge ratios of the transitions of interest, *m/z* 442.4 → m/z 295.1 for folic acid and *m/z* 447.4 → 495.1 for 13C folic acid, were monitored using an AB Sciex QTRAP 5500 triple quadrupole MS system (Agilent 1290 UHPLC system; Agilent Technologies).

The interbatch accuracy and precision were determined with the use of National Institute of Standards and Technology (NIST) 1950 Standard Reference Material with a certified value of 1.51 ± 0.45 ng/mL. Each group of samples was analyzed along with an aliquot of the reference material NIST1950 (Metabolites in Frozen Human Plasma); the mean (±SD) obtained for 12 batches of samples was 1.80 ± 0.21 ng/mL, with a CV of 11.9%. Some UMFA concentrations were below the limit of detection (n = 39, 7%) and were set to the midpoint between 0 and the detection limit for analysis.

Serum folate

Serum folate concentrations were determined using the microbiological method based on the technique of O'Broin and Kelleher, using standardized kits from the US CDC (44). This method uses 96-well microplates, 5-methyl tetrahydrofolate (Merck) as the calibrator, and chloramphenicol-resistant *Lactobacillus rhamnosus* (ATCC®

TABLE 1 Descriptive statistics for maternal and infant characteristics[1]

	Values
Maternal characteristics	
Age, y	32.9 ± 4.8
Allergic disease	517/560 (92.3)
Positive skin-prick test	482/537 (89.8)
Atopy	467/560 (83.4)
European Caucasian ethnicity	493/544 (90.6)
Further education after secondary school	417/558 (74.7)
Dog and/or cat ownership	334/559 (59.7)
Parity >1	296/560 (52.9)
Late-pregnancy vitamin use	457/528 (86.6)
Dietary folate intake at 32–36 wk of gestation, mg/d	265 (212–329)
Vaginal delivery	372/550 (67.6)
Infant characteristics	
GA at birth (n = 550), wk	39.3 ± 1.1
Birth weight (n = 559), g	3502 ± 434
Female sex	271/561 (48.3)

[1]Values are means ± SDs or medians (IQR) for continuous measures and n/total n (%) for categorical measures; n = 561 (unless otherwise indicated). GA, gestational age.

TABLE 2 Associations between maternal serum unmetabolized folic acid concentration (continuous) and infant allergic disease outcomes[1]

Outcome	Unadjusted OR (95% CI)	*P*	Adjusted[2] OR (95% CI)	Adjusted *P*
Medically diagnosed eczema	0.99 (0.89, 1.09)	0.77	1.02 (0.90, 1.15)	0.76
Steroid treated eczema	1.02 (0.93, 1.13)	0.66	1.04 (0.92, 1.18)	0.54
Atopic eczema	1.05 (0.94, 1.17)	0.39	1.06 (0.92, 1.22)	0.44
IgE-mediated food allergy	1.04 (0.93, 1.16)	0.52	1.05 (0.91, 1.21)	0.52
Allergen sensitization	1.02 (0.92, 1.13)	0.74	1.02 (0.90, 1.16)	0.77
Medically diagnosed wheeze	1.08 (0.96, 1.21)	0.22	1.13 (0.96, 1.34)	0.14

[1] ORs describe the effect of a 10-nmol/L increase in unmetabolized folic acid; goodness of fit confirmed using Hosmer-Lemeshow tests.
[2] Adjusted for maternal age, further maternal education after high school, maternal Caucasian ethnicity, infant sex, infant birth weight, infant gestational age at birth, maternal cat/dog ownership, parity >1, and vaginal delivery.

27773TM) as the test organism. High- and low-quality controls for serum folate provided by the CDC were run in quadruplet on every plate.

Statistical analysis

Associations between the maternal folate measures and infant allergy outcomes were evaluated using logistic regression, with effects described as ORs with 95% CIs. UMFA and serum folate concentrations were treated as continuous exposures in the main analysis, with the assumption of a linear association with the log odds of each allergic disease outcome assessed using Hosmer-Lemeshow tests. For completeness, additional analyses were also performed with the UMFA and folate measures grouped into quartiles and treated as categorical exposures. For each outcome variable and UMFA and folate measure, both unadjusted and adjusted analyses were performed, with adjustment for maternal age, further maternal education after high school, maternal Caucasian ethnicity, maternal cat/dog ownership, maternal parity >1, vaginal delivery, infant sex, infant birth weight, and infant gestational age at birth (45). UMFA concentrations below the limit of detection were set to the midpoint between 0 and the detection limit for analysis. All statistical analyses were performed using Stata version 16.0 (StataCorp LP).

Results

Study population

A total of 561 mother–infant pairs with complete maternal data, maternal blood samples, and at least 1 of the infant allergic disease outcome measures were included in this analysis (Figure 1). Maternal and infant characteristics for the mother–infant pairs are summarized in Table 1. The majority of the participating women were European Caucasian (91%) and most had completed postsecondary school education (75%). All infants had at least 1 immediate family member (first-degree relative) with a history of allergic disease and 92% had maternal allergic disease.

Maternal serum UMFA and folate concentrations

In late gestation, UMFA was detectable in 520 of 559 (93.0%) of maternal serum samples, with concentrations ranging from 0.03 to 244.7 (median: 1.6; IQR: 0.6–4.7) nmol/L. Maternal serum folate concentration ranged from 4.3 to 185.0 (median: 53.2; IQR: 32.6–74.5) nmol/L. The Spearman rank correlation coefficient between the UMFA and serum folate concentrations was 0.50 (95% CI: 0.44, 0.57).

Infant allergic disease outcomes

Of the infants, 194 of 561 (34.6%) had medically diagnosed eczema and 150 of 561 (26.7%) had eczema requiring steroid treatment during the first year of life. The allergen sensitization rate was 26.4% (146/552), with 14.9% (83/558) of infants classified as having atopic eczema (medically diagnosed eczema and allergen sensitization) and 14.9% (83/558) of infants with an IgE-mediated food allergy. Only 8.7% (49/561) infants had medically diagnosed wheeze during infancy.

No associations were found between maternal UMFA (Table 2) or folate concentrations (Table 3) and infant allergic disease outcomes. In the case of UMFA, results were not sensitive to the method used to deal with values below the detection limit (set to the limit, set to 0, or excluded from analysis, results did not change). Similarly, we did not find any associations between maternal UMFA quartiles (Table 4) or folate quartiles (Table 5) and any of the infant allergic disease outcomes in additional adjusted and unadjusted (not shown) analyses.

Discussion

This is the first prospective cohort study to examine the association between maternal late pregnancy UMFA status and multiple allergic disease outcomes in a "high risk" infant population. We found no evidence of associations between

TABLE 3 Associations between maternal serum folate concentrations (continuous) and infant allergic disease outcomes[1]

Outcome	Unadjusted OR (95% CI)	*P*	Adjusted[2] OR (95% CI)	Adjusted *P*
Medically diagnosed eczema	0.99 (0.94, 1.05)	0.81	1.00 (0.95, 1.06)	0.93
Steroid-treated eczema	1.01 (0.96, 1.07)	0.69	1.01 (0.95, 1.07)	0.76
Atopic eczema	1.04 (0.97, 1.11)	0.27	1.03 (0.96, 1.10)	0.43
IgE-mediated food allergy	0.95 (0.88, 1.02)	0.16	0.93 (0.86, 1.00)	0.07
Allergen sensitization	1.00 (0.95, 1.06)	0.87	0.99 (0.94, 1.05)	0.78
Medically diagnosed wheeze	1.02 (0.94, 1.11)	0.57	1.03 (0.95, 1.12)	0.51

[1] ORs describe the effect of a 10-nmol/L increase in serum folate; goodness of fit confirmed using Hosmer-Lemeshow tests.
[2] Adjusted for maternal age, further maternal education after high school, maternal Caucasian ethnicity, infant sex, infant birth weight, infant gestational age at birth, maternal cat/dog ownership, parity >1, and vaginal delivery.

TABLE 4 Associations between maternal serum unmetabolized folic acid concentration (quartiles) and infant allergic disease outcomes[1]

Outcome and quartile[2]	*n*/Total *n* (%)	Adjusted[3] OR (95% CI)	*P*
Medically diagnosed eczema			
1	53/141 (37.6)	1 (Reference)	0.63*
2	48/139 (34.5)	0.98 (0.58, 1.65)	0.94
3	52/140 (37.1)	1.05 (0.62, 1.77)	0.86
4	41/139 (29.5)	0.76 (0.44, 1.29)	0.31
Steroid-treated eczema			
1	44/141 (31.2)	1 (Reference)	0.71*
2	34/139 (24.5)	0.74 (0.42, 1.29)	0.29
3	35/140 (25.0)	0.78 (0.45, 1.36)	0.38
4	37/139 (26.6)	0.78 (0.45, 1.35)	0.37
Atopic eczema			
1	19/140 (13.6)	1 (Reference)	0.65*
2	23/138 (16.7)	1.39 (0.68, 2.86)	0.37
3	21/139 (15.1)	1.47 (0.71, 3.04)	0.30
4	20/139 (14.4)	1.07 (0.51, 2.25)	0.85
IgE-mediated food allergy			
1	20/141 (14.2)	1 (Reference)	0.36*
2	27/137 (19.7)	1.47 (0.74, 2.92)	0.27
3	18/140 (12.9)	0.91 (0.43, 1.93)	0.81
4	18/138 (13.0)	0.82 (0.39, 1.72)	0.59
Allergen sensitization			
1	35/139 (25.2)	1 (Reference)	0.74*
2	42/137 (30.7)	1.27 (0.71, 2.26)	0.42
3	33/137 (24.1)	0.98 (0.54, 1.78)	0.94
4	36/137 (26.3)	0.94 (0.52, 1.70)	0.84
Medically diagnosed wheeze			
1	8/141 (5.7)	1 (Reference)	0.12*
2	11/139 (7.9)	1.55 (0.56, 4.30)	0.40
3	14/140 (10.0)	2.33 (0.84, 6.45)	0.10
4	16/139 (11.5)	3.07 (1.15, 8.21)	0.03

[1]**P* values for the global null hypothesis that the log odds of allergic disease are the same in the 4 quartiles.
[2]Quartile 1, <0.66 nmol/L; quartile 2, 0.66 to 1.65 nmol/L; quartile 3, 1.65 to 4.69 nmol/L; quartile 4, >4.69 nmol/L.
[3]Adjusted for maternal age, further maternal education after high school, maternal Caucasian ethnicity, infant sex, infant birth weight, infant gestational age at birth, maternal cat/dog ownership, parity >1, and vaginal delivery.

maternal UMFA or folate concentrations and any infant allergic disease outcomes.

One other cohort study has examined the association between UMFA and childhood allergy outcomes using cord blood to measure exposure (34). In contrast to our findings, McGowan et al. (34) reported that children whose cord blood concentrations were in the highest quartile of UMFA (n = 14/33) had an 8.5-fold (95% CI: 1.7, 42.8) increased risk of confirmed food allergy compared with the lowest quartile. However, interestingly, there was no association between UMFA and food allergen sensitization. Furthermore, atopic dermatitis/eczema outcomes (the most common allergic disease in infancy) were not reported. Given that there was only 6.6% (33/502 children with cord blood UMFA) of the children with confirmed food allergy in the McGowan et al. publication, and the wide CI, there is a possibility of this being a chance finding. Our Australian-based study differed from this US study in several ways. Notably, our cohort had higher rates of offspring food allergy (14.9% vs. 6.6%). In addition, our population included mostly women of European Caucasian ethnicity (91%), whereas the Boston Birth Cohort in the study by McGowan et al. were predominantly of non-Hispanic Black ethnicity (only 7% White). We cannot compare our UMFA concentrations in maternal serum with those reported in cord blood samples in the study by McGowan et al. However, the prevalence and range of detectable concentrations of UMFA in our maternal study population (93%; range: undetectable to 245 nmol/L) are similar to those reported by Plumptre et al. (16) from blood samples collected from women in early pregnancy (56% Caucasian) and taking a folic acid–containing supplement (97%; range undetectable to 244 nmol/L).

We also found no association between maternal serum folate at 36–40 wk of gestation and infant allergic disease outcomes, even though we did find higher median maternal serum folate concentrations (53.2; IQR: 32.6–74.5 nmol/L) compared with a previous Western Australian cohort study (n = 435), where average serum folate concentrations were 37.2 nmol/L (IQR: 25.6–50.5 nmol/L) after 28 wk of gestation (32). The previous Western Australian cohort study also did not find any associations between maternal serum folate concentrations and infant eczema outcomes, but did find that cord blood folate concentrations <50 nmol/L (OR: 3.02; 95% CI: 1.16–7.87) and >75 nmol/L (OR: 3.59; 95% CI: 1.40–9.20) were associated with greater infant allergen sensitization risk than cord blood folate concentrations between 50 and 75 nmol/L (32).

Previous findings from observational studies examining associations between folate exposure in pregnancy from food and/or supplement use and offspring allergic disease have been equivocal, the majority reporting an increased risk (27–37), some no association (46, 38), and others suggesting a

TABLE 5 Associations between maternal serum folate (quartiles) and infant allergic disease outcomes[1]

Outcome and quartile[2]	*n*/Total *n* (%)	Adjusted[3] OR (95% CI)	*P*
Medically diagnosed eczema			
1	48/141 (34.0)	1 (Reference)	0.59*
2	48/140 (34.3)	1.13 (0.66, 1.92)	0.66
3	53/140 (37.9)	1.38 (0.82, 2.33)	0.23
4	45/140 (32.1)	1.01 (0.60, 1.71)	0.97
Steroid-treated eczema			
1	36/141 (25.5)	1 (Reference)	0.78*
2	35/140 (25.0)	1.06 (0.59, 1.89)	0.84
3	40/140 (28.6)	1.33 (0.76, 2.32)	0.32
4	39/140 (27.9)	1.12 (0.64, 1.97)	0.68
Atopic eczema			
1	21/141 (14.9)	1 (Reference)	0.91*
2	18/138 (13.0)	0.96 (0.46, 2.00)	0.91
3	19/139 (13.7)	0.96 (0.47, 1.98)	0.92
4	25/140 (17.9)	1.20 (0.60, 2.38)	0.61
IgE-mediated food allergy			
1	27/140 (19.3)	1 (Reference)	0.29*
2	20/138 (14.5)	0.76 (0.38, 1.52)	0.43
3	18/140 (12.9)	0.62 (0.31, 1.24)	0.18
4	18/140 (12.9)	0.52 (0.25, 1.05)	0.07
Allergen sensitization			
1	38/140 (27.1)	1 (Reference)	0.86*
2	37/134 (27.6)	1.09 (0.61, 1.96)	0.77
3	35/138 (25.4)	0.91 (0.51, 1.62)	0.74
4	36/140 (25.7)	0.86 (0.48, 1.52)	0.60
Medically diagnosed wheeze			
1	13/141 (9.2)	1 (Reference)	0.74*
2	10/140 (7.1)	0.82 (0.31, 2.13)	0.68
3	11/140 (7.9)	0.95 (0.39, 2.32)	0.91
4	15/140 (10.7)	1.33 (0.59, 3.04)	0.49

[1] **P* values for the global null hypothesis that the log odds of allergic disease are the same in the 4 quartiles.
[2] Quartile 1, <32.6 nmol/L; quartile 2, 32.6 to 53.2 nmol/L; quartile 3, 53.2 to 74.5 nmol/L; quartile 4, >74.5 nmol/L.
[3] Adjusted for maternal age, further maternal education after high school, maternal Caucasian ethnicity, infant sex, infant birth weight, infant gestational age at birth, maternal cat/dog ownership, parity >1, and vaginal delivery.

protective effect of folate (26, 39). Two studies suggested a "U-shaped distribution" association where both high and low folate exposure increased allergy risk (43, 30); however, we could not find any similar associations in our study. The inconsistency in results may be due to heterogeneity of populations studied, including genetic differences and background folate intakes from food. Study design differences including timing and classification (i.e., diet, supplement use, or folate biomarkers) of folate/folic acid exposure, as well as offspring age and types of allergic disease outcomes assessed may also confound the results. Many of the observational studies showing associations between folic acid and allergic disease have relied on maternal reported folic acid supplementation or dietary assessment methods as the exposure, which can be subject to reporting bias and measurement error. Furthermore, folic acid supplements are usually consumed as part of a multivitamin and mineral supplement and hence it is impossible to determine the independent effect of folic acid. In our study, there was no association between late-pregnancy supplement use and risk of allergic disease, despite 86% of participants taking folic acid–containing supplements.

A strength of our study is the objective measurement of serum UMFA and folate concentrations, which reduces potential reporting bias due to reliance on participant memory of dietary intake of folate from foods or supplement use. However, the pregnant women in our study were not fasted and the timing of the maternal blood sample collection in relation to the last ingestion of maternal folic acid supplementation is unknown. UMFA increases rapidly after ingesting folic acid and decreases steadily over time. The time taken to clear folic acid is not known, but it likely varies by dose of folic acid, background serum and tissue folate concentrations, and genetic factors. The activity of liver DHFR, the enzyme needed to convert folic acid to tetrahydrofolate, is low and highly variable between individuals (20). The latter may be due to common polymorphisms in the gene encoding for DHFR, which results in variable enzyme activity (21).

Our cohort of infants at high hereditary risk of allergic disease increased our incidence of allergy outcomes compared with studies in a general population. However, we cannot exclude the fact that these infants with a genetic tendency towards atopy may have a predetermined disposition to an allergic phenotype that may not be modifiable by maternal folic acid concentration.

Although we controlled for a number of important risk factors for allergic disease, as with any epidemiological study the possibility of residual confounding remains. Potential confounders include socioeconomic status, which has been associated with both allergic disease (47) and folic acid supplement use (48), and intake of other nutrients during

pregnancy. Despite our large sample size and focus on a population at high risk of allergic disease, we also cannot rule out type 2 errors. However, except for medically diagnosed wheeze where case numbers were small ($n = 46$), the adjusted ORs were ~1 (range: 0.93–1.06) and the CIs were narrow. Consequently, it seems unlikely any strong associations were missed due to the sample size.

In conclusion, we found no associations between maternal late-gestation serum UMFA or folate concentrations and risk of infant allergic disease at 1 y of age in a population with high hereditary risk of atopy. Further work, including randomized controlled trials with objective biomarkers, is needed to confirm that high folic acid exposure during late pregnancy, largely driven by the combination of food fortification and prenatal folic acid supplement use, does not increase the risk of childhood allergic disease in the general population.

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Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request to researchers who provide a methodologically sound research proposal following review and approval by the trial steering committee and completion of a signed data-access agreement. Following approval, de-identified, individual participant data that underlie the results reported in this article (text, tables, figures, and appendices) and/or dataset(s) will be limited to those participants and variables that are necessary for completion of the approved research proposal. Data-sharing requests for de-identified raw data should be made to the trial steering committee and can be submitted to karen.best@sahmri.com.

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Appendix 2

Samson KLI, Loh SP, Lee SS, **Sulistyoningrum DC**, Khor GL, Mohd Shariff ZB, Ismai IZ, Makrides M, Hutcheon JA, Roche ML, Green TJ, Karakochuk CD. The Inclusion of Folic Acid in Weekly Iron-Folic Acid Supplements Confers no Additional Benefit on Anemia Reduction in Nonpregnant Women: A Randomised Controlled Trial in Malaysia. J Nutr. 2021 Aug 7;151(8):2264-2270. doi: 10.1093/jn/nxab115. PMID: 33978167.

My contribution to the paper includes: 1) creating a data management system using REDCap; 2) training of the research assistants in the field to use the data management system, run a clinical trial, and collect biological specimen properly.

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The Inclusion of Folic Acid in Weekly Iron–Folic Acid Supplements Confers no Additional Benefit on Anemia Reduction in Nonpregnant Women: A Randomized Controlled Trial in Malaysia

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ABSTRACT

Background: Weekly iron–folic acid (IFA) supplements are recommended for all menstruating women in countries where anemia prevalence is ≥20%; however, it is unknown whether the inclusion of folic acid in weekly IFA supplements reduces anemia.

Objectives: We examined whether the inclusion of folic acid in weekly IFA supplements conferred any benefit on hemoglobin (Hb) concentration, anemia reduction, or iron status [ferritin and soluble transferrin receptor (sTfR)], over iron alone.

Methods: In this secondary analysis of a randomized controlled trial in Malaysia, $n = 311$ nonpregnant women (18–45 y old) received 60 mg Fe with either 0, 0.4, or 2.8 mg folic acid once-weekly for 16 wk. Fasting blood was collected at baseline and 16 wk. A generalized linear model (normal distribution with identity link) was used to assess Hb concentration at 16 wk (primary outcome).

Results: At baseline, 84% of women had low folate status (plasma folate < 14 nmol/L). At 16 wk, marginal mean (95% CI) Hb was 131 (130, 133), 131 (129, 132), and 132 (130, 133) g/L; ferritin was 58.2 (53.9, 62.5), 56.5 (52.2, 60.9), and 58.0 (53.7, 62.3) μg/L; and sTfR was 5.8 (5.5, 6.1), 5.8 (5.5, 6.1), and 5.9 (5.6, 6.2) mg/L in the 0, 0.4, and 2.8 mg/wk groups, respectively, with no differences between groups ($P > 0.05$). Baseline plasma folate concentration did not modify the effect of treatment on Hb concentration at 16 wk. Among all women, the risks of anemia [risk ratio (RR): 0.65; 95% CI: 0.45, 0.96; $P = 0.03$] and iron deficiency based on ferritin (RR: 0.30; 95% CI: 0.20, 0.44; $P < 0.001$) were lower at 16 wk than at baseline.

Conclusions: Despite the low folate status among these nonpregnant Malaysian women, the inclusion of folic acid in weekly IFA supplements did not reduce anemia or improve iron status, over iron alone. However, the benefits of folic acid for neural tube defect prevention still warrant its retention in weekly IFA supplements. This trial was registered at www.anzctr.org.au as ACTRN12619000818134. *J Nutr* 2021;151:2264–2270.

Keywords: folic acid, iron–folic acid, anemia, public health, women's health, iron deficiency, supplementation

Introduction

Anemia, a hemoglobin (Hb) concentration <120 g/L in nonpregnant women, is a serious public health concern that is estimated to affect one-third (~33%) of nonpregnant women of reproductive age (1). One strategy that the WHO recommends for anemia reduction is intermittent iron–folic acid (IFA) supplementation. In populations where the prevalence of anemia is ≥20% among nonpregnant women of reproductive age, women are recommended to receive 60 mg Fe and 2.8 mg folic acid once-weekly for 3 mo, followed by 3 mo of no supplementation, or supplementation aligned with the school calendar year for adolescent girls (2).

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Folate deficiency is characterized by an initial decrease in plasma folate concentrations and subsequent increase in homocysteine concentrations; eventually, RBC folate concentrations decrease and RBCs become megaloblastic (3). Megaloblastic anemia is likely to appear at plasma folate concentrations < 7 nmol/L and RBC folate concentrations < 227 nmol/L (4, 5). However, the current consensus is that folate deficiency is not a significant cause of anemia in nonpregnant women (6) and the global prevalence of folate deficiency anemia is low (7), further bringing some to question the inclusion of folic acid in these supplements for the purpose of anemia prevention. Trials conducted during pregnancy have suggested that folic acid in addition to iron confers no benefit over iron alone in anemia reduction (8, 9); however, this has yet to be proven in nonpregnant women.

However, the inclusion of folic acid might prevent a neural tube defect if a woman were to become pregnant. Globally, the most commonly used weekly IFA supplement contains 60 mg elemental iron and 0.4 mg folic acid, because until recently the 2.8 mg dose was not available or accessible in many countries (10). In a double-blind randomized trial in Malaysia, we recently showed that weekly IFA with 2.8 mg folic acid, compared with 0.4 mg, was 7 times more likely to increase RBC folate to a concentration associated with a low risk of neural tube defects (11).

The question that remains is: does the addition of folic acid in weekly IFA supplements increase Hb concentration or reduce anemia in nonpregnant women, over iron alone? Moreover, does baseline folate status modify the effect of IFA supplementation on Hb concentration at 16 wk and does the dose of folic acid matter?

The objective of this secondary data analysis was to compare the effects of 16 wk of weekly iron supplementation (60 mg), with 0, 0.4, and 2.8 mg folic acid/wk, on Hb concentrations in nonpregnant women, using secondary data obtained in our recent folate trial in Malaysia. The primary outcome was Hb concentration at 16 wk. We also examined the effect of these interventions on anemia prevalence and other biomarkers of iron status, including ferritin and soluble transferrin receptor (sTfR) concentrations.

Methods

Study design and participants

This is a secondary analysis of a parallel double-blind randomized trial; the original trial has been published in full elsewhere (11, 12). This study was conducted at Universiti Putra Malaysia in Selangor, Malaysia. Malaysia was chosen because the prevalence of anemia is ≥20% among women of reproductive age, meeting the WHO criteria for weekly IFA supplementation, and mass fortification strategies were not being implemented (2). Ethical approval was granted by the Ethics Committee for Research Involving Human Subjects of Universiti Putra Malaysia (JKEUPM-2018-255) and The University of British Columbia Clinical Research Ethics Board (H18-00768). This trial was registered with the Australian New Zealand Clinical Trial Registry (ACTRN12619000818134).

To be eligible to participate, women had to be between 18 and 45 y old, nonpregnant (self-reported), not planning on becoming pregnant, not taking folic acid–containing supplements, and not participating in any other nutrition study. All participants gave written informed consent.

Procedures

Potential participants were recruited through advertisements, word-of-mouth, and classroom presentations. Those eligible and willing to participate were asked to attend a morning clinic at the university health center. After reconfirming eligibility and obtaining written consent, sociodemographic, health, and anthropometric data were recorded. A fasting venous blood sample was collected into 2 evacuated tubes containing EDTA as an anticoagulant. After this, women were randomly assigned by trained research assistants using REDCap to receive 60 mg of elemental iron as fumarate with either 0, 0.4, or 2.8 mg folic acid/wk for 16 wk (13, 14).

At the baseline visit, women were asked to take their first tablet at the clinic and then 1 tablet every week on the same day for 16 wk. To encourage adherence and monitor any adverse events, participants were texted weekly by research assistants. After 16 wk, women returned to the clinic after an overnight fast to provide another blood sample. Adherence was assessed by counting the number of remaining tablets in the bottles at 16 wk.

Laboratory analysis

After blood collection, 1 EDTA-containing tube was sent to Clinipath Malaysia Sdn. Bhd. for a full blood count determination using an automated hematology analyzer (Sysmex XP-100, Sysmex Corporation). An aliquot of whole blood from the remaining tube was removed, diluted to 1:11 in 1% ascorbic acid, and incubated for 30 min at 38°C for future whole-blood folate analysis. The remaining blood was centrifuged at ~1400 × *g* for 10 min at 4°C. The resulting plasma was removed and divided into aliquots. From the plasma aliquots, ferritin (μg/L), sTfR (mg/L), α-1 acid glycoprotein (AGP; g/L), C-reactive protein (CRP; mg/L), and retinol-binding protein (μmol/L) were assessed using a sandwich-ELISA (15). All samples were stored at −80°C until analysis.

Statistical analyses

Descriptive comparisons of baseline and 16-wk biomarker indexes were completed, with values reported as mean ± SD, median [IQR], or *n* (%).

Plasma ferritin and sTfR concentrations were adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) method (16, 17). As per BRINDA recommendations, variables were ln-transformed to achieve normality before analysis and internal regression coefficients were applied. Because the assay's limit of detection was ≤0.1 mg/L for CRP and ≤0.5 g/L for AGP, the BRINDA external deciles were used as the reference values. In women of reproductive age, these non-logged reference values are 0.16 mg/L for CRP and 0.54 g/L for AGP (16, 17). The adjustment formulas were only applied to individuals with observed AGP or CRP values greater than their respective reference values. Once applied, values were back-transformed to give the adjusted ferritin and sTfR values. No adjustments were made for Hb concentrations because this population was nonpregnant, nonsmoking, and living at altitudes below 1000 m (18).

Comparisons of proportions between treatment groups were conducted with chi-square or Fisher's exact tests. Generalized linear models with normal distribution and identity link, and adjustment for baseline values, were used to assess the effects of each treatment group on Hb, inflammation-adjusted ferritin, and inflammation-adjusted sTfR concentrations at 16 wk. Marginal means (95% CIs) were predicted for each treatment group. Because this was a secondary data analysis, we did not adjust our outcomes for multiple comparisons. Interaction terms were included in the models to determine if the outcomes were modified

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Abbreviations used: AGP, α-1 acid glycoprotein; BRINDA, Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia; CRP, C-reactive protein; Hb, hemoglobin; IFA, iron–folic acid; RR, risk ratio; sTfR, soluble transferrin receptor.

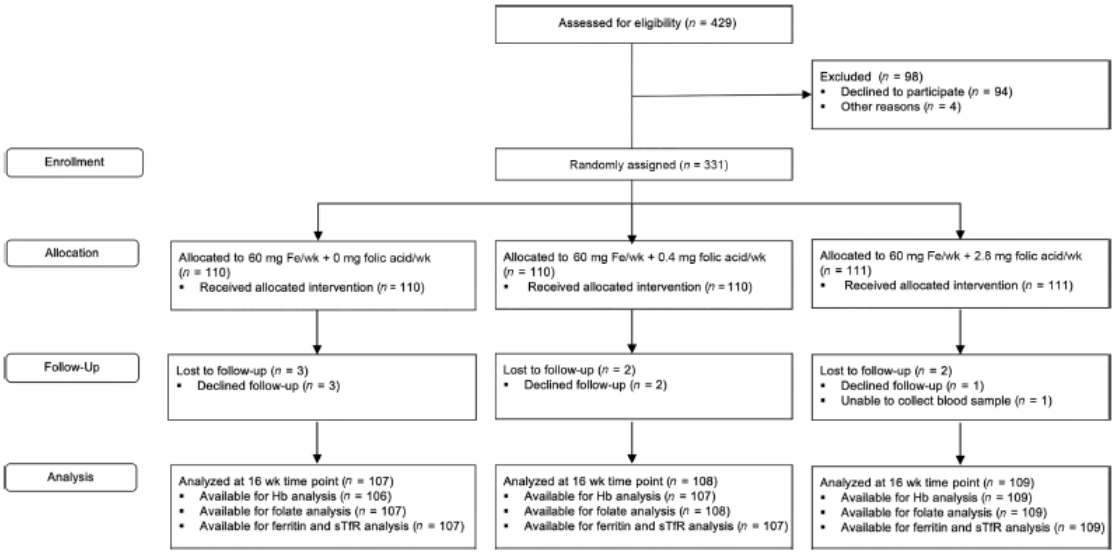

FIGURE 1 Trial flow diagram. Hb, hemoglobin; sTfR, soluble transferrin receptor.

by baseline plasma folate, ferritin, or sTfR concentrations. All groups were combined to assess the overall temporal changes in Hb, ferritin, and sTfR concentrations, as well as temporal changes in anemia and iron deficiency prevalence over the 16-wk period. Generalized linear models with normal distribution and identity link, and adjustments for treatment group, were used to estimate marginal means (95% CIs) for Hb, ferritin, and sTfR concentrations at baseline and 16 wk. Generalized linear models with binomial distribution and log link, with adjustments for treatment group, were used to estimate risk ratios (RRs) and 95% CIs for anemia, iron deficiency, and iron deficiency anemia prevalence at 16 wk. Statistical significance was set at $P < 0.05$ (thus, $P < 0.0167$ for pairwise comparisons between the 3 treatment groups). Statistical analyses were completed using Stata 15 (StataCorp LLC).

Results

Figure 1 shows the trial flow diagram. Baseline biochemical indexes are outlined in Table 1 and were similar across groups. At baseline, the overall prevalence of anemia was 17.5%

TABLE 1 Baseline biochemical indexes of enrolled Malaysian women (aged 18–45 y) by treatment group[1]

	60 mg Fe/wk + 0 mg folic acid/wk	60 mg Fe/wk + 0.4 mg folic acid/wk	60 mg Fe/wk + 2.8 mg folic acid/wk	Overall
Enrolled, *n*	110	110	111	
Age, y	18 [18–18]	18 [18–18]	18 [18–19]	18 [18–18]
Biochemical indexes				
Hemoglobin, g/L	129 ± 12.1	128 ± 11.6	128 ± 10.8	129 ± 11.5
	129 [123–137]	131 [123–136]	130 [122–136]	130 [123–136]
Plasma ferritin,[2] μg/L	34.0 ± 32.2	39.3 ± 30.5	37.3 ± 32.0	36.8 ± 31.6
	23.6 [9.8–41.5]	32.7 [14.2–54.7]	25.7 [12.0–52.3]	26.9 [12.0–50.8]
Plasma sTfR,[2] mg/L	7.0 ± 3.5	7.4 ± 4.5	6.5 ± 3.6	7.0 ± 3.9
	5.9 [4.9–8.1]	5.7 [5.0–7.5]	5.6 [4.4–7.3]	5.8 [4.8–7.6]
Plasma RBP, μmol/L	1.14 ± 0.27	1.12 ± 0.28	1.18 ± 0.30	1.15 ± 0.28
	1.11 [0.96–1.29]	1.09 [0.96–1.24]	1.13 [0.98–1.29]	1.11 [0.97–1.27]
Plasma AGP, g/L	0.57 ± 0.21	0.54 ± 0.17	0.57 ± 0.24	0.56 ± 0.21
	0.53 [0.43–0.69]	0.53 [0.41–0.64]	0.53 [0.43–0.65]	0.53 [0.43–0.65]
Plasma CRP, mg/L	1.09 ± 2.52	0.83 ± 2.04	1.62 ± 4.50	1.18 ± 3.21
	0.19 [0–0.90]	0.18 [0–0.68]	0.18 [0–0.75]	0.18 [0–0.76]
RBC folate, nmol/L	466 ± 136	474 ± 136	507 ± 168	482 ± 148
	444 [369–551]	471 [391–546]	494 [400–574]	469 [376–561]
Plasma folate, nmol/L	10.1 ± 4.0	10.3 ± 4.3	11.6 ± 6.6	10.7 ± 5.1
	9.3 [7.5–12.0]	9.5 [7.6–11.7]	9.9 [7.8–13.5]	9.5 [7.6–12.4]

[1]Values are raw mean ± SD or median [IQR] unless otherwise indicated. AGP, α-1 acid glycoprotein; CRP, C-reactive protein; RBP, retinol-binding protein; sTfR, soluble transferrin receptor.
[2]Adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia method (16, 17).

TABLE 2 Prevalence of anemia, folate deficiency, iron deficiency, and iron deficiency anemia among enrolled Malaysian women (aged 18–45 y) by treatment group at baseline and 16 wk[1]

	n[2]	60 mg Fe/wk + 0 mg folic acid/wk	60 mg Fe/wk + 0.4 mg folic acid/wk	60 mg Fe/wk + 2.8 mg folic acid/wk	*P* value
Anemia prevalence (Hb < 120 g/L)					
Baseline	331	18 (16.4)	20 (18.2)	20 (18.0)	0.93
Week 16	322	9 (8.5)	15 (14.0)	13 (11.9)	0.44
Iron deficiency anemia (Hb < 120 g/L and ferritin < 15 μg/L)[3]					
Baseline	331	14 (12.7)	13 (11.8)	14 (12.6)	0.98
Week 16	321	4 (3.8)	6 (5.7)	3 (2.8)	0.53
Iron deficiency (ferritin[4] < 15 μg/L)					
Baseline	331	38 (34.6)	29 (26.4)	33 (29.7)	0.41
Week 16	323	14 (13.1)	9 (8.4)	6 (5.5)	0.15
Iron deficiency (sTfR[4] > 8.3 mg/L)					
Baseline	331	24 (21.8)	21 (19.1)	14 (12.6)	0.19
Week 16	323	7 (6.5)	11 (10.3)	10 (9.2)	0.61
Plasma folate deficiency (<7 nmol/L)[3]					
Baseline	331	22 (20.0)	19 (17.3)	18 (16.2)	0.75
Week 16	324	21 (19.6)	8 (7.4)	2 (1.8)	<0.001
Plasma folate deficiency (<14 nmol/L)[5]					
Baseline	331	94 (85.5)	96 (87.3)	89 (80.2)	0.32
Week 16	324	90 (84.1)	54 (50.0)	5 (4.6)	<0.001
RBC folate deficiency (<227 nmol/L)[3]					
Baseline	331	1 (0.9)	2 (1.8)	1 (0.9)	0.85
Week 16	324	2 (1.9)	0 (0)	0 (0)	0.11
RBC folate deficiency (<305 nmol/L)[6]					
Baseline	331	7 (6.4)	11 (10.0)	9 (8.1)	0.62
Week 16	324	13 (12.2)	3 (2.8)	0 (0)	<0.001

[1] Values are *n* (%). Chi-square tests or Fisher's exact tests were used to compare prevalence rates across treatment groups. Hb, hemoglobin; sTfR, soluble transferrin receptor.
[2] $n = 324$ women completed the 16-wk study visit; however, owing to small volume $n = 2$ samples were prioritized for folate analysis (no blood count completed) and $n = 1$ ELISA value was missing owing to inadequate sample volume.
[3] Based upon the cutoff to define megaloblastic anemia (5, 19).
[4] Adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia method (16, 17).
[5] Deficiency defined based on rising homocysteine concentration as a metabolic indicator (5).
[6] Based upon the appearance of hyper-segmented neutrophils. Lack of megaloblastic changes in subjects with RBC folate concentrations > 140 ng/mL (~317 nmol/L) (5, 19).

($n = 58$ of 331). The overall prevalence of baseline plasma folate deficiency based on the risk of megaloblastic anemia (<7 nmol/L) was 17.8% ($n = 59$ of 331), whereas the prevalence of plasma folate deficiency based on rising homocysteine as a functional indicator (<14 nmol/L) was 84.3% ($n = 279$ of 331) (5). The overall baseline prevalence of RBC folate deficiency (<227 nmol/L) was 1.2% ($n = 4$ of 331).

At baseline, overall ~6% ($n = 19$ of 331) of women had acute inflammation (CRP > 5 mg/L) and ~3% ($n = 11$ of 331) had chronic inflammation (AGP > 1 g/L). Overall baseline iron deficiency prevalence based on ferritin (<15 μg/L) was 27.2% ($n = 90$ of 331) before BRINDA adjustment for inflammation; this increased to 30.2% ($n = 100$ of 331) after BRINDA adjustment. Overall baseline iron deficiency prevalence based on sTfR concentrations > 8.3 mg/L was 19.6% ($n = 65$ of 331) before adjustment and 17.8% ($n = 59$ of 331) after adjustment. Overall baseline prevalence of iron deficiency anemia, classified as Hb < 120 g/L and ferritin < 15 μg/L, was 12.4% ($n = 41$ of 331).

After 16 wk of weekly IFA supplementation, there were no significant differences in the prevalence of anemia, iron deficiency, or iron deficiency anemia between groups (Table 2). At 16 wk, median [IQR] Hb was 132 [125–139], 133 [125–138], and 133 [125–137] g/L; median [IQR] ferritin was 43.3 [22.5–82.4], 46.9 [30.6–87.8], and 50.7 [31.2–82.8] μg/L; and median [IQR] sTfR was 5.3 [4.4–6.3], 5.5 [4.5–6.5], and 5.0 [4.3–6.0] mg/L in the 0, 0.4, and 2.8 mg/wk groups, respectively. At 16 wk, no significant or clinically meaningful differences were detected in predicted marginal mean Hb, ferritin, or sTfR concentrations between groups (Table 3).

No significant interactions were found between baseline plasma folate concentration and treatment for Hb concentration at 16 wk ($P = 0.93$ and $P = 0.30$ for 0.4 mg/wk and 2.8 mg/wk, respectively). There was also no significant interaction between baseline ferritin concentration and treatment for ferritin concentration at 16 wk ($P = 0.86$ and $P = 0.97$ for 0.4 mg/wk and 2.8 mg/wk, respectively). However, for sTfR concentrations at 16 wk, there was a significant interaction between baseline sTfR and treatment detected in the 0.4 mg/wk group ($P < 0.001$) but not in the 2.8 mg/wk group ($P = 0.23$). In other words, baseline sTfR concentration modified the effect of the intervention on sTfR concentrations at 16 wk in the 0.4 mg/wk group, but not the 2.8 mg/wk group. There were no significant differences between treatment groups for any of the outcome variables.

Because no differences were detected in predicted marginal mean Hb, ferritin, or sTfR concentrations at 16 wk between groups, all women were combined into 1 group to assess the overall temporal changes in Hb, ferritin, and sTfR concentrations, as well as temporal changes in anemia and iron deficiency prevalence over the 16-wk period. Table 4 presents the marginal means (95% CIs) for Hb, ferritin, and sTfR concentrations at baseline and 16 wk. Table 5 presents the estimated RRs (95% CIs) for anemia, iron deficiency, and iron

TABLE 3 Predicted marginal mean (95% CI) Hb, ferritin, and sTfR concentrations by treatment group at 16 wk[1]

	n	60 mg Fe/wk + 0 mg folic acid/wk	60 mg Fe/wk + 0.4 mg folic acid/wk	60 mg Fe/wk + 2.8 mg folic acid/wk
Hb, g/L	322	131 (130, 133)	131 (129, 132)	132 (130, 133)
Ferritin,[2] μg/L	323	58.2 (53.9, 62.5)	56.5 (52.2, 60.9)	58.0 (53.7, 62.3)
sTfR,[2] mg/L	323	5.8 (5.5, 6.1)	5.8 (5.5, 6.1)	5.9 (5.6, 6.2)

[1]Values are marginal means (95% CIs). Generalized linear models with adjustments for baseline values were used to estimate the marginal mean (95% CI) for each of the outcome variables. No statistically significant differences were found between treatment groups for any of the outcome variables. Hb, hemoglobin; STfR, soluble transferrin receptor.
[2]Adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia method (16, 17).

deficiency anemia prevalence at 16 wk. At 16 wk, women had a reduced risk of anemia, iron deficiency, and iron deficiency anemia, as compared with baseline (Table 5).

Discussion

The inclusion of folic acid to intermittent iron supplementation conferred no additional benefit on anemia reduction in our study population of young Malaysian women with a high prevalence of folate deficiency. There were no differences in Hb concentration or anemia prevalence across treatment groups at 16 wk. The low prevalence of anemia in our study population at baseline (~17.5%) may have been a factor contributing to why we observed little effect after supplementation (2).

It was also of note that the Hb response to folic acid in our study did not depend on baseline plasma folate status. Owing to the lack of folic acid fortification in Malaysia (20), this population of women likely has one of the lowest population folate statuses globally—as shown by 84% of women presenting with plasma folate deficiency (<14 nmol/L) and a mean ± SD plasma folate concentration of 10.7 ± 5.1 nmol/L at baseline. This is a much lower concentration than we have observed among women of reproductive age in other low-resource settings, such as the Democratic Republic of the Congo (38 and 22 nmol/L in South Kivu and Kongo Central, respectively) or Vietnam (24.9 and 22.0 nmol/L in Hai Duong Province and Hanoi, respectively) (21, 22). Thus, it was surprising that we did not observe an additional benefit of folic acid on Hb concentrations and anemia. The fact that baseline folate status did not mediate the effect of treatment on Hb concentrations suggests that in populations with higher baseline folate status, such as those with mandatory fortification, the inclusion of folic acid in weekly iron supplements would not provide any benefit on anemia outcomes as measured by Hb. However, it should be noted that the duration of our trial was only 16 wk; conversely, intermittent IFA programs are recommended to start at menarche and continue through to menopause (2).

We also found that there were no significant differences in ferritin or sTfR concentrations, nor iron deficiency prevalence, between treatment groups at 16 wk. This is not surprising, because all groups received the same 60 mg/wk dosage of elemental iron. Further, macrocytosis was not detected in any women in our study (all women had a mean corpuscular volume < 98 fL). However, it was interesting that in the model for sTfR, we detected an interaction between baseline sTfR and treatment for the 0.4 mg/wk group ($P < 0.001$) but not for the 2.8 mg/wk group ($P = 0.23$) for sTfR concentration at 16 wk. We cannot explain this interaction on the basis of biological mechanisms. However, a lower dosage of folic acid (0.4 compared with 2.8 mg/wk) in weekly IFA supplements may be an important factor that modifies the response of sTfR concentrations to weekly IFA treatment.

Because we observed no significant differences between treatment groups in any of the examined outcomes, we combined the sample to explore temporal changes in outcome biomarkers and deficiency prevalence rates, adjusting for treatment group. Given that all women received iron, we predicted a temporal increase in ferritin concentrations over the 16-wk period. Indeed, we observed that women had a 20.7-μg/L (95% CI: 15.4, 26.1 μg/L) increase in mean ferritin concentrations over the 16 wk. This treatment effect is much higher than reported in a 2019 Cochrane review assessing the effect of intermittent iron supplementation when compared with no supplementation or placebo (7.46 μg/L; 95% CI: 5.02, 9.90 μg/L) (23). For Hb, the treatment effect was less substantial than ferritin and was less than expected. We observed that women had a 2.6-g/L (95% CI: 1.0, 4.3 g/L) increase in mean Hb concentrations over the 16 wk [as compared with 5.19 g/L (95% CI: 3.07, 7.32 g/L) in the 2019 Cochrane review] (23).

Interestingly, baseline ferritin concentrations were relatively low in our study population (median: 26.9 μg/L) and we observed a substantially large temporal increase in ferritin concentration over the 16 wk. However, this did not translate

TABLE 4 Temporal changes in Hb, ferritin, and sTfR concentrations over 16 wk in all enrolled Malaysian women (aged 18–45 y)[1]

	n	Baseline	Week 16	Mean difference
Hb, g/L	322	129 (127, 130)	131 (130, 132)	2.6 (1.0, 4.3)
Ferritin,[2] μg/L	323	36.8 (33.1, 40.6)	57.6 (53.8, 61.4)	20.7 (15.4, 26.1)
sTfR,[2] mg/L	323	7.0 (6.6, 7.3)	5.8 (5.4, 6.2)	−1.2 (−1.7, −0.7)

[1]Values are marginal means (95% CIs). Generalized linear models with adjustments for treatment group were used to estimate the marginal mean difference (95% CI) for each of the outcome variables. Hb, hemoglobin; STfR, soluble transferrin receptor.
[2]Adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia method (16, 17).

TABLE 5 Temporal changes in anemia, iron deficiency, and iron deficiency anemia prevalence over 16 wk in enrolled Malaysian women (aged 18–45 y)[1]

	Baseline	Week 16	RR[2]	*P* value
Anemia prevalence (Hb < 120 g/L)	58 (17.5)	37 (11.5)	0.65 (0.45, 0.96)	0.03
Iron deficiency (ferritin[3] < 15 μg/L)	100 (30.2)	29 (9.0)	0.30 (0.20, 0.44)	<0.001
Iron deficiency (sTfR[3] > 8.3 mg/L)	59 (17.8)	28 (8.7)	0.48 (0.32, 0.74)	0.001
Iron deficiency anemia (Hb < 120 g/L and ferritin[3] < 15 μg/L)	41 (12.4)	13 (4.1)	0.33 (0.18, 0.60)	<0.001

[1]Values are raw *n* (%) unless otherwise indicated. Hb, hemoglobin; RR, risk ratio; sTfR, soluble transferrin receptor.
[2]Generalized linear models with a binomial distribution and a log link, with adjustments for treatment group, were used to estimate the RR (95% CI) for each of the outcome variables. RRs are relative to the baseline prevalence of the outcome variable.
[3]Adjusted for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia method (16, 17).

to a comparatively large increase in Hb concentration, suggesting that even in the absence of a marked change in Hb concentration, there is still the potential to ameliorate iron deficiency.

The strengths of this trial include the randomized, double-blind design, as well as the high retention (~98%, $n = 324$ of 331) and adherence (~94%, $n = 312$ of 331) rates at 16 wk. Some limitations include a low baseline prevalence of anemia (~18%) that fell below both the Malaysian Ministry of Health's estimated prevalence of anemia among Malaysian women of reproductive age (~30%) and the current WHO population-level recommended prevalence of anemia for women of reproductive age that warrants weekly IFA supplementation (20%) (2, 24). We acknowledge that a study period of 16 wk may not be long enough in duration to observe changes in Hb concentration; however, the duration of weekly IFA supplementation in our study (16 wk) was longer than the 12-wk period as recommended in the WHO guideline. We also acknowledge the possibility that we were underpowered to detect significant differences in Hb between groups at 16 wk because this was an exploratory analysis; however, the narrow CIs of our estimates and the very small effect size observed (only an ~1-g/L difference in Hb concentrations between groups at 16 wk) suggest that this is unlikely. Because the primary aim of this trial was to examine the effects of once-weekly folic acid supplementation on RBC folate concentrations, all women received the standard 60 mg Fe/wk (we did not include an iron control group). We acknowledge the limitation that we did not adjust our outcomes for multiple comparisons, as this was a secondary data analysis. However, we note that all of our outcome measures across groups were nonsignificant, both with and without adjustment for multiple comparisons. Finally, we did not measure dietary intakes to be able to assess the major dietary sources of iron, folate, and vitamin B-12 in this population and we did not measure potential additional causes of anemia in this population such as hemoglobinopathies and infections. However, it should be noted that there is a low risk of malaria in urban and coastal areas of peninsular Malaysia (25).

Although we did not find evidence to suggest that the inclusion of folic acid in weekly IFA supplements is beneficial for anemia reduction, the benefits of 2.8 mg folic acid/wk for neural tube defect prevention warrant its retention in these supplements (11). The WHO guidelines for "Intermittent iron and folic acid supplementation in menstruating women" should be updated to reflect these findings.

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Data Availability

Data described in the article, code book, and analytic code will be made available upon request pending approval.

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Appendix 3

Samson KLI, Loh SP, Lee SS, **Sulistyoningrum DC**, Khor GL, Shariff ZBM, Ismai IZ, Yelland LN, Leemaqz S, Makrides M, Hutcheon JA, Roche ML, Karakochuk CD, Green TJ. Weekly iron-folic acid supplements containing 2.8 mg folic acid are associated with a lower risk of neural tube defects than the current practice of 0.4 mg: a randomised controlled trial in Malaysia. BMJ Glob Health. 2020 Dec;5(12):e003897. doi: 10.1136/bmjgh-2020-003897. PMID: 33272946; PMCID: PMC7716666.

My contribution to the paper primarily with overseeing the management of the clinical trial from the beginning to the end. In addition to my contribution as stated in Appendix 2, I also trained and supervised the primary author of this paper in analysing the plasma and red blood cell folate concentrations. I conducted the validation for the microbiological assay in particular to choose the right dilution and validation of internal and external standards. I was also in the field for a total of one month; two weeks at the beginning of the clinical trial and towards the end of the clinical trial to provide technical supports.

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Weekly iron–folic acid supplements containing 2.8 mg folic acid are associated with a lower risk of neural tube defects than the current practice of 0.4 mg: a randomised controlled trial in Malaysia

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ABSTRACT

Introduction Weekly iron–folic acid (IFA) supplements are recommended for all menstruating women in countries where anaemia prevalence is >20%. Anaemia caused by folate deficiency is low worldwide, and the need to include folic acid is in question. Including folic acid might reduce the risk of a neural tube defect (NTD) should a woman become pregnant. Most weekly supplements contain 0.4 mg folic acid; however, WHO recommends 2.8 mg because it is seven times the daily dose effective in reducing NTDs. There is a reluctance to switch to supplements containing 2.8 mg of folic acid because of a lack of evidence that this dose would prevent NTDs. Our aim was to investigate the effect of two doses of folic acid, compared with placebo, on red blood cell (RBC) folate, a biomarker of NTD risk.

Methods We conducted a three-arm double-blind efficacy trial in Malaysia. Non-pregnant women (n=331) were randomised to receive 60 mg iron and either 0, 0.4, or 2.8 mg folic acid once weekly for 16 weeks.

Results At 16 weeks, women receiving 0.4 mg and 2.8 mg folic acid per week had a higher mean RBC folate than those receiving 0 mg (mean difference (95% CI) 84 (54 to 113) and 355 (316 to 394) nmol/L, respectively). Women receiving 2.8 mg folic acid had a 271 (234 to 309) nmol/L greater mean RBC folate than those receiving 0.4 mg. Moreover, women in the 2.8 mg group were seven times (RR 7.3, 95% CI 3.9 to 13.7; p<0.0001) more likely to achieve an RBC folate >748 nmol/L, a concentration associated with a low risk of NTD, compared with the 0.4 mg group.

Conclusion Weekly IFA supplements containing 2.8 mg folic acid increases RBC folate more than those containing 0.4 mg. Increased availability and access to the 2.8 mg formulation is needed.

Trail registration number This trial is registered with the Australian New Zealand Clinical Trial Registry (ACTRN12619000818134).

Key questions

What is already known?

- Women should take folic acid supplements prior to and during early pregnancy to reduce the risk of neural tube defects (NTDs). However, many pregnancies are unplanned and women may not take supplements.
- In countries where anaemia prevalence is >20%, WHO recommends all menstruating women 15–49 years of age take a weekly supplement containing 60 mg iron and 2.8 mg folic acid to prevent anaemia and reduce the risk of NTDs, but most supplements currently available in countries implementing programmes contain only 0.4 mg of folic acid.
- Evidence is needed to determine whether 2.8 mg would be more effective than 0.4 mg folic acid weekly to reduce NTDs.

What are the new findings?

- In this efficacy trial, we showed that weekly iron–folic acid (IFA) supplements containing 2.8 mg folic acid increased red blood cell (RBC) folate, a biomarker of NTD risk, four times as much as 0.4 mg compared with 0 mg.
- Sixty-eight per cent of women receiving 2.8 mg folic acid per week achieved a RBC folate concentration >748 nmol/L, a level associated with a low NTD risk, compared with 8% in the 0.4 mg group.

What do the new findings imply?

- Weekly IFA supplements containing the WHO recommended 2.8 mg dose of folic acid should be made more widely available.

INTRODUCTION

WHO recommends blanket weekly iron–folic acid (IFA) supplementation for all non-pregnant adolescent girls and women (15–49

years) of reproductive age to prevent anaemia in areas where the prevalence of anaemia is higher than 20%.[1] Globally, WHO estimates that 50% of anaemia is caused by iron deficiency.[2] However, the burden of anaemia caused by folate deficiency is very low, and the need to include folic acid in the weekly supplement is in question. Folic acid taken before and during early pregnancy can reduce the incidence of neural tube defects (NTD),[3–5] birth defects such as spina bifida and anencephaly. As standard practice, WHO recommends that all women, from the moment they begin trying to conceive until 12 weeks of gestation, take a daily 0.4 mg folic acid supplement.[6] Unfortunately, many pregnancies are unplanned, especially among adolescent girls, and the neural tube closes early in pregnancy (~28 days) before most women know they are pregnant. At least 10 million unplanned pregnancies occur each year among adolescent girls aged 15–19 years in low-income and middle-income countries.[7] Therefore, a potential benefit of retaining folic acid in weekly IFA supplements is that if a woman were to have an unplanned pregnancy, it might reduce her risk of an NTD-affected pregnancy.

To help prevent NTDs, WHO recommends weekly IFA supplementation with 2.8 mg folic acid.[1] This dose of folic acid was chosen because it is seven times the daily 0.4 mg dose found to be effective in reducing NTDs in controlled trials.[5] Most weekly supplements currently available contain 60 mg iron and 0.4 mg folic acid, as this formulation is the standard for anaemia prevention and is readily available.[8] Moreover, 0.4 mg folic acid with 60 mg of iron is what is recommended daily during pregnancy,[6] thus, the 2.8 mg requires a different formulation. Of the 4.2 million IFA packs distributed by UNICEF in 2019, only 284 000 contained the WHO recommended folic acid dose of 2.8 mg (Personal communication, Andreas Tjornehoj, UNICEF Supply Division, Copenhagen, 2020). There is a reluctance to switch to IFA supplements containing 2.8 mg of folic acid because of a lack of evidence this dose, or any weekly dose, would prevent NTDs. New folic acid trials with NTD as an outcome are unlikely. Fortunately, cohort studies have found that red blood cell (RBC) folate in early pregnancy is inversely associated with subsequent NTD risk. RBC folate is now a well-accepted biomarker of NTD risk at the population level.[9 10] While the optimal RBC folate for NTD prevention is not known with certainty, WHO has recommended a concentration >748 nmol/L for women of reproductive age as desirable at the population level.[11] However, the relationship between RBC folate and NTD risk is continuous, and any increase in RBC folate would be expected to decrease NTD risk. Although the relationship is continuous, there appears to be little additional benefit above this threshold, as the reduction in risk approaches an asymptote at concentrations of ~1058–1216 nmol/L (calibrator adjusted).[10]

Our primary aim was to determine the effect of 16 weeks of weekly iron (60 mg) with 0 mg, 0.4 mg or 2.8 mg of folic acid on RBC folate concentrations in women of reproductive age. We also examined the percentage of women by treatment group who achieved an RBC folate concentration >748 nmol/L, the concentration associated with a low risk of NTDs. As IFA supplementation is commonly initiated during adolescence in school settings, we secondarily assessed the effect of a 4-week washout period on RBC folate concentrations in an attempt to mimic the effect of school holiday periods.

METHODS

Study design

Full details of the study design are in the published trial protocol.[12] The study was a parallel design, double-blind placebo-controlled randomised efficacy trial. It was conducted at Universiti Putra Malaysia in Selangor, Malaysia. Malaysia was chosen because the prevalence of anaemia is >20% among women of reproductive age, vitamin supplement use is low, and there is no folic acid fortification.[13–15] To be eligible, women had to be: between 18 and 45 years; non-pregnant (self-reported); not planning on becoming pregnant; not taking folic acid containing supplements; not participating in another nutritional intervention; and not taking any medications known to inhibit folate status (methotrexate, certain anticonvulsants or sulfasalazine). The primary outcome was RBC folate at 16 weeks postrandomisation. Secondary outcomes included plasma folate at 16 weeks and RBC folate and plasma folate at 20 weeks following a 4-week washout period in which women did not take supplements. Plasma and RBC folate were measured using a microbiological method described below.

All participants gave written informed consent.

Patient and public involvement

The development of the research question and outcome measures were not informed by the participants' priorities, experience and preferences. Participants were not involved in the design of this study.

Procedures

Women were recruited through advertisements, word-of-mouth, and classroom presentations at Universiti Putra Malaysia. Women expressing an interest were given a participant information sheet and pre-screened for eligibility. If women were eligible and willing to participate, they were asked to attend a morning clinic at the university health centre following an overnight fast. After reconfirming eligibility and obtaining written consent, a blood sample was collected by venepuncture into two evacuated tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. After sociodemographic, health and anthropometric data were recorded, women were randomised. Women were asked to take one tablet at the clinic and then one tablet every week on the same day for 16 weeks; they were also asked not to take any nutritional supplements during the study other than the investigational products provided. Participants were reminded weekly by text message to take

their supplement, to encourage adherence, and asked if they had experienced any adverse events. After 16 weeks, women returned to the clinic following an overnight fast and another blood sample was collected. Participants were instructed not to consume the supplement in the 48 hours preceding the blood draw. Adherence was assessed by counting the number of remaining tablets in the bottles at 16 weeks. Participants discontinued their supplements and returned to the clinic 4 weeks later for a final fasting blood draw.

Supplements, randomisation and masking

Supplements were manufactured by Unison Nutraceutical Sdn Bhd (Ayer Keroh, Malacca, Malaysia) and approved by the National Pharmaceutical Regulatory Agency in Malaysia. The company had no other role in the trial. Supplements were manufactured as tablets containing 60 mg of iron as ferrous fumarate and either 0, 0.4 or 2.8 mg folic acid as the active ingredients. Inactive ingredients included microcrystalline cellulose, polyvinylpyrrolidone, colloidal silicon dioxide, magnesium stearate and pregelatinised starch. The supplements were sent blinded to an external lab for independent folic acid testing (Factors Group of Nutritional Supplements, BC, Canada); the 0, 0.4 and 2.8 mg tablets had a measured value of 0, 0.38 and 2.72 mg, respectively. The supplements and the opaque glass bottles they were packed in were identical in appearance except for a coloured sticker to identify treatment group.

The randomisation schedule was prepared by an independent statistician using ralloc.ado version 3.7.6 in Stata V.15.1 (Stata Corp). Randomly permuted blocks of size six were used to assign participants to one of six colour codes; two colour codes were used per treatment to assist with blinding. Participants, outcome assessors and data analysts were blinded to treatment group. Participants were randomised using a secure web application (Research Electronic Data Capture) by trained research assistants.[16 17]

Laboratory analysis

After each blood collection, one EDTA tube was sent to Clinipath Malaysia Sdn. Bhd. (Selangor, Malaysia) for a full blood count determination using an automated haematology analyser (Sysmex XP-100, Sysmex). An aliquot of whole blood from the remaining tube was removed, diluted to 1 in 11 in 1% ascorbic acid, and incubated for 30 min at 38°C. The remaining blood was centrifuged at 3000 rpm for 10 min at 4°C. The resulting plasma was removed and aliquoted. All samples were stored at −80°C until analysis.

Blood samples were shipped on dry ice to Adelaide, Australia for folate analysis. Whole blood and plasma folate concentrations were determined using the microbiological method based on the method of O'Broin and Kelleher, using standardised kits from the US Centres for Disease Control and Prevention (US CDC; Atlanta, GA).[18–20] This method uses 96 well microplates, 5-methyl tetrahydrofolate (Merck) as the calibrator, and chloramphenicol resistant Lactobacillus rhamnosus (ATCC 27773TM) as the test organism. High and low quality controls (QC) for each of whole blood folate and plasma folate, provided by the US CDC, were run in quadruplets on every plate. RBC folate was calculated by subtracting plasma folate from whole blood folate and correcting for haematocrit.

As per US CDC instructions[21]: if all QC results were within mean (2 SD) limits, the assay was accepted; if more than one of the QC results were outside of the mean (2 SD) limits or any of the QC results were outside of the mean (3 SD) limits, then the assay was rejected. Results from assay runs that passed QC were recorded only when the quadruplets were below 15%. If the coefficient of variation (CV) of the quadruplets was above 15%, the largest outlier was removed and the results recorded as long as the CV of the remaining triplicates was below 10%; otherwise, the sample measurement was repeated.

At the population level, WHO recommends RBC folate concentrations be >906 nmol/L in women of reproductive age to prevent NTDs. This RBC folate value was generated using folic acid as the calibrator.[9 19] We used a newer method recommended by the US CDC that uses 5-methyl tetrahydrofolate as the calibrator. Since 5-methyl tetrahydrofolate gives lower RBC folate concentrations than folic acid, we used a cut-off of >748 nmol/L to define the optimal RBC folate concentration for NTD risk reduction.[22–24]

Statistical analyses

A sample size of 63 participants per treatment group was required to detect a clinically meaningful difference of 100 nmol/L in mean RBC folate concentrations across groups at the end of the intervention period (16 weeks), while adjusting for baseline RBC folate concentration, with 80% power and two-sided α of 0.0167 for pairwise comparisons between the three treatment groups (overall α=0.05). The sample size assumes an SD of 202 nmol/L,[25] a correlation between RBC folate concentrations at baseline and 16 weeks of 0.6, and a drop-out rate of up to 10%. We aimed to recruit 100 participants per group to allow for some uncertainty in the assumed values.

A descriptive comparison of the randomised groups was conducted on all baseline demographic characteristics and baseline measures of the outcomes. The primary analysis was performed on the available data according to treatment allocation at randomisation (intention-to-treat analysis). A secondary 'per-protocol' analysis was also performed, including only women who completed the study and were >80% adherent to the treatment regime. Continuous outcomes were analysed using linear regression models, and binary outcomes were analysed using log-binomial regression models, or log Poisson regression models with robust variance estimation if convergence issues occurred. Adjustment was also made for baseline body mass index (BMI) category in an unplanned sensitivity analysis after observing a chance

imbalance in BMI between the treatment groups. Treatment group, time point (16 or 20 weeks), and a treatment group by time point interaction were included as predictors and analyses were adjusted for the baseline measure of the outcome. The generalised estimating equation method with an exchangeable working correlation structure was used to account for the correlation between outcomes at 16 and 20 weeks. Global interaction tests were performed, and the difference in means or the relative risk (with two-sided 95% CI and p value) comparing each pair of treatment groups was estimated for each time point separately. Statistical significance was set at $p<0.05$ for interaction tests and $p<0.0167$ for pairwise comparisons between treatment groups. Analyses followed a prespecified statistical analysis plan and were completed using Stata SE V.15.1 (StataCorp).

RESULTS

Recruitment commenced 20 August 2019, and finished 12 September 2019, with 429 women screened for eligibility. Of these, 94 declined to participate mainly due to a fear of giving blood (n=24) or their parents did not want them to participate (n=21). Four women were excluded before randomisation because a blood sample could not be obtained. The first study visit was conducted on 3 September 2019, and the final study visit occurred 13 February 2020. Of the 331 women randomised, 110, 110 and 111 were allocated to the 0, 0.4 and 2.8 mg folic acid groups, respectively. Overall, 98% (n=324) of participants returned for the 16-week visit and 94% (n=311) returned for the 20-week visit (figure 1). The study ended when the last participant who remained in the trial completed her final visit on 13 February 2020.

Overall, the median (IQR) age of the participants was 18 (18, 18) years, with ~95% of the 331 participants between the ages of 18 and 21 years. Eighty-nine per cent of participants were Malay (n=295), >99% had never been married, and 77% were in a Foundation year at Universiti Putra Malaysia. More than half of the participants had a healthy BMI (table 1). The overall mean±SD baseline plasma folate concentration was 10.7±5.1 nmol/L, and the overall mean±SD baseline RBC folate concentration was 482±148 nmol/L.

Overall, 94% (n=312/331) participants were adherent, consuming >80% of the required tablets throughout the 16 weeks of intervention. In the 2.8 mg folic acid group, 95% (n=106/111) were adherent, while 93% (n=102/110) and 95% (n=104/110) were adherent in the 0.4 mg and 0 mg groups, respectively. Thirty-five women (11%) reported experiencing at least one side effect (n=10 in the 0 mg group, n=10 in the 0.4 mg group and n=15 in the 2.8 mg group). Common side effects included: nausea (n=9), diarrhoea (n=10), vomiting (n=4), gas (n=8) and constipation (n=7).

After 16 weeks of supplementation, the mean RBC folate was significantly higher in the groups receiving

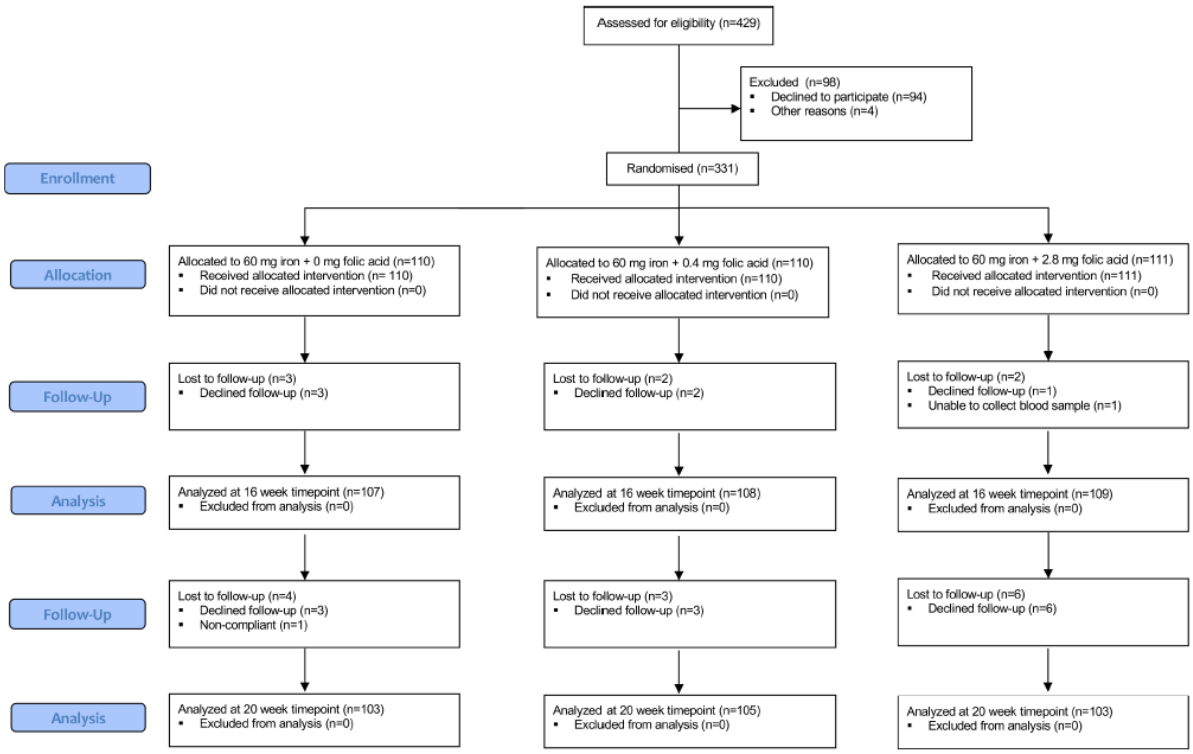

Figure 1 Flow diagram of trial enrolment.

Table 1 Baseline characteristics of enrolled Malaysian women by treatment group*

	0 mg folic acid (n=110)	0.4 mg folic acid (n=110)	2.8 mg folic acid (n=111)
Age, years	18 (18,18)	18 (18,18)	18 (18,19)
Ethnicity			
Malay	96 (87%)	99 (90%)	100 (90%)
Chinese	9 (8%)	7 (6%)	8 (7%)
Indian	1 (1%)	2 (2%)	2 (2%)
Other	4 (4%)	2 (2%)	1 (1%)
Never married	110 (100%)	107 (97%)	110 (99%)
People living in household	5 (4,6)	5 (4,6)	5 (5,6)
Current level of schooling			
Foundation year	84 (76%)	87 (79%)	83 (75%)
Bachelor's degree	22 (20%)	19 (17%)	22 (20%)
Completed Bachelor's degree or higher	4 (3.6%)	4 (4%)	6 (5%)
Monthly household income, US$			
Less than US$250	5 (5%)	3 (3%)	10 (9%)
US$250–US$450	18 (16%)	20 (18%)	12 (11%)
US$450–US$900	22 (20%)	18 (16%)	14 (13%)
US$900–US$1400	21 (19%)	21 (19%)	17 (15%)
US$1400 and above	33 (30%)	30 (27%)	52 (47%)
Declined	11 (10%)	18 (16%)	6 (5%)
BMI category (kg/m^2)			
Underweight, <18.5	19 (17%)	25 (23%)	16 (14%)
Normal weight, 18.5–24.9	64 (58%)	61 (56%)	74 (67%)
Overweight, 25–29.9	13 (12%)	15 (14%)	11 (10%)
Obese, ≥30	14 (13%)	9 (8%)	10 (9%)
Baseline Hb, g/L	129±12	128±12	128±11
Anaemia prevalence, Hb <120 g/L	18 (16%)	20 (18%)	20 (18%)

*Values are mean±SD, median (IQR), or n (%).
BMI, body mass index; Hb, haemoglobin.

2.8 mg and 0.4 mg folic acid than 0 mg (mean difference (MD) 355 (95% CI 316 to 394) and 84 (95% CI 54 to 113) nmol/L, respectively (p<0.0001)) (table 2). RBC folate was 271 (95% CI 234 to 309) nmol/L higher on average in the group receiving 2.8 mg than those receiving 0.4 mg (p<0.0001). Following washout, mean RBC folate remained significantly higher in the groups receiving 2.8 mg and 0.4 mg folic acid per week than 0 mg (MD 293 (95% CI 259 to 327) and 82 (95% CI 52 to 113) nmol/L, respectively (p<0.0001)). After 16 weeks of supplementation, mean plasma folate was significantly higher in the group receiving 2.8 mg folic acid than the 0.4 mg and 0 mg groups (MD 14.9 (95% CI 12.0 to 17.8) and 19.6 95% CI (16.9 to 22.4) nmol/L, respectively (p<0.0001)). Following washout, mean plasma folate remained significantly higher in the group receiving 2.8 mg folic acid than the groups receiving 0.4 mg and 0 mg (MD 8.0 (95% CI 5.7 to 10.3) and 9.5 (95% CI 6.8 to 12.3) nmol/L, respectively (p<0.0001)). The per-protocol analysis produced similar findings (online supplemental table 1), as did an unplanned sensitivity analysis adjusting for baseline BMI category due to a chance imbalance between the treatment groups (data not shown).

At 16 weeks, the 2.8 mg group was more likely to have RBC folate concentrations >748 nmol/L than the 0.4 mg (relative risk (RR) 7.3, 95% CI 3.9 to 13.7) and the 0 mg folic acid group (RR 16.0, 95% CI 6.1 to 42.3), while there was no evidence of a difference between the 0.4 and 0 mg groups. Following the washout period, the 2.8 mg group was still more likely to have RBC folate >748 nmol/L than the 0.4 mg (RR 4.2, 95% CI 2.4 to 7.3) and 0 mg group (RR 14.6, 95% CI 5.2 to 41.1). The difference between the 0.4 and 0 mg groups at 20 weeks did not reach statistical significance after adjustment for multiple comparisons (table 3).

DISCUSSION

Here, we provide the first evidence that the recommended weekly dose of 2.8 mg folic acid, rather than the

Table 2 RBC and plasma folate concentrations by treatment group at 16 and 20 weeks

Outcome	0 mg (n=110)*	0.4 mg (n=110)*	2.8 mg (n=111)*	2.8 vs 0 mg		0.4 vs 0 mg		2.8 vs 0.4 mg	
				Mean difference (95% CI)†	P value‡	Mean difference (95% CI)†	P value‡	Mean difference (95% CI)†	P value‡
RBC folate, nmol/L§									
Baseline	466±136	474±136	507±168						
16 weeks	466±158	554±146	851±208	355 (316 to 394)	<0.0001	84 (54 to 113)	<0.0001	271 (234 to 309)	<0.0001
20 weeks	443±134	534±155	766±183	293 (259 to 327)	<0.0001	82 (52 to 113)	<0.0001	211 (173 to 249)	<0.0001
Plasma folate, nmol/L§									
Baseline	10.1±4.0	10.3±4.3	11.6±6.6						
16 weeks	10.7±4.8	15.6±7.2	31.5±14.5	19.6 (16.9 to 22.4)	<0.0001	4.8 (3.5 to 6.0)	<0.0001	14.9 (12.0 to 17.8)	<0.0001
20 weeks	13.5±10.3	15.2±6.2	24.2±11.0	9.5 (6.8 to 12.3)	<0.0001	1.5 (−0.5 to 3.6)	0.15	8.0 (5.7 to 10.3)	<0.0001

*Values are mean±SD based on the raw data of the intention-to-treat population.
†Mean differences are adjusted for the baseline value of the outcome.
‡Statistical significance set at p<0.0167 for pairwise comparisons of treatment groups.
§P<0.0001 for treatment group by time point interaction test.
RBC, red blood cell.

commonly used 0.4 mg weekly dose, is more effective at increasing RBC folate. Therefore, we would expect the 2.8 mg dose to be more protective against NTDs should a woman or adolescent girl become pregnant. After 16 weeks of treatment, 68% of women in the 2.8 mg group attained an RBC folate >748 nmol/L, compared with only 8% in the 0.4 mg weekly group. Moreover, at 16 weeks, women receiving 0.4 mg folic acid were not more likely to achieve RBC folate >748 nmol/L than women receiving 0 mg—further highlighting the minimal impact of providing 0.4 mg once weekly.

With respect to the reduction in NTD, Crider *et al* estimated a risk reduction from 35.9 (95% CI 28.1 to 46.2) NTDs per 10 000 births to 14.6 (12.4 to 17.0) NTDs per 10 000 births following a 300 nmol/L increase in RBC folate among US women with RBC folate concentrations in the fifth percentile.[10] Given that women in our trial receiving 2.8 mg folic acid per week had a similar increase in RBC folate concentrations, a large risk reduction, such as the one demonstrated above, would be predicted—especially among women with low baseline status. Overall, weekly IFA supplements that contain 60 mg of elemental iron and 2.8 mg of folic acid could not only prevent anaemia but also reduce the risk of NTD if a woman were to become pregnant. The 2.8 mg dose of folic acid would be particularly benefit adolescent girls beause of their high rate of unplanned pregnancies.[7]

While no studies have compared iron with different weekly folic acid doses on RBC folate, weekly dosing has been compared with daily supplementation in at least two trials. Unsurprisingly, daily dosing is more effective than weekly at increasing blood folate indices. Among New Zealand women (18–40 years), daily supplementation with 0.4 mg folic acid was more effective than 2.8 mg weekly at increasing RBC folate after 12 weeks (MD (95% CI) 411 (325 to 504) and 265 (192 to 345) nmol/L, respectively) compared with the 0 mg group.[25] Nevertheless, the authors concluded that 2.8 mg folic acid weekly would still be expected to decrease NTD risk. Moreover, in the New Zealand study, over half of the women taking the weekly folic acid supplement achieved an RBC folate concentration associated with a low risk of NTD at week 12 compared with nearly three-quarters in the daily 0.4 mg group. Compared with their respective placebo groups, the MD in RBC folate among women receiving 2.8 mg folic acid weekly in the New Zealand study was not as great as the difference reported in our study (MD 265 vs 355 nmol/L). The greater difference in mean RBC folate observed in our study may be due to the longer duration of our study compared with the New Zealand study (16 vs 12 weeks), as baseline RBC folate concentrations were similar after calibrator adjustment.

Hao *et al* compared the effect of 4.0 mg folic acid weekly vs 0.4 mg folic acid daily on RBC folate concentrations in Chinese women (24–42 years).[26] After 3 and 6 months of supplementation, RBC folate concentrations increased by ~171 and ~278 nmol/L, respectively, in the 4.0 mg weekly group compared with ~310 and ~430 nmol/L in

Table 3 RBC folate concentrations >748 nmol/L by treatment group at baseline, 16 and 20 weeks

Treatment	N	Baseline*	16 weeks*	Relative risk (95% CI)†	P value‡	20 weeks*	Relative risk (95% CI)†	P value‡
2.8 mg folic acid§	111	9 (8%)	74 (68%)	16.0 (6.1 to 42.3)	<0.0001	55 (53%)	14.6 (5.2 to 41.1)	<0.0001
0.4 mg folic acid	110	4 (4%)	9 (8%)	2.2 (0.7 to 6.8)	0.17	12 (11%)	3.5 (1.1 to 10.7)	0.03
0 mg folic acid	110	3 (3%)	4 (3%)	Reference		3 (3%)	Reference	

*Values are n (%) of women with RBC folate concentrations greater than 748 nmol/L based on the raw data of the intention-to-treat population.
†Relative risks are relative to the 0 mg folic acid group and are adjusted for baseline RBC folate levels.
‡Statistical significance set at p<0.0167 for pairwise comparisons of treatment groups.
§RBC folate relative risk for 2.8 mg vs 0.4 mg was 7.3 (95% CI 3.9 to 13.7; p<0.0001) at 16 weeks and 4.2 (95% CI 2.4 to 7.3; p<0.0001) at 20 weeks. P=0.31 for treatment group by time point interaction test.
RBC, red blood cell.

the group receiving 0.4 mg folic acid daily. The increase seen when supplementing with 4.0 mg folic acid weekly was half that in our study at a similar time point (12 and 16 weeks), despite using a higher dose. The women in the study by Hao *et al* had a high prevalence (>30%) of the TT variant of the methylenetetrahydrofolate reductase (MTHFR) 677C→T genotype, an enzyme polymorphism that is associated with altered folate metabolism. The increase in those with the TT variant receiving the weekly supplement was only half that of those with the CC wildtype at 3 months (~123 vs 220 nmol/L, respectively).[27] The global prevalence of this variant is estimated at less than 10%.[28 29] In our recent study in a similar group in Malaysia (n=75), only one woman carried the TT variant.[30] Thus, this Chinese population may not be representative of the wider global population.

Plasma folate concentrations at week 16 by treatment group generally paralleled RBC folate. Plasma folate is affected by recent dietary intake, especially folic acid supplement use, and is less stable than RBC folate, which better reflects tissue folate concentrations.[24] Moreover, WHO recommends that plasma folate not be used as a biomarker for NTD prevention, and no sufficiency threshold for plasma folate is given.[11]

Strengths of this trial include the randomised placebo controlled design and high retention (98% at 16 weeks) and adherence rates (94% at 16 weeks). Further, there is no fortification with folic acid in Malaysia and the prevalence of anaemia among women of reproductive age is greater than 20%,[13] suggesting that WHO recommended intermittent IFA programming is warranted in this population for non-pregnant women. We used a well-accepted biomarker of NTD risk, RBC folate, which is inversely associated with NTD risk in diverse populations, including Irish and Chinese women.[9 10] Moreover, the finding that folic acid fortification, which typically provides women with less than 0.4 mg/daily, has increased RBC folate and lowered NTD risk in countries where it has been implemented further supports the use of this biomarker.[31–33] The work was carried out in Malaysia because it had anaemia rates high enough to justify a weekly IFA programme and the necessary infrastructure and expertise to carry out a high-quality efficacy trial. However, we are confident that our results are generalisable to other countries where weekly IFA supplements are used and will reduce the burden of these devastating birth defects.

Limitations include that no trial has shown that weekly folic acid, at any dose, lowers NTDs. Evidence supporting weekly folic acid comes from México, where a 50% reduction in NTDs was found following a public health campaign that recommended women take 5 mg folic acid weekly.[34] Moreover, the 16-week duration of our intervention likely underestimates the full potential of the intervention effect on RBC folate concentrations. It has been previously shown that women consuming 0.4 mg folic acid daily for 40 weeks had still not yet reached a steady-state of RBC folate concentrations. Houghton *et al* estimated that it would likely require 96 weeks for women to achieve 90% of the estimated steady-state when supplementing with 0.4 mg folic acid per day.[35] While our intervention was short, intermittent IFA supplementation is recommended to commence after menarche and continue through to menopause,[1] meaning that adolescent girls and women participating in IFA programmes could be consuming the supplements for long periods and will reach a steady-state. Also, our secondary analyses examining the dichotomized outcome presented for RBC folate >748 does not have the same degree of statistical precision as our pre-specified primary outcome on which the trial's sample size was based, RBC folate as a continuous variable (which had excellent statistical precision (table 2)).

Finally, we did not measure MTHFR genotype nor account for differences in efficacy based on BMI. The MTHFR TT variant is associated with lower plasma and RBC folate concentrations[27]; however, the prevalence of the TT variant is thought to be relatively low in the Malay population. A higher BMI has been shown to alter distributions of plasma and cellular folate, leading to lower plasma concentrations and higher RBC folate concentrations.[36–38] WHO guideline states that weekly IFA supplementation is to be targeted to all menstruating adolescent girls and women in populations where the prevalence of anaemia in non-pregnant women of reproductive age is 20% or higher.[1] In practice, there is unlikely to be different doses of IFA supplements for populations depending on their BMI or MTHFR genotype.

Daily supplementation with folic acid remains the best practice to prevent NTDs in women planning a pregnancy. Where women are not planning to become pregnant, or do not take a daily supplement, our findings show that weekly IFA supplements containing the WHO recommended dose of 2.8 mg folic acid are more effective at improving RBC folate concentrations and reducing the risk of NTDs than the dose currently used in practice (0.4 mg folic acid per week). In order to achieve success in a real world setting, weekly IFA programmes must be designed using evidence-based best practices that encourage maximal coverage and adherence.[39 40] We recommend that countries currently using weekly IFA supplements containing 0.4 mg folic acid be enabled and supported to switch to 2.8 mg.

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Contributors MLR, CDK and TJG conceived the trial and proposed the initial trial design. KLIS, SPL, GLK, ZBMS, IZI, MM, MLR, CDK and TJG revised and finalised the protocol; LNY, SL and JAH advised on sample size calculations, trial design and analysis; statistical analyses were completed by LNY; KLIS, SPL, SSL, DCS and IZI were responsible for organisation and conduct of the study; KLIS, SSL and DCS collected data. KLIS, DCS and TJG conducted the blood folate analyses; KLIS, CDK and TJG drafted the manuscript, all authors reviewed the manuscript and approved the final submission.

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Competing interests MM reports being on the Scientific Board of Trajan Nutrition, outside the submitted work. LNY reports grants from Australian National Health and Medical Research Council, during the conduct of the study. MLR is an employee of the sponsor.

Patient consent for publication Not required.

Ethics approval Ethical approval was received from the Ethics Committee for Research Involving Human Subjects of Universiti Putra Malaysia (JKEUPM-2018–255) and The University of British Columbia Clinical Research Ethics Board (H18-00768).

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Data availability statement Data are available on request. Deidentified data will be made available 12 months after publication. Person(s) requesting the data must provide a methodologically sound research proposal which will reviewed by the trial steering committee. If approved, a signed data access agreement will be required before the data is released. Please contact the corresponding author for access to the data.

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Appendix 4

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My contribution to the paper was to provide advice on the technical aspect of running a clinical trial from data management, randomisation, blinding, masking, and collecting biological specimen.

BMJ Open

Effect of once weekly folic acid supplementation on erythrocyte folate concentrations in women to determine potential to prevent neural tube defects: a randomised controlled dose-finding trial in Malaysia

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ABSTRACT

Introduction Folic acid (0.4 mg) taken prior to and during early pregnancy reduces the risk of neural tube defects (NTDs). Because these birth defects occur early in pregnancy, before women may know they are pregnant, many countries have mandated the addition of folic acid to food staples. In countries where fortification is not possible, and weekly iron folic acid programmes exist to reduce anaemia, the WHO recommends that 2.8 mg (7×0.4 mg) folic acid be given instead of the current weekly practice of 0.4 mg. Currently, there is a lack of evidence to support if the 2.8 mg folic acid per week dose is sufficient to raise erythrocyte folate concentrations to a level associated with a reduced risk of a NTD-affected pregnancy. We aim to conduct a three-arm randomised controlled trial to determine the effect of weekly folic acid with iron on erythrocyte folate, a biomarker of NTD risk.

Methods and analysis We will recruit non-pregnant women (n=300; 18–45 years) from Selangor, Malaysia. Women will be randomised to receive either 2.8, 0.4 or 0.0 (placebo) mg folic acid with 60 mg iron weekly for 16 weeks, followed by a 4-week washout period. The primary outcome will be erythrocyte folate concentration at 16 weeks and the mean concentration will be compared between randomised treatment groups (intention-to-treat) using a linear regression model adjusting for the baseline measure.

Ethics and dissemination Ethical approval was obtained from the University of British Columbia (H18-00768) and Universiti Putra Malaysia (JKEUPM-2018-255). The results of this trial will be presented at scientific conferences and published in peer-reviewed journals.

Trial registration numbers ACTRN12619000818134 and NMRR-19-119-45736.

Strengths and limitations of this study

- This is the first study to determine the optimal dose of folic acid to be included in weekly iron folic acid supplements to reduce neural tube defect (NTD) risk.
- Women of reproductive age will be randomised to a weekly iron (60 mg) supplement with either 0.0 mg, 0.4 mg (current practice) or 2.8 mg (WHO recommendation) folic acid.
- The primary outcome is erythrocyte folate, a biomarker of NTD risk, concentration after 16 weeks by treatment group.
- Erythrocyte folate is inversely associated with NTD risk.
- NTD as an outcome would not be ethical or feasible.

INTRODUCTION

In 2015, an estimated 260 000 infants were born globally with a neural tube defect (NTD).[1] NTDs, such as anencephaly and spina bifida, are caused by the failure of the neural tube to close properly around 28 days postconception and are a significant cause of mortality and morbidity. Evidence from controlled trials has shown that up to 80% of NTDs could be prevented if women were to take folic acid supplements prior to and during early pregnancy.[2–4] As such, the WHO recommends that women planning to become pregnant take 0.4 mg folic acid per day.[5] Because many pregnancies are unplanned and the neural tube closes early in pregnancy,[6 7] before women may know they are pregnant, many countries have mandated the addition of folic acid to wheat flour or other grain staples.[8] This has led to a reduction of NTDs in these countries.[9 10] In many countries where wheat flour is not the staple, or where milling of staple grains is at the

village or household level, fortification is difficult.[11] Alternative strategies are needed to deliver folic acid to women of reproductive age.

In countries where the prevalence of anaemia is ≥20%, the WHO recommends blanket intermittent supplementation of all women of reproductive age with weekly iron (60 mg) and folic acid (2.8 mg). The dose of folic was chosen as it is seven times the 0.4 mg daily dose found to be effective in reducing NTDs in controlled trials.[4] However, there is a reluctance to change the dose of folic acid to 2.8 mg weekly for non-pregnant women due to the lack of evidence that this higher dose will reduce NTDs. Further, the global prevalence of anaemia due to folate deficiency is low and there have been calls to remove folic acid from these supplements altogether.[12]

Further randomised trials with NTDs as an outcome would be unethical. However, erythrocyte folate in women of reproductive age is accepted as a proxy indicator of NTD risk. In a case control study from Dublin, Ireland, erythrocyte folate measured early in pregnancy was inversely associated with NTD risk, with an erythrocyte folate >906 nmol/L considered desirable for maximal prevention.[13] There are no studies which compare the current effect of weekly 0.4 mg folic acid, the dose currently contained in most weekly supplements on the market, with the recommended weekly 2.8 mg folic acid on erythrocyte folate concentration in non-pregnant women of reproductive age.

The primary aim of this randomised placebo-controlled trial is to compare the effect of the WHO recommended weekly folic acid dose of 2.8 mg versus the current weekly 0.4 mg folic acid practice or no folic acid (placebo) on erythrocyte folate concentrations after 16 weeks of treatment. Weekly iron folic acid programmes are often started when women are in secondary school.[14] Women in school have several periods of school break during the year. Thus, we will include a 4-week washout period to determine the effect that a school break would have on erythrocyte folate concentrations. This research is needed by policy makers across the globe and will inform WHO guidelines on the optimal weekly dose of folic acid, if any, needed for NTD prevention.

METHODS AND ANALYSIS

Trial design

This is a three-arm, parallel-group, randomised controlled trial with a 16-week intervention period followed by a 4-week washout period. Participants will attend three clinic visits: baseline (0 weeks), endline (16 weeks) and washout (20 weeks). This trial started in September 2019 and is projected to run until February 2020.

Location

This study will take place at Universiti Putra Malaysia in Selangor, Malaysia. Malaysia was chosen for the study location as there is significant technical infrastructure in place and the overall prevalence of anaemia in Malaysia in women of reproductive age is above 20%,[15 16] falling within the criteria of the current WHO guideline for intermittent iron folic acid supplementation.[14] Moreover, there is no folic acid fortification programme in Malaysia and folic acid supplement use is not widespread.[17–19]

Study population

A total of 300 women (18–45 years) from Selangor, Malaysia will be recruited.

Eligibility criteria

Potential participants will be approached by research staff at Universiti Putra Malaysia.

Inclusion criteria

Women must meet the following criteria to be enrolled in the study:

- Be between the ages of 18 and 45 years.
- Not be pregnant.
- Plan on living, working or studying near Universiti Putra Malaysia for 4 months following enrolment.
- Be able to give informed consent.

Exclusion criteria

Women must not have any of the following criteria to be enrolled in this study:

- Are pregnant (self-reported) or planning to become pregnant.
- Taking folic acid containing supplements.
- Participating in another nutritional intervention.
- Taking any medication known to inhibit folate status (methotrexate, certain anticonvulsants or sulphasalazine).

Study treatments

The supplements will be packaged in bottles containing 30 tablets. Each supplement will contain 60 mg of elemental iron as ferrous fumarate and either 0.0 mg, 0.4 mg or 2.8 mg of folic acid to be taken weekly. Supplements have been produced to US Pharmacopeia standards. Supplements have been manufactured by Unison Nutraceutical Sdn. Bhd. in Ayer Keroh, Malacca, Malaysia and approved by the National Pharmaceutical Regulatory Agency in Malaysia. Supplements will be dispensed in glass opaque bottles containing 30 tablets. The tablets, bottles and labels on the bottle will be identical except for a coloured sticker which identifies the different treatments.

Monitoring adherence to study treatment

Study staff will be responsible for storage and dispensing of the supplements. Supplements will be administered by study staff at the baseline clinic visit . Participants will be asked to take their first supplement at the clinic and then asked to take the supplement at the same time each week. Participants will be reminded weekly by short message service (SMS) to take their supplement to encourage adherence and asked to reply by SMS when they have taken the supplement. If they do not reply, research assistants will contact them by phone. If the participant fails

Table 1 Assessment time points

	Assessment time points			
Visit (V)	Screening	V1	V2	V3
Time per study site session				
Week		Week 0	Week 16	Week 20
Enrolment and randomisation				
Eligibility assessment	X			
Randomisation		X		
Implementation				
Questionnaire		X		
Blood collection		X	X	X
Adverse event reporting			X	X
Supplementation				
Tablet distribution		X		
Tablet count			X	

to take the supplement within the 5 days following their designated day, the supplement will not be taken, and the participant will be considered to have missed treatment that week. Adherence will be assessed by trained research staff by counting remaining tablets in the bottles at 16 weeks.

Outcome measures

The primary outcome will be erythrocyte folate concentration at 16 weeks.

Key secondary outcomes:

- Erythrocyte folate concentration at 20 weeks.
- Plasma folate concentration at 16 and 20 weeks.

Participant timeline

Screening period

Women who are interested in participating in the study will be given a participant's information sheet and research assistants will answer any questions they may have. Written informed consent will be obtained from each woman who meets the screening criteria and is willing to participate in the study. The assessment time points are summarised in table 1.

Visit 1 (baseline)

Participants will be asked to attend a clinic at Universiti Putra Malaysia following an overnight fast (since midnight). After reaffirming consent, blood samples will be collected by venipuncture into two evacuated tubes containing EDTA as an anticoagulant. Sociodemographic, health and anthropometric data will be recorded by the researchers. Women with severe anaemia (defined as a haemoglobin concentration <80 g/L) will be contacted within 3 days and referred to a local health centre for follow-up, but will not be excluded from the study unless their medical practitioner recommends withdrawal.[20]

Visit 2 (~16 weeks)

Participants will attend a second clinic visit following an overnight fast. The second visit will be scheduled with a +2-week allowance to accommodate participant schedules. Participants will be advised to take their last pill no less than 48 hours before the blood draw and up to 1 week beforehand. The blood sample will be collected as described in the baseline visit. Adverse events will be recorded, and adherence determined via counting the remaining tablets.

Visit 3 (~20 weeks)

Following 4 weeks (+2 weeks) of not taking any supplements, women will return for a fasting blood sample. Adverse events will be recorded.

Sample size

To detect a clinically meaningful difference of 100 nmol/L in mean erythrocyte folate concentrations across treatment groups at the end of the intervention period (16 weeks), a sample size of 63 participants per group is required, assuming a SD of 202 nmol/L with 80% power while adjusting for baseline erythrocyte folate concentration.[21] A two-sided α of 0.0167 will be used for pairwise comparisons between the three treatment groups (0.0 mg, 0.4 mg and 2.8 mg) for an overall α of 0.05. The sample size assumes a correlation between erythrocyte folate concentrations at baseline and 16 weeks of 0.6 and allows for a dropout rate of 10%. We will recruit 100 participants per arm to allow for some uncertainty in the assumed values.

Recruitment

Potential participants will be approached by trained research staff and informed about the purpose of the study, the protocol and potential risks and benefits of participation. Trained research staff will gather informed

consent, voluntary and free from coercion, to ensure that information about the trial is understood.

Randomisation

Following confirmation of eligibility and enrolment, participants will be randomised after the first blood draw using a web-based randomisation service. Participants will be randomly assigned to receive 60 mg of iron as ferrous fumarate and either 0.0, 0.4 or 2.8 mg of folic acid weekly. A computer-generated randomisation schedule will be prepared using ralloc.ado in Stata by an independent statistician who is not involved with trial participants or data analysis. The randomisation procedure will use randomly permuted blocks of size six to assign participants to one of six colour codes in the ratio 1:1:1:1:1:1. Two colour codes will be assigned to each treatment by an independent individual who will be responsible for labelling the study products but have no further involvement in the trial.

Blinding

Blinding will only be broken during the trial in the event of an emergency, when the investigator deems that a participant cannot be adequately treated without knowledge of the participant's treatment arm. The principal investigator will be contacted. In order to break the blinding for the affected participant, the investigator must contact the randomisation personnel. All attempts to avoid study withdrawal will be made, although participants can cease treatment as appropriate. Trial arms will only be unblinded once all data have been collected and entered in the study database and analysis of the primary and secondary outcomes has been completed.

Data collection, access and storage

Up to seven research staff, as needed, will be on site to obtain informed consent, collect data, randomise and distribute tablets. All efforts to ensure data quality will be taken. Study data will be collected and managed using REDCap electronic data capture tools hosted at the South Australian Health and Medical Research Institute (SAHMRI).[22 23] Eleven investigators and research staff will have access to the data during the data collection period. All co-investigators will have access to the data at all stages of analyses and interpretation of data. Responsibilities concerning privacy and confidentiality will be reminded and discussed with all co-investigators and data entry staff.

Electronic data files will be stored on encrypted and password protected computers using secure servers. Any hard copies of data, consent forms, questionnaires or other papers containing data will be stored in locked filing cabinets in locked research rooms at Universiti Putra Malaysia in Selangor, Malaysia.

Blood collection and processing

A fasting venous blood sample will be collected at weeks 0, 16 and 20 of the trial into evacuated tubes containing EDTA.

Table 2 A summary of study blood analytes and methods

Analyte	Methods
Plasma folate	Microtiter technique with chloramphenicol-resistant *Lactobacillus casei* as the test micro-organism[25]
Erythrocyte folate	Calculated from whole blood folate by subtracting plasma folate and correcting for haematocrit
Plasma vitamin B_{12}	Elecsys 2010 (Roche Diagnostics, Switzerland) automated electrochemiluminescence immunoassay
Plasma ferritin, sTfR, AGP, CRP and RBP	Single sandwich-enzyme linked immunosorbent assay (s-ELISA)[26]

AGP, α–1 acid glycoprotein; CRP, C-reactive protein; RBP, retinol binding protein; sTfR, soluble transferrin receptor.

Whole blood for haematocrit determination

One EDTA tube will be sent to Clinipath Malaysia Sdn. Bhd. (Selangor, Malaysia) for a full blood count determination using an automated haematology analyser.

Whole blood for plasma and erythrocyte folate, buffy coat and erythrocytes

The other tube will be inverted gently 8–10 times to ensure the blood is properly mixed. For erythrocyte folate, 100 µL of whole blood and 1000 µL of 1% ascorbic acid (~1 in 11), will be added to three separate tubes and then vortexed for 5 seconds. Samples will then be incubated at 38 for 30 min and subsequently placed on ice for storage at –80°C. Samples will be centrifuged at 3000 rpm for 10 min at 4°C. Plasma will be aliquoted into three labelled microtubes and stored at –80°C. Buffy coat will be aliquoted into a single labelled microtube and stored at –80°C.

Blood analyses

Deidentified blood samples will be shipped on dry ice to SAHMRI, Adelaide, Australia where plasma folate (nmol/L) and erythrocyte folate (nmol/L) concentrations will be determined using the folate microbiological assay harmonised by the Centers for Disease Control and Prevention.[24] Plasma ferritin (µg/L), soluble transferrin receptor (mg/L), α–1 acid glycoprotein (g/L), C-reactive protein (mg/L) and retinol binding protein (µmol/L) will be analysed at the VitMin lab in Germany (table 2).

Statistical analysis

The primary analysis will be performed on an 'intention-to-treat' basis, according to treatment allocation at randomisation. A secondary 'per-protocol' analysis will also be performed including only women who complete the study and are >80% adherent to the treatment regime. Continuous outcomes, including the primary outcome erythrocyte folate concentration at 16 weeks, will be analysed using linear regression models and binary outcomes will be analysed using log binomial regression models.

Independent variables will include randomised treatment group, time point (16 or 20 weeks) and a treatment group by time point interaction. Treatment effects (0.4 mg vs 0.0 mg, 2.8 mg vs 0.0 mg and 2.8 mg vs 0.4 mg) will be estimated for each time point separately (16 and 20 weeks) along with 95% CIs and two-sided p values. Clustering due to repeated measurements on the same individuals at different time points will be considered using generalised estimating equations. Adjustment will be made for the baseline measure of the analysed outcome as this is expected to be strongly related to the outcome; no adjustment for other baseline variables or subgroup analyses are planned. Missing data will be addressed using multiple imputation to create 100 complete datasets for analysis, with a sensitivity analysis performed on the available data. All analyses will follow a prespecified statistical analysis plan.

Dissemination

This trial is registered with the Australian New Zealand Clinical Trials Registry and the Malaysian National Medical Register.

Confidentiality

Participant confidentiality will be maintained throughout the trial. Confidentiality will extend to the biological testing of samples and additional medical information. The protocol and all study documents will be held in strict confidence. No information about the study or its data will be released to unauthorised third parties. Any medical information of the participants will not be released without the permission of the participant.

Use of data and publication policy

Publication of information regarding this protocol or its data in formats including, but not limited to, conference abstracts, posters or presentation, seminars, journal articles, public reports and internet postings. Approval of these activities must have the permission of all co-investigators before the event. The results of this trial will be published in peer-reviewed scientific journals and presented at conferences. Additionally, all results will be relayed to the relevant stakeholders, including the Ministry of Health and the WHO.

Patient and public involvement

The development of the research question and outcome measures were not informed by the participants' priorities, experience and preferences. Participants were not involved in the design of this study. Participants will be provided with a summary of the trial findings and their personal results. Results will also be disseminated to policy makers and the Ministry of Health through briefing papers containing summaries of the main findings.

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Contributors SPL, GLK, MLR, ZMS, MM, LMD-R, TG and CDK conceived the trial and proposed the trial design; LY, SL and JAH advised on sample size calculations, trial design and analysis; DCS advised on analytical methodology; KLIS, JJY, TG and CDK drafted the protocol, all authors reviewed the protocol and approved the final submission.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The Ethics Committee for Research Involving Human Subjects of Universiti Putra Malaysia (JKEUPM-2018-255) and The University of British Columbia Clinical Research Ethics Board (H18-00768) approved the research.

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Appendix 5

Probst Y, **Sulistyoningrum DC**, Netting MJ, Gould JF, Wood S, Makrides M, Best KP, Green TJ. Estimated Choline Intakes and Dietary Sources of Choline in Pregnant Australian Women. Nutrients. 2022; 14(18):3819. https://doi.org/10.3390/nu14183819

As this paper is stemmed from the RCT in Chapter 4, I have contributed in conducting the RCT and the methodological aspect of collecting food frequency questionnaire for dietary intake.

nutrients

MDPI

Brief Report

Estimated Choline Intakes and Dietary Sources of Choline in Pregnant Australian Women

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Abstract: (1) Background: Despite the postulated importance of choline during pregnancy, little is known about the choline intake of Australians during pregnancy. In this study, we estimated dietary intakes of choline in early and late pregnancy, compared those intakes to recommendations, and investigated food sources of choline in a group of pregnant women in Australia. (2) Methods: 103 pregnant women enrolled in a randomized controlled trial. In early pregnancy (12–16 weeks gestation) and late pregnancy (36 weeks gestation), women completed a food frequency questionnaire designed to assess dietary intake over the previous month. (3) Results: Choline intakes and sources were similar in early and late pregnancy. Median choline intake in early pregnancy was 362 mg/day. Of the women, 39% and 25% had choline intakes above the Australian National Health and Medical Research Council (NHMRC) adequate intake (AI) of >440 mg/day and the European Food Safety Authority (EFSA) AI of >480 mg/day for choline in pregnancy, respectively. Eggs, red meat, nuts, legumes, and dairy accounted for 50% of choline intake, with eggs being the most significant contributor at 17%. (4) Conclusions: Few pregnant women in our study met the AI recommended by the NHMRC and EFSA. In Australia, choline intake in pregnancy may need to be improved, but further work to define choline requirements in pregnancy is required.

Keywords: choline; intakes; diet sources; pregnancy; Australia

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1. Introduction

Choline is an essential nutrient required for synthesizing the neurotransmitter acetylcholine and the methyl group donor betaine; it is a component of phospholipids [1]. Choline can be synthesized from phosphatidylcholine in the body, but endogenous synthesis may be insufficient to meet requirements, especially during periods of rapid growth [2]. During pregnancy, choline requirements increase as the fetus requires large amounts of choline for brain development [3]. A lack of choline during pregnancy has been associated with poorer cognitive outcomes in cohorts [4]. However, high-quality randomized controlled trial data are lacking.

In 2005, the Australian National Health and Medical Research Council (NHMRC) adapted the 1997 U.S. Institute of Medicine adequate intakes (AI) for choline [5,6]. For pregnancy, this was set at 10 mg/day lower than the US at 440 mg/day. Based on more recent evidence, the European Food Safety Organization (EFSA) set the AI at 480 mg/day for pregnancy in 2016 [7]. Most surveys during pregnancy suggest that intakes are well

below the AI [8]. The only Australian data available were collected during the National Nutrition and Physical Activity Survey (NNPAS) 2011–2012, and indicates that less than 1% of pregnant women had choline intakes exceeding the NHMRC AI [9,10]. However, this survey was conducted over ten years ago, so its findings were not compared against the newer EFSA recommendations for choline, nor did it consider food sources of choline during pregnancy [7].

As part of a randomized controlled trial (RCT) of folic acid supplementation, we obtained estimates of choline intakes using a food-frequency questionnaire at study entry (12–16 weeks) and ~36 weeks' gestation [11]. Here we report the dietary choline intakes and related nutrients in early and late pregnancy, compare choline intakes to recommendations, and explore food sources of choline in a group of Australian pregnant women.

2. Materials and Methods

The data described here were collected as part of an RCT. Details of the study design have been described previously [11]; here, we highlight aspects of the study methods relevant to the choline sub-study.

2.1. Study Participants and Procedures

The term "pregnant women" in this study refers to any person with the potential to be pregnant, including trans men and non-binary people [12]. To be eligible, women had to live in Adelaide, South Australia, be between 12–16 weeks gestation carrying a singleton pregnancy, currently taking a folic acid supplement, and planning to continue taking a folic acid-containing supplement throughout pregnancy. Participants were recruited in person at their first antenatal appointment or through a trial recruitment company (TrialFacts, Melbourne, Australia) utilizing an online digital marketing campaign. Participants were randomized to a prenatal micronutrient supplement with (800 µg) or without (0 µg) folic acid. The supplement did not contain choline. An online self-administered food frequency questionnaire was completed at study entry (early pregnancy; 12–16 weeks) and late pregnancy (34–36 weeks). At study entry, women also reported age, income, and pre-pregnancy body mass index (BMI). All participants provided informed consent, and the study was approved by the Women's and Children's Health Network Research Ethics Committee—HREC/19/WCHN/018 and Flinders Medical Centre—SSA/20/SAC/61.

2.2. Choline Intakes

Choline intakes were determined using the online version of the Dietary Questionnaire for Epidemiological Studies (DQES v3.2) developed by the Cancer Council of Victoria [13]. The DQES v3.2 is an 80-item semiquantitative food-frequency questionnaire (FFQ). It has undergone validation in several populations, including various ethnic backgrounds, including young adults [14], women of childbearing age [15], and people living with diabetes [16]. The DQES was initially developed to assess dietary intake over the previous year but was modified to assess intake over the prior month. In a study of young adults, the DQES showed acceptable validity and reproducibility over one month [14]. As the DQES 3.2 does not include data for choline, we used choline food-composition values recently added to the AUSNUT 2011–2013 database by Probst et al. [9]. In brief, the choline data was created from a systematic literature search of published studies and food composition data. The data were matched to AUSNUT food codes; foods not specified in detail and composite items were informed by the Food Standards Australia New Zealand (FSANZ) recipe files [17]. The DQES includes proprietary information for determining the nutrient values, and choline values were assigned to each of the 80 DQES food items. Choline values previously matched to food items from the AUSNUT 2011–2013 database were considered. All relevant items from AUSNUT were mapped to the DQES food items. For example, the DQES item 'full cream milk' was mapped to 15 'full cream milk' food items in AUSNUT. Food entries not specified were excluded due to the use of mean data to determine these values. Box plots were created to confirm the relevance of all mapped

foods to the food items, with outliers' food items considered individually for relevancy and, if necessary, excluded (11%).

2.3. Statistics

Descriptive statistics are reported as means ± SD, median (IQR), *N* (%), and % (95% CI) as appropriate. Statistical analyses were completed using SPSS 28.0 (I.B.M. Corp., Armonk, NY, USA).

3. Results

3.1. Participants

Pregnant women were recruited and enrolled in the trial between December 2019 and June 2020. Of the 639 women assessed for eligibility, 103 were enrolled and randomized. Of the 103 participants, 93 completed the DQES FFQ at early pregnancy (12–16 weeks), and 84 completed the FFQ at late pregnancy (34–36 weeks). Eighty women completed both FFQs. Mean gestational age ± SD at study entry was 13.2 ± 1.2 weeks. The mean maternal age was 31 years, and more than 85% of the participants were of European ethnicity. More than 87% of participants had completed secondary education, and 60% had an annual household income higher than AUD 105,001 (Table 1). Of the participants, 55% had previously given birth to one or more children. Most participants had a BMI in the healthy range.

Table 1. Characteristics of participants at study entry.

Characteristic	Mean SD or *N* (%) [1]
Age (years)	31.1 ± 4.8
Gestational age at study entry	
12–<14 weeks	64 (62)
≥14–16 weeks	39 (38)
Pre-pregnancy BMI (kg/m^2) [2]	24.1 ± 4.7
Healthy (18.5–24.9)	68 (72)
Overweight (25.0–29.9)	12 (13)
Obese (30.0 and above)	14 (15)
European ethnicity	85 (83)
Completed secondary education	90 (87)
Annual household income (AUD)	
$70,000 or less	18 (17)
$70,001–$105,000	19 (18)
$>105,001	61 (60)
Prefer not to disclose	5 (5)
Parity	
0	47 (46)
1	45 (44)
>1	11 (11)

[1] *N* = 103. [2] Body Mass Index (*N* = 94).

3.2. Choline Intakes

Median choline intakes were similar in early and late pregnancy, 394 and 418 mg/day, respectively (Table 2). In early pregnancy, 39% of participants exceeded the National Health and Medical Research Council's (NHMRC) AI for choline during pregnancy (>440 mg/day), which rose to 51% by late pregnancy. Using The European Food Safety Authority's (EFSA) higher AI (>480 mg/day) for choline, only 25% and 33% of participants exceeded the AI in early and late pregnancy, respectively.

Table 2. Median choline intake and percentage of women consuming less than the National Health and Medical Research Council (NHMRC) and European Food Safety Authority (EFSA) adequate intake (AI) for choline in early and late pregnancy.

	Early Pregnancy (*N* = 93)	Late Pregnancy (*N* = 84)
Choline intake (mg/day)	362	414
Interquartile range	298–484	(303–509)
<NHMRC AI [1] % (95% CI)	61 (51, 70)	49 (39, 59)
<EFSA AI % (95% CI)	75 (65, 84)	67 (56, 77)

[1] 440 mg/day choline.

3.3. Sources of Choline

Food sources of choline were similar for early and late pregnancy. Food or food groups ranked by their contribution to daily choline intakes during pregnancy are shown in Table 3. Eggs were the most significant contributor to choline intake at both time points, providing 72–76 mg/day and approximately 17% of total choline intake. Eggs, red meat, nuts and legumes, dairy, vegetables, and chicken accounted for around 70% of choline intake. The remaining food or food groups account for 5% of choline intake or less.

Table 3. Choline intake by reported DQES food or food group in early and late pregnancy [1].

Food or Food Group	Early Pregnancy (*N* = 93)		Late Pregnancy (*N* = 84)	
	mg/Day	% Intake [2]	mg/Day	% Intake [2]
Eggs	72 ± 55	17 ± 11	76 ± 59	17 ± 10
Red Meat	49 ± 39	12 ± 11	58 ± 42	14 ± 9
Nuts and legumes	46 ± 58	11 ± 13	50 ± 61	11 ± 12
Dairy	40 ± 29	10 ± 7	43 ± 27	11 ± 6
Vegetables	35 ± 22	9 ± 5	30 ± 20	8 ± 5
Chicken	30 ± 19	8 ± 6	29 ± 19	8 ± 5
Fruit	20 ± 13	5 ± 4	19 ± 14	5 ± 4
Pasta	18 ± 11	5 ± 3	19 ± 12	5 ± 3
Fish	16 ± 16	4 ± 4	18 ± 16	4 ± 4
Bread	12 ± 8	3 ± 3	12 ± 7	3 ± 3
Breakfast cereals	12 ± 10	3 ± 3	13 ± 15	3 ± 3
Beverages	10 ± 14	2 ± 3	13 ± 12	3 ± 3
Other savory foods	10 ± 6	3 ± 2	8 ± 5	2 ± 1
Processed meat	7 ± 7	2 ± 2	9 ± 8	2 ± 2

[1] Values are Mean ± SD. [2] Mean daily percentage of choline from food or food group. DQES: Dietary Questionnaire for Epidemiological Studies vs. 3.2.

4. Discussion

Between 50–70% of pregnant participants in our study had choline intakes below NHMRC and EFSA recommendations. Choline intake was similar in early and late pregnancy, averaging 401 mg/day over both periods. More participants exceeded the NHMRC AI in late (51%) than early (39%) pregnancy. Indeed, the lowest intakes published [8] were in pregnant women in the Australian NNPAS 2011–2012 [9], where the mean choline intake was only 251 mg/day, and <1% of women met the NHMRC AI.

There are several reasons why the choline intakes were higher in our study than in NNPAS 2011–2012. We used an FFQ which assessed usual nutrient intakes over the month prior, whereas the method used in NNPAS 2011–2012 relied on 24-h recalls on two different days per individual. Compared to FFQs, 24-h recalls may underestimate intakes of infrequently consumed foods such as eggs, which may be eaten once or twice per week and contain high amounts of choline [18]. Conversely, the FFQ, due to its long lists of foods, may overestimate the consumption of food items because there is a tendency to report a specific food as being eaten more than it has [18]. In the NNPAS 2011–2012, 24-h-recall

energy intakes were under-reported by 22% of subjects (NNPAS underestimation), leading to all nutrients being under-estimated.

Furthermore, a higher pre-pregnancy BMI is also known to increase the under-reporting of energy intake, leading to the under-reporting of other nutrients, including choline, during the later stages of pregnancy [19]. Pre-pregnancy BMIs were not given in the NNPAS; however, the BMIs of non-pregnant participants of childbearing age were higher than pre-pregnancy BMIs in our study. The phenomenon of under-reporting, known to occur with 24-h recalls, may have been counteracted by the overestimation of energy and nutrient intake inherent with FFQs [18], though this requires further confirmation.

Derbyshire et al. have recently published a comprehensive review of 'habitual' choline intakes across childbearing years [8]. Although the choline intakes observed in our study were higher than those reported in others in this review, globally, there is considerable variation in reported intake. In the USA, the NHANES mean intake was ~320 mg/day despite using 24-h recalls [20]. In contrast, studies using an FFQ, in Vancouver (Canada) and Eastern Massachusetts reported mean intakes for pregnant participants of 383 and 344 mg/day, respectively [21,22]. All studies have reported considerable variations in choline intake within a population; in many cases, the standard deviation is larger than the mean. This variation is not surprising given that choline is found in high quantities in certain foods that may or may not be eaten during pregnancy, such as eggs or fish.

In the NNPAS 2011–2012, as in our study, eggs were the highest contributor (9%) to choline intakes for all Australian population groups [9]. However, the contribution of eggs to the choline intake of Australian pregnant women was not detailed. Most other studies of choline intake in women of childbearing age have not reported food sources of choline. One exception is the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort, where the authors reported that dairy was the number one source of choline at 21%, followed by eggs at 12% [23].

A limitation of our study was the small sample size and the use of a cohort from an existing randomized controlled trial. However, our participants were not dissimilar to the broader population of Australian pregnant women. In 2019, the mean age of first-time mothers in Australia was 29.4 years, slightly lower than the mean age of 31.1 years in our study [24]. However, over 50% of our participants had given birth to at least one previous child. Of our participants, 83% described themselves as of European ethnicity; data showing maternal country of birth suggest that this is higher than the national average [24]. Sixty percent of participants had an annual family income of over AUD 105,000, similar to the median average Australian family income (2020) of AUD 120,000 [25]. A second limitation of our study is that choline only has an AI, not an estimated average requirement. When a population exceeds an AI, we can be confident that the population is receiving an adequate amount of the nutrient; however, being below an AI does not mean the population is deficient.

In conclusion, few pregnant women in our study met the AI recommended by the NHMRC and EFSA. Choline intake in Australia may need to be improved; however, more data are required on the clinical consequences of inadequate choline intake during pregnancy.

Author Contributions: K.P.B., T.J.G., D.C.S., and M.M. conceived the original folic acid trial and proposed the trial design; S.W. and T.J.G. designed the prenatal supplement; S.W. coordinated the manufacture of the supplements; K.P.B., T.J.G., and J.F.G. conceived the choline study; Y.P. updated the FFQ for choline data; T.J.G., Y.P., and M.J.N. interpreted the data and drafted the manuscript. All authors provided critical input into the manuscript and approved the final version. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Women and Children's Health Network Human Research Ethics Committee and HREC/19/WCHN/018 and Flinders Medical Centre—S.S.A./20/S.A.C./

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Appendix 6

Cohort Studies Investigating the Associations between Maternal Folate and Allergy Risk in Offspring

Study, year	Population/ Number of Participants, Country	Maternal exposure and assessment	Offspring outcome assessment	Association between exposure and allergic disease outcome/s	(≠), (+), or (-)	Covariates (confounding) adjustment
Alfonso et al., 2018	2543 pregnant women from the University of California Los Angeles Environment and Child Health Outcomes Study, USA 2003	Self-reported FACS use 1st, 2nd, 3rd trimester or never.	Parent reported symptoms of wheeze using ISAAC at 3 y.	Timing of FA supplement initiation was not associated with wheeze. Initiation of FA supplementation in late pregnancy associated with increased risk of wheeze in children born to mothers with history of atopy.	(-)	Maternal race/ethnicity age, education, preconception vitamins, initiation of prenatal care, alcohol, environmental tobacco smoke, pre-pregnancy BMI, marital status, primary source of payment for prenatal care, parity, birth outcome, maternal history of atopy, duration of exclusive breastfeeding, child attendance day care/preschool, infection during pregnancy, and housing characteristics.
Bekkers et al., 2012	3,963 pregnant women from the Prevention and Incidence of Asthma and Mite Allergy study. The Netherlands, 1996-1997	FACS pre-pregnancy and during pregnancy by maternal questionnaire at 30 to 36 weeks GA.	Parental reported asthma and eczema symptoms using ISAAC from 1 to 8 y. Bronchial hyperresponsiveness and sensitisation at 8 y.	No overall (1 – 8 years) association between maternal use of FACS and asthma and eczema symptoms. FACS use associated with higher rate of wheeze at 1 y and with eczema at 7 y. No association with sensitisation (IgE) or bronchial hyperresponsiveness at 8 y.	(≠) (-)	Maternal age, education, allergy, BMI, smoking during pregnancy, vitamin supplements use other than folic acid-only. Child sex, birth weight, gestational age, number of older siblings, breast feeding duration, smoking in the home, type of day care and region.
Best et al., 2021	561 mother-infant pairs from the Western Australia prospective cohort study.	Maternal serum UMFA and folate concentration between 36-40-weeks GA.	Physician-diagnosed eczema, IgE-mediated food allergy, and skin prick test (SPT) at 1 y.	No association between late gestation maternal UMFA or folate concentrations and infant allergic disease outcomes.	(≠)	Maternal age, further maternal education after high school, maternal Caucasian ethnicity, maternal cat/dog ownership, maternal parity, vaginal delivery,

	Australia 2011 to 2016,					infant sex, infant birth weight, and infant gestational age at birth.
denDekker et al., 2018	5653 children and their mothers Generation R Study. The Netherlands, 2002 to 2006.	Maternal FA supplement use before 18 weeks GA (>10 weeks or preconception); maternal folate concentration in early pregnancy; cord blood folate concentration.	Physician-diagnosed asthma and wheezing in the past 12 mos at 10 y.	No association with FA supplement use and current asthma. No significant association between maternal folate concentrations in early pregnancy or in cord blood and current asthma.	**(≠)**	Maternal age, parity, history of asthma, eczema or allergy and educational level. Maternal weight and height at enrollment. Maternal smoking and alcohol use during pregnancy. Child's sex, gestational age at birth and birthweight.
Dunstan et al., 2012	628 pregnant women recruited in last trimester. The Infant Fish Oil Supplementation Study (IFOS). Australia, 2005 to 2008.	Folate intake from diet and supplement use using SQ-FFQ at >26 weeks GA; maternal serum folate in 3rd trimester and cord blood folate.	Physician-diagnosed eczema, food allergy, and allergic sensitisation; IgE-mediated food allergy, eczema, SPT and asthma at 1 y.	No association between cord blood folate or maternal folate intake from foods and infant eczema. FA supplementation (>500µg vs <200 µg/day). associated with increased risk of eczema. Cord blood folate <50nmol/L and >75 nmol/L associated with greater sensitisation risk.	**(-)** **(U)**	Maternal age, maternal allergic disease (and sensitisation), parity, socioeconomic status, education level, daycare attendance, infection history, postnatal dietary intervention, pet keeping, breastfeeding, infant dietary patterns.
Fortes et al., 2019	395 pregnant women delivering at G. B. Grassi Hospital Italy, 2018	FA only or FA and iron supplement use in the 1st & 2nd trimesters via hospital clinical records.	Parent reported AD based on the UK Diagnostic Criteria for atopic dermatitis at 6 y.	No association with FA only supplements and AD. FA and iron supplement use associated with decreased risk of AD.	**(≠)**	Maternal age, education, passive smoking, family history of AD, food supplement use, food antigen avoidance, dietary intake of fruits and vegetables, BMI, maternal psychological distress, breastfeeding, birth weight, infant's sex, day care attendance, the presence of domestic animals, early introduction of weaning foods, indoor and outdoor allergen exposure.
Granell et al., 2008	5364 mother/child pairs from the Avon	Maternal dietary folate intake at 32	Atopy in the child assessed by skin prick test 7-8 y;	Maternal folate intake was not associated with childhood atopy.	**(≠)**	Prenatal and post-natal smoking, maternal education and social class

	Longitudinal Study of Parents and Children (1991 to 1992). United Kingdom, outcome assessed 1998 to 1999.	weeks GA from 3-day FFQ. FA supplement use at 18 and 32 weeks GA.	asthma defined as physician diagnosis and current symptoms at 7 ½ y.	The study did not report any findings related to maternal folate intake with asthma. It was reported that childhood asthma did not differ between child's MTHFR C677T genotypes (CC, CT, and TT; p=0.94).		
Haberg et al., 2009	32 077 pregnant women- from The Norwegian Mother and Child Cohort Study, Norway, 2000-2005.	Maternal intake of FA supplements from 0-30 weeks GA using the sstudy's own questionnaire.	Parent reported wheeze between 6 and 18 mos of age using the study's own questionnaire.	FA supplements in the first trimester associated with increased risk of wheeze between 6 to 18 mos.	**(-)**	Intake of other supplements in pregnancy, infant sex, birthweight, month of birth, maternal atopy, maternal educational level, parity, maternal smoking in pregnancy, type of day care, parental smoking in first 3 months after birth, breast feeding at 6 months, and exposure to vitamin supplements or cod liver oil at 6 months of age.
Kiefte-deJong et al., 2012	8742 mother-child pairs from the Generation R study, The Nertherlands, between 2002-2006	Maternal report of periconceptional or early pregnancy (0-10 weeks) FACS use and maternal plasma folate analysis at 13.5 ± 2.0 weeks GA.	Annual parental report of AD and wheeze symptoms using ISAAC to 48 mos.	No association with maternal FACS use on AD or wheeze to 48 mos. Maternal plasma folate 16.21 to 23.20 nmol/L and >= 23.21 nmol/L associated with increased risk of atopic dermatitis but no association with wheeze.	**(≠)** **(-)**	Maternal age, BMI, maternal educational level & ethnicity, parity, infant's sex, infant's birth weight and gestational age at birth; any maternal smoking & alcohol consumption during pregnancy; duration of breastfeeding; attendance of day care, parental atopic constitution.
Kim et al., 2015	917 mother-child pairs from the Mothers and Children's Environmental Health Study. South Korea 2006-2011.	Maternal serum folate concentrations during mid-pregnancy (12-28 GA weeks) and late pregnancy.	Cord blood IgE at birth and 24 moss; AD and asthma symptoms using ISAAC at 6, 12 and 24 mos.	Maternal serum folate in mid-pregnancy associated with a decreased risk of AD at 24 mos. Asthma data excluded from the analysis due to lack of statistical power resulting from low asthma prevalence.	**(+)**	Infant sex, birth weight, gestational age, duration of breastfeeding, maternal age, maternal history of allergic disease; urinary cotinine levels in mid- and late-pregnancy as a marker of exposure to nicotine, pre-pregnancy BMI.

Liu et al., 2020	9100 mother–child pairs randomly selected from a community intervention trial (n=247 831). China, 1993-1996.	Self-reported folic acid intake during the 1st trimester of pregnancy vs. no folic acid use via questionnaire.	Parent reported allergy and asthma symptoms using the Child Behaviour Checklist at 4-6 y.	Maternal folic acid supplementation vs no supplementation was not associated with risk of allergy symptoms at 4-6 y.	(≠)	Maternal age at childbirth, education, occupation and parity. Because rates of childhood allergy symptoms and asthma differ between northern and southern China, further analyses stratified by region was performed.
Magdelijns, et al. 2011	2834 healthy pregnant women from the KOALA Birth Cohort Study. The Netherlands, 2002	Self-reported FA use (stand-alone and/or multivitamin supplement).	Eczema and wheeze at 3, 7, 12, and 24 mos, 4 to 5 y, and 6 to 7 y using ISAAC; Nurse assessed eczema using the UK Working Party criteria and total & specific IgE levels at 2 y; asthma & lung function at 6 to 7 y.	Overall, no association with maternal folic acid supplement use and risk of wheeze, asthma or AD.	(≠)	Maternal antibiotic use, smoking & alcohol use in pregnancy, mode and place of delivery, birth weight, infant sex, infant antibiotics during the first 6 mos of life, exposure to environmental tobacco smoke and domestic animals, breastfeeding, maternal education level, family history of atopy, siblings, day care attendance, and multivitamin or other supplement use during pregnancy.
Martinussen et al., 2012	1499 women from the Perinatal Risk of Asthma in Infants of Asthmatic Mothers USA, 2003-2007.	Self-reported folic acid intake 1 month prior to conception through to the 3rd month of pregnancy.	Parental Maternal report of physician diagnosis of asthma, wheezing or whistling in the chest in the last 12 mos at 6 y. Questions asked: ""as the child ever been diagnosed by a doctor or health professional as having asthma?"" and ""as your child had wheezing or whistling in the chest	No association between maternal folic acid intake and asthma at 6 y.	(≠)	Maternal, ethnicity, marital status, household income, parity, asthma, smoking during pregnancy, use of other vitamins (C, D, and E), iron use, and calcium use in the first trimester.

			in the last 12 mos?"" A positive answer to both these questions was considered a positive definition of current asthma.			
McGowan et al., 2020	1,394 children from the Boston Birth Cohort who participated in the follow up study, the Children's Health Status. USA, 2018.	Maternal plasma total folate at delivery*; cord blood (CB) plasma UMFA and 5-MTHF * Referring to Wang et al. blood was taken AFTER delivery (48-72 hrs after).	Food-Specific IgE using ImmunoCAP, food sensitisation (FS) defined by food specific IgE and food allergy (FA) in early childhood, 0-5 y.	There was no association between increasing quartiles of maternal total folate at delivery and FS at (on average) 2.4 y. Maternal total folate concentrations at delivery between those whose children later developed FA vs not developed FA at (on average) 2.4 y, were 30.2 vs. 35.3 nmol/L. Maternal total folate concentrations in the third quartile (30.4 – 44.8 nmol/L) vs. in the first (6.64 – 19.7 nmol/L) was associated lower odds of developing FA at (on average) 2.4 y. There was no association seen between increasing quartiles of CB UMFA concentrations and FS in multiple logistic regression models. Increasing UMFA quartiles was associated with FS using sensitivity analyses examining a higher threshold for sIgE (0.7 kU/L) at (on average) 2.4 y. Increasing quartiles of CB UMFA concentrations was associated with an increased odds of developing FA in a dose-response manner (test for trend p value = 0.001). No significant association was seen between cord blood 5-MTHF concentrations and the development of FS or FA.	**(≠)** **(+)** **(+)** **(≠)** **(-)** **(U)** **(≠)**	Information on demographics, in utero, and post-natal exposures, such as race/ethnicity, infant feeding, maternal smoking, prenatal folic acid intake, and maternal history of atopy were collected from the mothers via questionnaire. Information was further collected from the medical record regarding mode of delivery, gestational age, maternal education, pre-pregnancy maternal BMI, and maternal age at delivery.

Miyake et al., 2011	763 healthy mother-child pairs recruited between 5th and 39th of GA Osaka Maternal and Child health Study (OMCHS), Japan, 2002 to 2003	Maternal folate intake during pregnancy using self-administered diet history	Maternal reported cchild'swheeze and asthma based on ISAAC questionnaire in infants aged 16-24 mos.	No association between maternal consumption of folate and risk of wheeze or eczema at 16-24 mos.	(≠)	Maternal age, GA & residential municipality at baseline, family income, parental education, parental history of asthma, atopic eczema, and allergic rhinitis, changes in maternal diet in the previous1 mo, season at data collection, maternal smoking during pregnancy, baby's older siblings, baby's sex, baby's birth weight, household smoking in the same room as the infant, breastfeeding duration, age at which solid foods were introduced, age of the infant, and maternal intake of DHA, n-6 PUFA, vit D, calcium, vit E, and b-carotene
Miyashita et al., 2020	6651 mother-child pairs (healthy women). Hokkaido Study on Environment and Children's Health. Japan, 2003 and 2012.	Maternal serum folate levels in the first-mid and third trimester	Wheezing symptoms of children at 1, 2, 4, and 7 y using a modified section of the ISAAC questionnaire.	Higher maternal folate levels (highest quartiles vs. second highest) increased adjusted ORs of childhood wheezing at 2 y, but not at 1, 4, and 7 y.	(-)	Maternal age, parity, delivery year, alcohol consumption during pregnancy, maternal cotinine level for nicotine marker, parental allergic history, annual household income, and child's sex.
Molloy et al., 2020	1074 mother-infant pairs. Barwon Infant Study (BIS). Australia, 2010 to 2013	Maternal RBC folate at 28-32 weeks GA by chemiluminescent immunoassay. Maternal dietary folate intake by dietary questionnaire for	Infant challenge-proven food allergy, food sensitisation and eczema at 1 y. Food sensitisation using a SPT test to: cow's milk, egg, peanut, cashew and sesame. All	There was no association between RBC folate and challenge-proven food allergy; food sensitisation (either ≥2 or ≥3 mm wheal size); and SPT wheel size when the SPT wheel size treated as continuous measures. There was no association between "high" "RBC folate (>1360 nmol/L) and offspring food allergy; food sensitisation ≥2 mm or ≥3mm wheal size; and eczema.	(≠) (≠) (≠)	Ethnicity, family history of allergy and # of siblings, smoking & alcohol consumption in pregnancy, markers of SES & SEIFA: parental education, household income, FACS, maternal age, pet ownership in pregnancy, infant birthweight and sex.

		epidemiological studies v.2 (DQES). Maternal folate supplementation recorded in trimester 1 and 2 questionnaires.	participants with SPT wheals ≥1 mm than the negative control were offered an in-hospital open food challenge. Eczema using questionnaires (modified from the UK working party criteria) at 1, 3, 6, 9 mos and 1 y & clinical assessments at 1 mo, 6 mos and 1 y.	There was no association between FACS in pregnancy and allergic outcomes in offspring.		
Nwaru et al., 2011	3523 mother-child pairs (3253 participants and 270 non-participants) Finnish Type 1 Diabetes Prediction and Prevention Nutrition Study. Finland, 1996 to 1997.	Maternal dietary folate during the 8th month of pregnancy using FFQ (checked by nutritionist and validated with 10-day food record).	Child's asthma, rhinitis or wheeze at age of 5 years based on the ISAAC questionnaire. Asthma was defined as doctor-diagnosed asthma. Children were followed up at 5 y.	There was no association between maternal dietary folate intake during pregnancy and the risk of asthma, rhinitis or eczema in the offspring. .	**(≠)**	Sex of child, place of birth, season of birth, GA at birth, maternal age at birth, maternal basic education, maternal smoking during pregnancy, mode of delivery, number of siblings, parental asthma, parental allergic rhinitis, pets at home at 1 year of age, and atopic eczema by 6 months of age, maternal intake of vit D and PUFA.
Ogawa et. al, 2018	310 singletons with their mother (healthy) The National Center for Child Health and Development (NCCHD). Japan, 2010 to 2013	Maternal folate rich vegetable using self-administered FFQ both during early pregnancy (until 16 weeks GA) and mid to late pregnancy (from	Maternal reported childhood wheeze at 2 y using the ISAAC questionnaire.	Highest vs lowest maternal intake of folate-rich vegetables was associated with lower prevalence of wheeze in the offspring at 2 y.	**(+)**	Self-reported pre-pregnancy height and weight, parity, parental asthmatic history, weight gain during pregnancy, maternal age, sex of the infant, mode of delivery, GA and birth weight from medical charts, annual household income and maternal education, breastfeeding and height and weight of the offspring.

		26 weeks GA to delivery)				
Parr et al., 2017	39,846 children for asthma based on Norwegian Prescription Database. 28.872 children for asthma and atopy on maternal questionnaire report. Norwegian Mother and Child Cohort (MoBa) Study. Norway, 2002 to 2006.	Maternal total folate intake (DFE) including food folate and FACS using FFQ administered at about 22 weeks GA. Maternal plasma folate concentrations measured approx. 18 weeks GA.	Current asthma in children examined using either at least two pharmacy dispensations of asthma medications or maternal report of the child ever having doctor-verified asthma plus either asthma symptoms or asthma medication use in the past year at 7 y in 2014. Current eczema or allergy to either pollen or animal hair (cat or dog) in the past year by maternal report.	Highest quantile (vs. lowest quintile) of maternal folate intake was associated with increased risk of asthma and asthma and atopy at 7 y.	**(-)**	Potential confounders and other covariates evaluated were based on data from the birth registry (maternal age at delivery, parity, child's sex, and birth weight) or MoBa questionnaires completed around gestational weeks 18 (baseline) and 30, and when the child was aged 6 or 18 mos.
Roy et al., 2018	858 healthy women with their offspring (852 with 2nd trimester and 818 with 3rd trimester plasma folate values). Conditions Affecting Neurocognitive Development in Early Childhood (CANDLE) study. USA, 2006 to 2011.	Plasma folate concentrations at 2nd and 3rd trimesters using folate microbiological assay.	Current wheeze within the past 12 mos captured at 3 y. Atopic dermatitis (AD) or eczema, doctor-diagnosed during 3rd year of life.	No associations between 2nd trimester folate as a continuous exposure with wheeze, AD or eczema. Plasma folate concentrations ≥20 ng/mL in 2nd trimester was associated with decreased odds of current wheeze at 3 y. There was no associations between 3rd trimester folate with wheeze, AD or eczema.	**(+)**	Maternal age at enrollment, self-reported race, education, prenatal smoking, asthma, pre-pregnancy body mass index, 2nd trimester vitamin D levels, parity, delivery route, and child sex and birthweight.

Trivedi et al., 2018	1279 mother-child pairs. Project Viva, an ongoing prospective cohort study. USA, 1999 to 2002.	Folic acid (ingested foods and dietary supplements) intake using SQ-FFQ at the 1st trimester visit (during this pregnancy) and at the 2nd trimester (during the past 3 months of pregnancy).	Physician-diagnosed current asthma at mid-childhood (9-11 y) based on the validated ISAAC questionnaire.	Higher folic acid intake was associated with lower odds of asthma in mid-childhood (9-11 y), in an unadjusted logistic regression model. This association was attenuated after adjusting for covariates.	(≠)	Maternal age, history of asthma, educational attainment, and household income, child's sex and date of birth from hospital records and determined gestational age at delivery, maternal reported child's race/ethnicity and breastfeeding duration. At the mid-childhood in-person visit, child age was recorded and we measured child height using a stadiometer.
Tuokkola et al., 2016	1922 mothers (with 4982 children). Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition study. Finland, 1997 to 2004.	Maternal diet (dietary folate and FACS) during pregnancy (8th month) using SQ-FFQ.	Physician-diagnosed ccow'smilk allergy (CMA) at 3 y.	Folate intake and folic acid supplement use were associated with an increased risk of CMA in the offspring at 3 y.	(-)	Energy intake, child's sex and birth weight, maternal age and education, maternal smoking status during pregnancy, duration of gestation, type of delivery, number of older siblings, season of birth, length of breast-feeding, atopic family history, study centre, urbanity of the living environment, visits to a stable and pet keeping during the 1st year of life were adjusted for in the analysis.
Veeranki et al., 2015	104,428 mother-child dyads. Tennesse Medicaid. USA, 1996 to 2005.	Folic acid containing prescriptions during pregnancy (no prescription filled, 1st trimester only, after 1st trimester, and both during and after 1st trimester) from the pharmacy files.	Early childhood asthma at ages 4.5-6 y as defined using a previously validated algorithm that uses asthma-specific healthcare visits (ICD-9 diagnosis) and asthma-specific medication fills.	Folic acid prescription in the 1st trimester only was associated with increased relative odds of asthma in children between 4.5-6 y. Folic acid prescription in the 1st trimester and beyond increased odds ratio for asthma diagnosis to 1.2 in children between 4.5-6 y.	(-)	Maternal race, age at delivery, education, smoking during pregnancy, marital status, year of pregnancy, history of asthma, region of residence, and adequacy of prenatal care. Child's gender, birth weight, estimated gestational age, and number of siblings.

Whitrow et al., 2009	557 children at completion of pregnancy phase (490 at 3.5 years follow up; 423 at 5.5 years follow up) with their mother. The Generation 1 Cohort Study in South Australia between. Australia, 1998-2000.	Maternal dietary folate in early and late pregnancy using FFQ and FACS questionnaire before pregnancy; and at both early and late pregnancy obtained through an interview by a trained research nurse.	Maternal report on child's physician-diagnosed asthma status at 3.5 y, as well as atopic and non-atopic asthma.	Folic acid taken in supplement form in late pregnancy was associated with an increased risk of childhood asthma at 3.5 y. There was no significant association between FACS in late pregnancy and asthma at 5.5 y.	(-)	Maternal education; maternal smoking; maternal intake of vit E, vit A, vit D, and zinc (assessed as per method for dietary and supplemental folate above, supplement only for vit D); GA; parity; gravida; breastfeeding duration; and maternal asthma.
Ye et al., 2020	456 pregnant women recruited at 12-14 weeks and their offspring. Maternal Key Nutritional Factors and Offspring's Atopic Dermatitis (MNKNFOAD) Study. China, 2016 to 2018.	Periconceptional FACS use in the 3 mos before pregnancy or in the 1st trimester of gestation through self-administered questionnaire. Fasting maternal RBC and serum folate concentrations at enrolment (between 12-14 weeks GA).	Infant atopic dermatitis (AD) that occurred within 6 mos (Primary outcome). Final diagnosis confirmed at 1 y by three experienced paediatric dermatologists through panel discussion (Secondary outcome).	Higher maternal RBC folate levels (per 100 ng/mL) were associated with an increased risk of AD. An RBC folate level ≥620 ng/mL was associated with increased infant AD by 91%. There was no association between fasting maternal serum folate at early gestation or periconceptional folic acid supplement intakes with AD.	(-) (≠)	Maternal age at enrolment, education level, preconception BMI, GA at enrolment, parity, self and family history of allergic disease (ie AD, allergic rhinitis, and asthma), alcohol consumption, smoke exposure, hair colouring, home decoration, and pet keeping for 3 months around conception. Child's birthweight and gender, delivery mode, and GA through electronic medical records.
Yu et al., 2017	1320 infant-mother pairs. China, 2011 to 2012.	Folic acid supplements 1 mo before pregnancy, early-stage pregnancy, middle and later stage of pregnancy	Physician-diagnosed wheezing in infants within 2 y of birth according to Zhu Futang Practice of Pediatrics	For infants whose mothers begin to take folic acid in middle and late stage of pregnancy, the risk of wheezing was 1.95 times greater (95%CI =1.05, 3.62; p=0.03) than that of infants whose mothers did not take folic acid. Among infants, with or without wheezing, whose mothers did not have family-specific constitution, there was	(≠)	No adjustment.

				no significant difference in the pregnancy stage of taking folic acid (p>0.05).		
Zetstra-van der Woude et al., 2014	35 604 children with their mother. The pregnancy database IADB.nl which contains pharmacy-dispensing data of mothers and children from community pharmacies. The Netherlands, 1994 to 2011.	Folic acid (FA) exposure defined as at least one dispension of high-dose FA during pregnancy. Preparation dispensed (FA 5 mg) or the number of daily doses prescribed and the number of days prescribed for. Timepoints: 3 mos before pregnancy, three trimesters, the first 3 mos and the period of 3-6 mos after pregnancy.	Dispense of asthma medication for the child was taken as a proxy for childhood asthma.	Exposure to maternal high-dose FA during pregnancy was associated with the risk of asthma medication (increased up to 26% for the recurrent dispension of inhalation corticosteroid [ICS]). Both high-dose FA only dispensed in the 1st trimester (n = 176) and high-dose FA only dispensed in the 3rd trimester (n = 563) versus no dispension showed an increased but non-significant risk for recurrent ICS. Maternal high-dose FA was associated with increase any asthma medication (1.34), recurrent asthma medication (1.36), any ICS (1.39), and for recurrent ICS (1.36) in only children aged 8 y and older. Timing of FA use during pregnancy have a significant effect on the associations studied.	**(-)**	Age of the mother, single or multiple pregnancy, maternal asthma medication, paternal asthma medication, medication associated with high-dose folic acid supplementation, dispension of iron supplements, antifolate medication, antidepressants, antihypertensives, antidiabetics, and benzodiazepines during pregnancy

BMI: body mass index, DHA: docosahexaenoic acid, DFE: dietary folate equivalents, FACS: folic acid containing supplement, FFQ: food frequency questionnaire, GA: gestational age; ISAAC: International Study of Asthma and Allergies in Childhood, RBC: red blood cell, PUFA: polyunsaturated fatty acids; SES: socio-economic status, SEIFA: Socio-Economic Indexes for Areas,
(≠): no associations
(-): negative association (higher maternal folate was associated with increased allergy risk in offspring)
(+): positive association (higher maternal folate was associated with decreased allergy risk in offspring)
(U): U-shaped association

Appendix 7

Randomised Controlled Trials (RCTs) Investigating Maternal Folate Interventions and Allergy Risk in Offspring

Study, year	Population/Number of participants, Country	Maternal intervention	Offspring outcome assessment	Results	(≠), (+), (-)	Covariates (confounding) adjustment
Dobó & Czeizel, 1998	625 (out of 800; 78%) mother-child pairs. receiving periconceptional multivitamin (MV) with or without 0.8mg folic acid and their children. The Hungarian Optimal Family Planning Programme (HOFPP). Hungary, 1984-1992.	The women entering the study were randomly assigned to receive either an MV (Elevit Pronatal, Hoffman-La Roche) or a placebo-like TE supplement as a single tablet each day for at least 1 mo before the planned conception and at least until the date of the second missed menstrual period. The MV supplement contained 0.8 mg folic acid. The TE supplement did not contain any folic acid.	Physician-diagnosed allergies: Asthma bronchiale, bronchitis obstructive, pseudocroup, food allergies, atopic dermatitis at 2 and 6 y.	The incidence of allergic diseases including atopic dermatitis was not significantly different at 2 (p=0.11) and 6 years of age (p=0.90), though the rate of atopic dermatitis was somewhat higher at 2 y in the MV group.	**(≠)**	
Su et al., 2020	502 infants with their mothers enrolled during gestational age 12-16 weeks. China, 2014 to 2015.	All mothers in the prevention group were treated with folic acid 600 IU/D every day for 3 mos after admission	Infant's bronchial asthma, wheezing, eczema, and rhinitis at 1-3 y. Diagnosis was based on the Respiratory Group, Society of Paediatrics, Chinese Medical Association.	The prevention group had significantly lower rates of wheezing and eczema during the follow-up period. The prevalence of wheezing and eczema was significantly lower in the prevention group compared to control (p=0.024 vs p=0.034, respectively).	**(+)**	No adjustment. No ORs.

Appendix 8

Ingredients of supplements for intervention (no folic acid) and control (800 µg folic acid/d) groups

Ingredients	**No folic acid**	**800 µg folic acid**	**unit**
folic acid	0	0.8	mg
calcium	250	250	mg
Iron	27	27	mg
thiamine	1.4	1.4	mg
riboflavin	1.4	1.4	mg
niacinamide	18	18	mg
vitamin B-6	1.9	1.9	mg
vitamin B-12	2.6	2.6	mcg
pantothenic acid	6	6	mg
biotin	30	30	mg
vitamin C	85	85	mg
vitamin E	13.5	13.5	IU
magnesium	50	50	mg
zinc	7.5	7.5	mg
manganese	2.0	2.0	mg
iodine	0.22	0.22	mg
copper	1	1	mg
selenium	30	30	mcg
Vitamin D3	10	10	mcg
b-carotene	2500	2500	IU

Appendix 9


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