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Feasibility of a randomized clinical trial comparing 5-methyltetrahydrofolate and folic acid prenatal multivitamins in couples with recurrent pregnancy loss

Carolyn Ledowsky[a,*], *Vanessa Scarf*[b], *Kris Rogers*[a], *Amie Steel*[a]

[a] *School of Public Health, University of Technology Sydney, Ultimo, Australia*
[b] *School of Nursing and Midwifery, University of Technology Sydney, Australia*

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ABSTRACT

To assess the feasibility of a randomized controlled trial (RCT) comparing 5-methyltetrahydrofolate (5-MTHF) and folic acid (FA) in couples with recurrent pregnancy loss. Pregnancy loss affects up to 15% of pregnancies, with over half of cases remaining unexplained. Emerging evidence suggests that folate metabolism, particularly in individuals carrying methylenetetrahydrofolate reductase polymorphisms such as C677T and A1298C variants, may influence reproductive outcomes. A double-blind, RCT feasibility trial was conducted in Australia with 22 reproductive dyads randomized to receive either 5-MTHF or FA prenatal multivitamins. Participants adhered to dietary restrictions, abstained from conception for two cycles, and completed regular assessments. Primary outcomes included feasibility, adherence, acceptability, and preliminary efficacy based on biochemical markers and pregnancy outcomes. The trial demonstrated high acceptability (86% in arm A [MTHF-A] and 94% in arm B [FA-B]) and adherence rates for supplement use over 78% in each arm. Unmetabolized FA concentration decreased in the 5-MTHF group but rose significantly in the FA group. A critical finding was the degradation of 5-MTHF in retained samples, highlighting formulation instability as a confounder. A fully online RCT comparing 5-MTHF

Abbreviations: ACTRN12622001577707, Australian New Zealand Clinical Trials Registry ID; ANCOVA, analysis of covariance; CONSORT, Consolidated Standards of Reporting Trials; DHFR, dihydrofolate reductase; DNA, deoxyribonucleic acid; FA, folic acid; FA-B, folic acid group in the study; IVF-ICSI, IVF with Intracytoplasmic Sperm Injection; MRC, Medical Research Council; MTHF, methyltetrahydrofolate; MTHF-A, methyltetrahydrofolate group in the study; MTHFR, methylenetetrahydrofolate reductase; NHMRC, National Health and Medical Research Council; NIH, National Institutes of Health; NSW, New South Wales; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT, randomized controlled trial; RPL, recurrent pregnancy loss; SD, standard deviation; THF, tetrahydrofolate; TS, thymidylate synthase; TTC, trying to conceive; UK, United Kingdom; UMFA, unmetabolized folic acid.

* *Corresponding author at*: School of Public Health, University of Technology Sydney, Level 8, Building 10, 235-253 Jones Street, Ultimo, NSW 2007, Australia.
E-mail address: Carolyn.j.ledowsky@student.uts.edu.au (C. Ledowsky).

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and FA is feasible. Future trials should address formulation stability and expand sample size to evaluate clinical efficacy and personalized folate strategies.

1. Introduction

An estimated 23 million miscarriages occur annually, accounting for approximately 15.3% of all pregnancies [1,2]. Recurrent pregnancy loss (RPL)—defined as two or more consecutive losses prior to 20 to 22 weeks gestation—affects a subset of these women, yet in more than 50% of cases, no identifiable cause can be found [3]. This idiopathic nature leads to significant emotional and financial stress [2], with many couples pursuing assisted reproductive technologies despite low success rates and substantial costs [4].

Folate supplementation is one of the most widely implemented strategies to reduce pregnancy loss [5]. Folic acid (FA), the synthetic and oxidized form of folate, is recommended globally to prevent neural tube defects [6,7] and is mandated for food fortification in several countries, including Australia [8]. However, this strategy has led to widespread exposure to FA, particularly in women of reproductive age who consume a prenatal multivitamin with FA in countries with mandatory fortification [9]. Recent research has raised concerns about the accumulation of unmetabolized FA (UMFA), particularly in populations consuming both fortified foods and supplements [10–12]. Although the long-term clinical implications of UMFA are still under investigation, associations with cancer [13], infertility [14,15], altered immune function [13,14], and metabolic disturbances have been reported [10].

FA is first reduced by the enzyme dihydrofolate reductase (DHFR) to THF and then converted by methylenetetrahydrofolate reductase (MTHFR) [16] to the active form, 5-methyltetrahydrofolate (5-MTHF) [13,17]. Genetic polymorphisms in MTHFR—particularly C677T and A1298C impair this latter step, reducing enzymatic activity and altering folate metabolism [18–20]. These variants are common, with C677T homozygosity present in up to 14% of Caucasians and heterozygosity in up to 25% of the general population [21]. Impaired folate metabolism due to MTHFR polymorphisms has been linked to infertility, miscarriage, and poor reproductive outcomes in both sexes [22–27].

5-MTHF is the biologically active form of folate that bypasses MTHFR and DHFR, directly supporting one-carbon metabolism and methylation, ensuring immediate metabolic usability [28]. Unlike FA, which depends on the slow and variable activity of DHFR and subsequent MTHFR conversion, 5-MTHF demonstrates superior bioavailability and does not lead to UMFA accumulation [17,28–32]. Clinical studies show that 5-MTHF raises plasma and red cell folate more effectively than FA, particularly in individuals with MTHFR C677T and A1298C variants [33]. It has been shown to improve folate biomarkers and reduce homocysteine concentration (which may contribute to vascular dysfunction and pregnancy loss) [34,35]. Despite this, few studies have evaluated its use in RPL populations, and even fewer have considered formulation stability, which may dilute the potency of the folate used as a potential confounder in clinical trials.

Preconception care has traditionally focused on women, despite evidence that male factors may contribute up to 70% of all infertility cases [36,37]. Most RPL studies exclude male participants [38–41] despite growing evidence that folate status and MTHFR polymorphisms also affect male fertility [42,43]. Pilot and feasibility studies are essential precursors to fully powered randomized controlled trials (RCTs), particularly for complex interventions. Their primary purpose is not to establish treatment efficacy but to determine whether a full-scale trial is practical and achievable. These studies address critical methodological questions such as recruitment rates, adherence, acceptability, intervention delivery, and data collection processes, acting as a "dress rehearsal" for the main trial [44,45]. Without this preliminary step, large trials risk failure due to unforeseen logistical or design challenges.

In reproductive health research, and especially in RPL, interventions often involve multiple variables, including genetic factors, nutritional components, and emotional burden. A feasibility study ensures that protocols are robust and ethically sound before substantial resources are committed [46]. Furthermore, pilot studies provide essential data for sample size estimation, confirm the suitability of outcome measures, and identify barriers to participation, which are critical for planning a definitive trial [44,45]. Reporting preliminary efficacy indicators, such as biomarker trends, is acceptable only to inform whether a full trial is warranted, not to claim effectiveness [44].

Given the absence of prior trials comparing 5-MTHF and FA in couples with RPL, this feasibility study represents an important first step toward developing a fully powered RCT.

Key messages: This pilot study aimed to assess the feasibility of conducting a fully powered RCT comparing prenatal multivitamins containing 5-MTHF vs FA in couples experiencing RPL. It also evaluated acceptability, adherence, preliminary efficacy, and the impact on biomarkers such as UMFA, serum folate, red cell folate, and MTHFR polymorphisms, while also identifying formulation stability as a critical factor influencing nutrient delivery and clinical interpretation.

2. Methods and materials

2.1. *Study design and setting*

This study is registered with the Australian New Zealand Clinical Trials Registry (Trial ID: ACTRN12622001577707, registered 20/12/2022). Utilizing an exploratory framework, this was a double-blind, randomized controlled feasibility trial delivered

entirely online. All aspects of the trial—including participant recruitment, screening, consent, communication, and follow-up—were facilitated via a secure online platform managed by the research team at the University of Technology Sydney. A detailed risk assessment and safety protocol were developed to ensure the welfare of all participants.

The study Recruitment commenced in March 2023 and concluded in October 2023. The trial continued until August 2024.

Study supplements were delivered by mail to participants' homes, and data were collected using electronic questionnaires, dietary records, and uploaded pathology results. Virtual support was provided to all participants via a regular scheduled monthly interview with the researchers to ensure adherence to the supplement protocol, respond to participant questions regarding diet and ensure participants were not experiencing distress or adverse events.

2.2. *Ethics and consent*

The study was approved by the University of Technology Sydney Human Research Ethics Committee (ETH22-7218 16/5/22 including amendments ETH-23-8188, ETH-23-8644).

Participants provided consent at multiple stages. Initial consent was obtained via a Qualtrics screening questionnaire for the collection of health-related data to assess eligibility. Those meeting inclusion criteria were invited to consent to further contact and to schedule a follow-up "Confirmation Interview" via Calendly. Final informed consent for participation in the pilot trial was obtained during the confirmation Interview.

2.3. *Recruitment strategy*

Participant recruitment for the study was conducted across multiple channels between March 2023 and October 2023. A comprehensive outreach approach was employed to ensure broad visibility and engagement with the target population across Australia. Recruitment advertisements were developed and shared via social media platforms, including Facebook, Instagram, and Twitter. Posts were strategically scheduled and targeted to reach individuals with lived experience relevant to the study. In addition to direct social media promotion, support was sought from relevant organizations and community groups. These groups either posted the recruitment advertisement on their websites or distributed it via their own social media channels and networks: Pink Elephant Support Network, Miscarriage Australia, HIPPP Study Group, Melbourne Mums Facebook group, Sydney Mums Facebook group, Mumma Tribe Facebook group, TTC Australia (trying to conceive) Facebook group, Ectopic and Miscarriage Support Group Facebook group. This multipronged strategy enabled the study to reach a wide and diverse audience across Australia. As a result, 766 individuals responded to the survey, and 101 met the eligibility criteria for inclusion in the study.

2.4. *Participants*

Eligible participants were heterosexual couples residing in Australia, in which the female partner had experienced two or more confirmed pregnancy losses before 22 weeks' gestation. Females were aged between 20 and 49 years; there were no age restrictions for male partners. Couples were excluded if either partner was currently pregnant or lactating, undergoing assisted reproductive technologies, using medications known to interfere with folate metabolism, or had a diagnosed medical or genetic condition likely to confound study outcomes.

2.5. *Randomization and blinding*

Following confirmation of eligibility and baseline data collection, couples were randomly assigned using computer-generated block randomization in a 1:1 ratio to either the intervention group (5-MTHF) or control group. The randomization list was prepared by the UTS statistician, who was not involved in participant recruitment or data collection. The sequence was uploaded into the REDCap electronic data capture system. Once a participant was enrolled and baseline data entered, REDCap automatically allocated the participant to a study arm according to the preloaded sequence. This process was software-driven and fully automated, ensuring allocation concealment and eliminating manual intervention.

Supplements were prepackaged by the manufacturer in identical capsules and containers, labelled only as "Prenatal Multivitamin A" or "Prenatal Multivitamin B." Both products were indistinguishable in appearance, size, and color. Investigators and participants remained blinded to group assignments throughout the trial. Unblinding occurred only after completion of data collection and primary analysis. This process ensured allocation integrity and minimized performance and ascertainment bias.

2.6. *Intervention*

Each multivitamin contained 2500 mcg of folate as either 5-MTHF or as FA. This dosage was derived from previous study data [47]. Participants were instructed to take three capsules daily during the first trimester, reducing to two capsules in the second trimester, and one capsule in the third trimester. Supplementation began prior to conception (at least two reproductive cycles) and continued throughout pregnancy, depending on conception timing during the study. Participants were also required to follow a dietary protocol avoiding FA-fortified foods (e.g., commercial breads and cereals) for the duration of the study. A food list was provided that included FA fortified and nonfortified foods. Dietary folate intake and compliance with food exclusions were assessed during the regular meetings with the researcher at multiple time points. Participants were required to abstain from conception attempts for the first 8 weeks (or two reproductive cycles) of the trial to allow for sufficient supplementation prior to pregnancy. A protocol change was approved after the study commenced, which allowed the use of niacin, if need be, to mitigate minor side effects of the folate, such as headaches, skin rash, and mild anxiety. The metabolism of niacin reduces methyl groups in the body, minimizing symptoms. The stopping guidelines for the trial included the inability of a participant to follow the dosage recommendations of the trial, which were three capsules trimester one, two capsules trimester two, one capsule trimester one, or a serious adverse event.

3. Outcome measures

3.1. Primary outcome

The primary outcome of this study was feasibility, and as such, outcomes were measured according to the recognized domains of feasibility studies [48,49]. Protocol acceptance was evaluated based on the number of eligible individuals who contacted the research team for study involvement compared with those who completed the study. This evaluation included reasons for study nonparticipant or withdrawal, and experiences of study participants at completion. Demand was measured by reported interest in a new treatment for RPL as reported by individuals interested in study participation. Implementation was measured by participant adherence to data collection requirements, adherence to supplementation intake, and avoidance of FA fortified foods, and adherence to abstinence of TTC for 2 reproductive cycles following the introduction of the multivitamin. Practicalities were a measure of the hours required by researchers, effectiveness of recruitment methods, blinding process, and use of online booking and meeting scheduling systems. Efficacy testing measured the rate of participants that achieve 22 weeks gestation, the difference in concentrations of UMFA over 2 months on the multivitamins, change in blood biomarkers, and MTHFR polymorphism variance amongst the cohort.

4. Data analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Technology Sydney. Analyses were primarily descriptive, given the small sample size ($n = 11$ couples per arm) and the feasibility focus of this trial. Continuous variables are presented as means, medians, and ranges, while categorical variables as counts and percentages.

Parametric tests (*t* tests for continuous variables and chi-square tests for continuous variables) were applied for exploratory purposes only and should not be interpreted as evidence of efficacy. These tests were used to illustrate potential trends rather than to draw statistical inferences. Nonparametric alternatives such as Mann–Whitney *U* and Fisher's exact tests will be considered in future adequately powered trials. All analyses were conducted using R (version 4.5, R Foundation for Statistical Computing, Vienna, Austria).

Following the completion of the trial and unblinding of the product allocations for arms A and B, the research team initiated stability testing of trial products in response to unexpected outcomes. This test was performed by ScienTEST Analytical Services, an independent laboratory specializing in the stability assessment of raw materials and finished products.

5. Results

5.1. Feasibility domain results overview

Findings are presented as descriptive summaries to illustrate feasibility outcomes and biomarker trends rather than to infer efficacy. Given the small sample size ($n = 11$ couples per arm), all statistical comparisons are exploratory and should be interpreted with caution. Means, medians, and ranges are reported for continuous variables, and proportions for categorical outcomes. Where *P* values are shown, they are provided for completeness but do not indicate statistical significance in this feasibility context.

5.2. Baseline characteristics of female participants

A total of 22 reproductive dyads (22 females and 22 males) were enrolled in the study (see Fig. 1), with 11 allocated to arm A methyl folate arm (MTHF-A) and 11 to arm B FA arm (FA-B). Participant demographics and baseline characteristics are summarized descriptively in Table 1.

5.3. Demographics

The mean age of female participants in MTHF-A was 37 years (standard deviation [SD] = 6), compared to 34 years (SD = 8) in FA-B. The mean age of male participants was similar across groups, with men in MTHF-A having a mean age of 36 years (SD 5) and those in FA-B a mean age of 35 years (SD 11). Other characteristics such as education level, employment status, and physical activity are presented as counts and percentages in Table 1.

5.4. MTHFR genotype distribution

Table 1 *also* summarizes the distribution of MTHFR polymorphisms among male and female participants in each study arm. These data are presented descriptively to characterize the sample and inform feasibility considerations for future trials. Genotypic analysis of MTHFR polymorphisms among the study participants revealed notable differences in distribution across gender and treatment arms. Males displayed a higher prevalence of homozygous MTHFR polymorphisms than females across both treatment arms. In MTHF-A, 11.1% of males were homozygous for either A1298C or C677T, while in FA-B, this rose to 36.3% (9.1% for A1298C and 27.2% for C677T). Only 11% of females in either arm were homozygous for any polymorphism.

5.5. Reproductive history of female participants

Table 2 summarizes the reproductive histories of the 22 female participants, including the number of previous pregnancies, live births, pregnancy losses, and history of high-strength folate prescription were recorded. These data are presented descriptively to characterize the sample and inform feasibility considerations. No statistical comparisons or clinical interpretations are made, as this study was not powered to assess associations between reproductive history and outcomes.

5.6. Acceptability

5.6.1. Recruitment

The CONSORT flow diagram above outlines participant recruitment, screening, eligibility, randomization, and follow-up

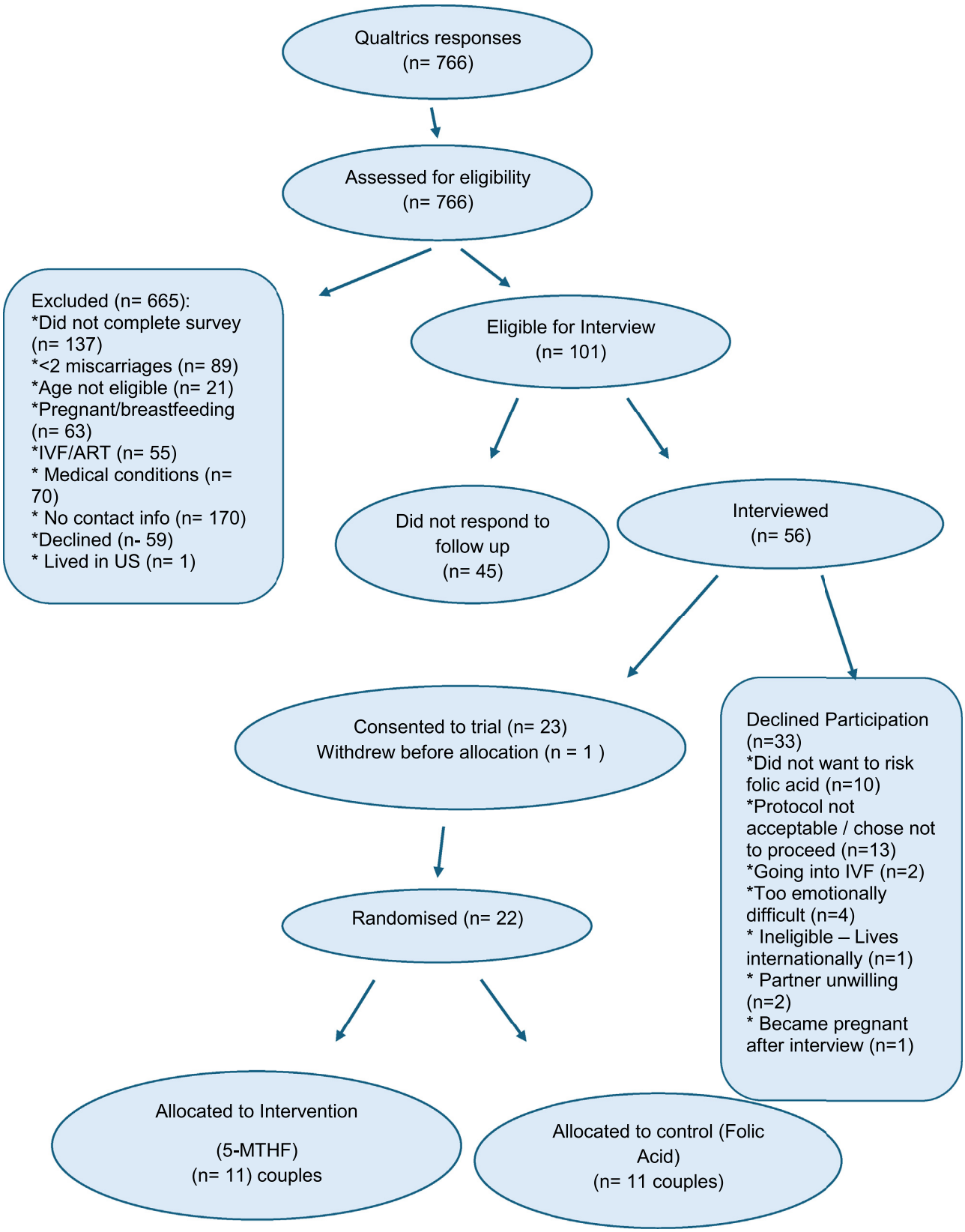

Fig. 1 – CONSORT flow diagram for a feasibility trial in couples with recurrent pregnancy loss. The figure illustrates participant flow through the study: 766 individuals screened, 101 eligible, and 23 couples enrolled. 22 couples were randomized 1:1 to prenatal multivitamins containing either 5-methyltetrahydrofolate (5-MTHF) or folic acid (FA). Completion rates were 55% in the 5-MTHF arm and 73% in the FA arm. Analyses were descriptive only and not powered for statistical significance. 5-MTHF, 5-methyltetrahdyrofolate; ART, assisted reproductive technology; IVF, in vitro fertilization.

Table 1 – Baseline demographic and genotype characteristics of male and female participants in a feasibility trial of prenatal multivitamins (5-methyltetrahydrofolate vs folic acid) in couples with recurrent pregnancy loss

Characteristic	Female participants		Male participants	
	MTHF-A ($n = 11$) *Mean (SD)*	FA-B ($n = 11$) *Mean (SD)*	MTHF-A ($n = 11$) *n (%)*	FA-B ($n = 11$) *N (%)*
Age (y)	37 (6)	34 (8)	36 (5)	35 (11)
Height (cm)	165 (8)	173 (11)	178 (10)	178 (9)
Missing	2	1	2	0
Weight (kg)	81 (16)	80 (24)	94 (26)	83
Missing	2	1	2	0
	n (%)	***n (%)***	***n (%)***	***n (%)***
Education level	($n = 11$)	($n = 10$)	($n = 7$)	($n = 8$)
Y 10	0 (0)	0 (0)	0 (0)	0 (0)
Y 12	0 (0)	0 (0)	1 (14)	2 (25)
Trade qualification	0 (0)	0 (0)	0 (0)	0 (0)
Diploma	5	2 (20)	0 (0)	5 (63)
Degree	3	8 (80)	6 (86)	0 (0)
PhD	3	0 (0)	0 (0)	1 (13)
Missing	2	1	4	3
Employment status	$n = 11$ (%)	$n = 11$ (%)	$n = 10$ (%)	$n = 11$ (%)
Full-time work (35 h per week)	3 (27)	6 (55)	9 (90)	11 (100)
Part-time work (<35 h per week)	3 (27)	4 (36)	1 (10)	0 (0)
Casual or temporary work	1 (9.1)	1 (9.1)	0 (0)	0 (0)
Currently looking for work	2 (18)	0 (0)	0 (0)	0 (0)
Not working	2 (18)	0 (0)	0 (0)	0 (0)
missing			1	0
Self-reported physical activity				
Sedentary	2 (18)	4 (36)	2 (20)	5 (45)
Moderate	9 (82)	7 (64)	7 (70)	3 (27)
Vigorous	0 (0)	0 (0)	1 (10)	3 (27)
Missing			1	0
Self-reported physical activity	($n = 11$)	($n = 11$)	($n = 10$)	($n = 11$)
Less than 5 h per week	8	6 (55)	5 (50)	5 (50)
5-10 h per week	3	5	3 (30)	4 (36)
More than 10 h per week	0 (0)	0 (0)	2 (20)	2 (18)
Missing			1	0
MTHFR polymorphism	$n = 9$ (%)	$n = 11$ (%)	$n = 10$ (%)	$n = 11$ (%)
MTHFR A1298C heterozygous	3 (33)	2 (18)	4 (40)	1 (9)
MTHFR A1298C homozygous	1 (11)	0 (0)	1 (10)	1 (9)
MTHFR C677T heterozygous	2 (22)	7 (64)	2 (20)	2 (18)
MTHFR C677T homozygous	0 (0)	1 (9.1)	1 (10)	3 (27)
MTHFR compound heterozygous	3 (33)	1 (9.1)	0 (0)	2 (18)
Nil polymorphisms	0 (0)	0 (0)	2 (20)	2 (18)

Data are presented as mean ± SD for continuous variables and *n* (%) for categorical variables. Analyses were descriptive only and not powered for statistical significance

Abbreviations: 5-MTHF, 5-methyltetrahydrofolate; FA, folic acid; RPL, recurrent pregnancy loss; SD, standard deviation.

throughout the pilot trial (Fig. 1). Of the 766 individuals who initially enquired about the study, 101 (13.2%) were deemed eligible based on initial screening criteria. From these, 56 participants (55.4% of those eligible) engaged in an interview, and ultimately 23 participants (22.8% of those eligible and 41.0% of these interviewed) consented and were enrolled in the trial. Among the 33 individuals who declined participation after interview, the most commonly cited reason was concern about the possibility of being allocated to the FA group (30.3%). A further 39.4% declined but gave no reason other than they did not wish to participate. Emotional burden also played a significant role, with 12.1% of those who declined, reporting that the topic of RPL was too emotionally difficult to engage with. Other reasons included plans to begin in vitro fertilization (IVF) (6.0%), lack of partner willingness to enter the trial (6.0%), ineligibility due to international residence (3.0%), and discovering a new pregnancy shortly after the interview (3.0%). There were five dropouts in MTHF-A, three in FA-B, and one prior to randomization. Reasons for drop out include (Dr. told her it was better to do in vitro fertilization, due to hospitalization of partner unrelated to the trial product, due to marriage issues [2 participants], cycle changes the participant contributed to trial product, lost to follow-up, supplement caused anxiety, left the country).

Table 2 – Reproductive history of female participants in a feasibility trial of prenatal multivitamins (5-methyltetrahydrofolate vs folic acid) in couples with recurrent pregnancy loss

Characteristic	MTHF-A ($n = 11$)	FA-B ($n = 11$)
Number of previous pregnancies	*n*	*n*
2-3	5 (45.5)	5 (45.5)
4-6	4 (36.3)	3 (27.2)
>6	2 (18.0)	3 (27.0)
Number of live births		
0	5 (45.0)	5 (45.0)
1-3	6 (54.5)	6 (54.5)
Number of previous pregnancy losses		
2-3	6 (54.5)	7 (63.6)
4-6	5 (45.0)	2 (18.0)
More than 6	0 (0.0)	2 (18.0)
Previously prescribed high-strength folate (≥5 mg) by fertility specialist	2 (18.0)	1 (9.1)

Data are presented as *n* (%) unless otherwise indicated. Analyses were descriptive only and not powered for statistical significance. Abbreviations: 5-MTHF, 5-methyltetrahydrofolate; FA, folic acid; RPL, recurrent pregnancy loss.

5.7. *Implementation and adherence*

5.7.1. *Adherence rates and protocol deviations*

Adherence to supplementation and dietary restrictions was high across both arms, with descriptive adherence rates reported in Table 3. These findings indicate strong protocol compliance.

5.8. *Acceptability*

5.8.1. *Acceptability of the trial to participants*

Overall, the trial demonstrated a high level of acceptability among participants across both study arms (Table 3). Completion rates were 55% in MTHF-A and 73% in FA-B. Acceptability scores (0-100 scale) and ease of compliance ratings are presented descriptively in Table 3.

5.9. *Efficacy*

5.9.1. *Pregnancy viability*

There were 2 successful pregnancies in MTHF-A and 3 in FA-B. Recorded miscarriages occurred in 3 participants in MTHF-A and none in FA-B. These outcomes are reported descriptively and should not be interpreted as evidence of efficacy.

5.9.2. *Adverse events*

No serious adverse events were reported in either arm (a serious adverse event was defined as an event that was life-threatening). More adverse events (defined as not life-threatening and included neurological symptoms, hormonal changes, skin symptoms, stomach/bowel issues or nausea or vomiting in pregnancy) were reported in FA-B vs MTHF-A (mean of 1.27 vs 0.64 per participant) mean difference –0.64 (95% CI: –1.5, 0.27), $P = .16$.

5.9.3. *Biomarker trends*

Observed biomarker changes from baseline to follow-up are summarized in Tables 4 and 5. At baseline, 5-MTHF and folate concentrations were significantly lower in those participants with the homozygosity in the MTHFR gene (Table 5), and homocysteine concentrations were higher. These comparisons are exploratory and not powered for statistical significance. 5-MTHF concentrations increased in both groups, with a greater rise observed MTHF-A (mean increase 50.0 nmol/L, SD 33.0) compared to FA-B (mean increase 28.9 nmol/L, SD 19.9). Holotranscobalamin (active vitamin B12) concentrations showed a downward trend in both groups, less pronounced in MTHF-A. The most significant patterns emerged in UMFA concentrations. UMFA concentrations decreased in the methyl folate group but rose markedly in the FA group, with some participants exceeding the 1 to 2 nmol/L threshold considered beyond physiological capacity for metabolism. These trends are descriptive and intended to inform hypotheses for future trials.

6. Discussion

This pilot study demonstrated that the trial design was broadly feasible and acceptable to participants, aligning with prior research emphasizing the value of participant-centered approaches in reproductive health interventions [42,50]. Participant satisfaction was high across both study arms, with slightly greater acceptability reported in the FA group, and no significant adverse effects were observed, supporting the established safety profiles of both FA and methyl folate in reproductive-aged populations [29,31,33,51–55]. Screening procedures effectively identified eligible participants but were resource-intensive, reflecting known challenges in early reproductive and miscarriage research, where emotional and logistical barriers can complicate recruitment [56,57]. Blinding was successfully maintained, minimizing performance and ascertainment bias [35], and digital tools for scheduling and data collection were well received, consistent with recent findings that online platforms can enhance engagement and reduce logistical burdens [58,59]. Retention was high, with only one participant lost to follow-up, and data collection tools demonstrated low variability, indicating strong protocol adherence and data reliability. However, while online recruitment was feasible, it may not fully address the psychological needs of individuals experiencing RPL, a population known to experience significant grief, anxiety, and trauma [56,60,61]. Studies suggest that a more personalized recruitment through clinical settings may better support ethical engagement and informed decision-making in this group [56,57]. Overall, the study procedures were robust, and with minor refinements, particularly in recruitment and screening, future trials may be further optimized for scale and participant support.

All couples in this study carried at least one MTHFR gene polymorphism, underscoring the potential clinical relevance of folate metabolism in reproductive outcomes, particularly in populations with RPL. The relatively uniform distribution of genotypes enhanced internal validity, while the notably higher prevalence of homozygous polymorphisms among male participants raises important considerations for male

Table 3 – Acceptability and adherence metrics for female participants in a feasibility trial of prenatal multivitamins (5-methyltetrahydrofolate vs folic acid) in couples with recurrent pregnancy loss

Characteristic	MTHF-A ($n = 11$)		FA-B ($n = 11$)	
	n (%)	***95% CI***	***n (%)***	***95% CI***
Completed study	6 (55)	25%, 82%	8 (73)	39%, 93%
Acceptability[a] (0-100) (missing 6/3, MTHF-A/FA-B)	***Mean (SD)***	***95% CI***	***Mean (SD)***	***95% CI***
Acceptability of how trial was conducted amongst all participants (missing 2/1)	86 (17)	73, 99	94 (11)	86, 101
Acceptability in completers only[d] (missing 5/3)	91 (11)	79, 103	92 (11)	83, 102
Acceptability in withdrawers only[d] *(missing 8/9)*	76 (25)		100 (0)	
Level of support in online meetings[b] *(missing 2/1)*	***Mean (SD)***		***Mean (SD)***	
Level of support in online meetings	1.22 (0.67)	0.71, 1.7	1.40 (0.97)	0.71, 2.1
Difficulty in completing tasks/protocol elements[c] *(missing 2/1)*	***Mean (SD)***		***Mean (SD)***	
Experience of completing forms before first meeting	1.44 (1.01)	0.67, 2.2	2.10 (1.60)	0.96, 3.2
Experience of completing forms before follow-up meetings	1.89 (1.05)	1.1, 2.7	2.20 (1.55)	1.1, 3.3
Experience of collecting forms from Dr.	2.56 (1.33)	1.5, 3.6	2.10 (1.37)	1.1, 3.1
Experience of blood collection tests	2.78 (1.72)	1.5, 4.1	3.00 (1.05)	2.2, 3.8
Experience of completion of diet diary	1.89 (1.27)	0.91, 2.9	2.00 (1.41)	0.99, 3.0
How easy was it to adhere to diet	2.22 (1.20)	1.3, 3.1	1.90 (0.74)	1.4, 2.4
How easy was trying not to conceive for first 2 cycles	2.22 (1.20)	1.3, 3.1	2.30 (1.25)	1.4, 3.2
How easy was it take the multivitamin	2.00 (1.32)	0.98, 3.0	1.90 (1.29)	0.98, 2.8
How easy was it to book and change meetings	2.00 (1.73)	0.67, 3.3	1.30 (0.95)	0.62, 2.0
How easy was logging into meetings	1.89 (1.27)	0.91, 2.9	1.40 (1.26)	0.50, 2.3
How easy was having everything online	1.89 (1.36)	0.84, 2.9	1.40 (1.26)	0.50, 2.3
Adherence to key protocol measures	***n (%)***		***n (%)***	
Adherence to abstinence for two reproductive cycles (missing 6/3)	5 (100)	46%, 100%	7 (88)	47%, 99%
Adherence to supplement in first six visits (missing 2/1)	7 (78)	40%, 96%	8 (80)	44%, 96%
Adherence to dietary restrictions in first six visits (missing 2/1)	9 (100)	63%, 100%	10 (100)	66%, 100%
Adherence to completed baseline assessment within 2 wk	8 (80)	44%, 96%	11 (100)	68%, 100%

Abbreviations: FA-B, arm B couples on the folic acid multivitamin; MTHF-A, arm A couples on the 5-methyletrahydrofolate multivitamin; SD, standard deviation.

Data are presented as mean ± SD unless otherwise indicated. Analyses were descriptive only and not powered for statistical significance. Confidence intervals for adherence and completion rates are provided in the text.

[a] Scale: 0 = not acceptable, 100 = very acceptable.

[b] Scale: 1 = very supported, 5 = not at all.

[c] Scale: 1 = easy, 5 = difficult.

[d] Response numbers may vary due to different stages of data collection or to missing data.

fertility. MTHFR variants, particularly C677T and A1298C, are known to impair folate metabolism, disrupt deoxyribonucleic acid (DNA) methylation, and elevate homocysteine concentrations [23,62], factors linked to oxidative stress, sperm DNA fragmentation, and impaired spermatogenesis [63]. The C677T homozygous genotype may reduce enzyme activity by up to 70% [23] and has been associated with reduced sperm quality and increased infertility risk, especially in Asian populations [64,65]. Similarly, A1298C homozygosity has been linked to implantation failure and embryonic aneuploidy [23]. However, findings remain inconsistent across studies, with some reporting no significant associations, likely due to ethnic variability, dietary folate intake, and methodological differences [23,66]. Epigenetically, MTHFR plays a central role in gametogenesis and early embryonic development through its regulation of DNA and histone methylation, with disruptions linked to infertility, miscarriage, and imprinting disorders [67]. These observations suggest a rationale for exploring MTHFR genotyping and targeted folate strategies in future research, but confirmation through adequately powered RCTs is essential before any clinical recommendations can be made.

6.1. *Folate metabolism and MTHFR polymorphisms*

Participants receiving 5-MTHF demonstrated greater improvements in folate biomarkers and maintained lower concentrations of UMFA compared to those receiving FA, suggesting a metabolically favorable profile for 5-MTHF. Unlike FA, 5-MTHF is the biologically active form of folate [29], and bypasses DHFR, thereby supporting one-carbon metabolism without contributing to UMFA accumulation [68,69]. In this study, UMFA concentrations decreased in the 5-MTHF group but rose significantly in the FA group, with some participants exceeding the 1 to 2 nmol/L threshold considered beyond physiological capacity for metabolism [70]. This aligns with previous findings that even low doses of FA (200-400 mcg/d) can result in UMFA accumulation due to limited DHFR activity [70,71].

Additionally, participants with the MTHFR C677T homozygous genotype exhibited significantly lower concentrations of 5-MTHF and serum folate, consistent with impaired folate metabolism and increased physiological demand for active folate forms [72]. These biomarker trends highlight a poten-

Table 4 – Exploratory biochemical outcomes at 2 months postrandomization for female participants in a feasibility trial of prenatal multivitamins (5-methyltetrahydrofolate vs folic acid) in couples with recurrent pregnancy loss

Blood methylation markers	MTHF-A			FA-B			Between groups		
	Baseline ($n = 9$)	Follow-up[a] ($n = 7$)[b]	Change from baseline	Baseline ($n = 11$)	Follow-up[a] ($n = 8$)	Change from baseline	Between arm difference at follow-up	95% CI	P value
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)		
Red cell blood folate (nmol/L) (missing 8/6 A/B)	1090 (90)			1162 (127)					
Serum folate >45 (nmol/L)	1/11 (9.1%)			2/11 (18%)					
Serum folate (nmol/L) Missing 2/1	41.7 (4.9)			40.2 (8.7)					
Vitamin B12 (pmol/L) Missing 2/1	349 (162)			341 (110)					
Homocysteine µmol/L	8.22 (2.47)	-	-	7.96 (2.51	-	-	-	-	-
Tetrahydrofolate nmol/L (Missing 2/1)	0.42 (0.27)	1.04 (0.22)	–0.60 (0.29)	0.37 (0.16)	0.82 (0.38)	–0.43 (0.29)	0.22	–0.14, 0.57	.21
5-MTHF nmol/L F/U $n = 6$ (A), $n = 8$ (B)	31 (16)	79.3 (30.6)	–50.0 (33.0)	31 (15)	60.6 (17.4)	–28.9 (19.9)	19	–0.14, 51	.22
Holotranscobalamin pmol/L	96 (32)	81.0 (45.8)	18.3 (62.0)	74 (27)	54.9 (46.0)	31.0 (58.9)	26	–25, 78	.29
Unmetabolized folic acid (UMFA) nmol/L Missing 2/0	0.61 (1.17)	0.39 (0.22)	0.32 (1.55)	0.41 (0.20)	2.78 (5.27)	–2.39 (5.38)	–2.4	–6.8, 2.0	.24

Abbreviations: 5-MTHF, 5-methyltetrahydrofolate; FA, folic acid; RPL, recurrent pregnancy loss; SD, standard deviation; UMFA, unmetabolized folic acid.

Data are presented as mean ± SD. Analyses were descriptive only and not powered for statistical significance.

[a] F/U—Follow-up blood samples were collected after participants completed 2 reproductive cycles on the multivitamin.

[b] Only six participants in MTHF-A had usable samples to measure 5-MTHF and UMFA at follow.

Table 5 – Baseline folate and homocysteine concentrations by MTHFR polymorphism in couples with recurrent pregnancy loss

Characteristic	Overall $N = 20$[a]	MTHFR C677T homozygous $N = 1$[a]	MTHFR A1298C homozygous $N = 1$[a]	MTHFR C677T heterozygous $N = 9$[a]	MTHFR A1298C heterozygous $N = 5$[a]	MTHFR compound heterozygous $N = 4$[a]
Red cell folate (nmol/L): baseline	1135 (114)	1199 (NA)	NA (NA)	1116 (162)	NA (NA)	1140 (52)
Serum folate (nmol/L) baseline	40.9 (7.0)	29.4 (NA)	31.6 (NA)	41.4 (8.4)	42.9 (3.9)	43.0 (3.1)
THF: baseline	0.39 (0.21)	0.71 (NA)	0.20 (NA)	0.41 (0.19)	0.36 (0.25)	0.37 (0.23)
5-MTHF baseline	31 (15)	19 (NA)	25 (NA)	36 (18)	26 (10)	30 (15)
Homocysteine (µmol/L): baseline	8.08 (2.43)	10.00 (NA)	11.70 (NA)	7.34 (2.61)	8.26 (2.00)	8.13 (2.56)

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; NA, not applicable (not collected); THF, tetrahydrofolate.

[a] Mean (SD).

tial area for further investigation in larger trials to determine whether targeted folate strategies, such as 5-MTHF supplementation, could improve metabolic outcomes in individuals with MTHFR polymorphisms.

Beyond fertility research, this feasibility trial has implications for other areas of folate science where concerns about excess FA exposure are emerging. Conditions such as gestational diabetes [73–79], neurodevelopmental disorders in offspring [80–83], and certain cancers [13,84–86]. By demonstrating the practicality of comparing 5-MTHF and FA in a controlled setting, this study provides a model for future investigations addressing these broader public health questions.

6.2. Stability of 5-MTHF within complex nutritional products

A critical and unexpected finding in this trial was the significant degradation of 5-MTHF in the methylated folate intervention arm, which likely confounded both biochemical and clinical outcomes. Independent analysis revealed that the actual 5-MTHF content per capsule had degraded from the intended 833 µg to as low as 199 µg in retained samples, while the FA comparator also showed partial degradation. This instability appears linked to the moisture-retaining properties of excipients, particularly calcium citrate, which can create a microenvironment conducive to hydrolysis and oxidation of labile nutrients. The susceptibility of 5-MTHF to degradation under heat, light, pH changes, and humidity is well documented [87], with different salt and crystal forms exhibiting variable stability profiles [88,89]. Hygroscopic ingredients such as choline salts may further exacerbate degradation by attracting moisture within the capsule matrix [90]. These findings underscore the importance of formulation integrity in trials involving methyl donors. Without adequate stability, the true efficacy of 5-MTHF may be underestimated, potentially leading to flawed conclusions. Future studies should avoid combining 5-MTHF with unstable excipients in multinutrient formulations and instead use moisture-controlled, individually packaged capsules with validated stability testing throughout the trial duration. This approach is essential to ensure accurate interpretation of nutrient efficacy and to support evidence-based recommendations in clinical practice.

This pilot trial is unique in several respects. To our knowledge, it is the first feasibility study directly comparing 5-MTHF and FA in a fertility context. Furthermore, it included male partners, demonstrating that men can successfully participate in preconception research, a critical but often overlooked aspect of reproductive health [39,91]. The trial also showed that dietary FA restriction is achievable, supporting the feasibility of implementing food-based protocols in future large-scale studies.

6.3. Vitamin B12 metabolism and clinical implications

This study also observed changes in vitamin B12 metabolism, with holotranscobalamin (active B12) concentrations declining in both intervention arms. However, the reduction was less pronounced among participants receiving 5-MTHF, suggesting a potential advantage of methyl folate in preserving active B12 status. This may reflect differences in cobalamin handling between FA and 5-MTHF, as proposed in metabolic studies [10,92]. The hypothesis that 5-MTHF may exert a protective effect on vitamin B12 metabolism warrants further investigation. While the FA group had a higher rate of successful pregnancies (27% vs 18%) and no recorded miscarriages, compared to miscarriages observed in the methyl folate group, the small sample size limits interpretation. These findings underscore the importance of cautious evaluation of clinical endpoints in future trials. While biomarker trends suggest potential differences between FA and 5-MTHF, this feasibility study was not powered to assess clinical efficacy. Future adequately powered trials are required to determine whether these differences translate into meaningful clinical outcomes.

6.4. Strengths and limitations

As a feasibility study, this pilot was designed to assess the practicality of the trial design and procedures rather than to determine clinical effectiveness. While it demonstrated several methodological strengths, including a randomized, double-blind design, high participant retention, and the successful use of digital platforms for recruitment and data collection, its small sample size and short intervention period limit the generalizability of findings. The study was not powered to detect significant differences in clinical or biochemical outcomes, and results should therefore be interpreted with caution. These findings primarily inform the refinement of study protocols and highlight considerations for future, adequately powered trials aimed at evaluating the clinical efficacy of FA vs 5-MTHF in reproductive health contexts.

7. Conclusion

This feasibility study demonstrated the practicality of conducting a randomized trial comparing 5-MTHF and FA in a reproductive-age population. Although limited by sample size and formulation instability, the study identified a notable rise in UMFA among participants receiving FA, warranting further investigation into its potential clinical relevance. These findings highlight key considerations for future trials, including the need for stable formulations, extended follow-up, and comprehensive biochemical profiling. The results also support continued investigation into personalized folate supplementation strategies, particularly in populations with genetic variants affecting folate metabolism. Only through rigorously designed studies can the comparative effects of FA and 5-MTHF on reproductive and metabolic health be fully understood, particularly in light of emerging concerns around excess FA exposure. This feasibility study cannot determine clinical efficacy. Observed biomarker patterns warrant further investigation in larger, well-controlled trials to evaluate whether 5-MTHF provides clinical advantages over FA.

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Author declarations

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CRediT authorship contribution statement

Carolyn Ledowsky: Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Vanessa Scarf:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Kris Rogers:** Writing – review & editing, Software, Formal analysis. **Amie Steel:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Data sharing

Data described in the manuscript, code book, and analytic code will be made available upon request pending approval (https://data.research.uts.edu.au/publication/c53120e062d611f0a60ca5014b61eed3).

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