UTS UNIVERSITY OF TECHNOLOGY SYDNEY

The Effectiveness of 5-methyltetrahydrofolate Versus Folic Acid Prenatal Multivitamins in Couples with a history of Recurrent Pregnancy Loss: A Randomised, Controlled Feasibility Trial

by Carolyn Jane Ledowsky

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Public Health)

under the supervision of Associate Professor Amie Steel and Associate Professor Vanessa Scarf.

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Certificate of original authorship

I, Carolyn Jane Ledowsky, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy (Public Health) in the Faculty of Health at the Australian Research Centre in Complementary and Integrative Medicine, at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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This work is more than just research, it is a promise. A promise to keep asking questions, to keep pushing for answers, and to never stop searching for ways to help those who need it most. My greatest hope is that this thesis serves as a stepping stone for future research and, ultimately, as a source of hope for couples facing the heartbreak of RPL.

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Disclosure of Interests

Carolyn Ledowsky declares that she is the founder of *MTHFR* Support Australia.

Thesis Structure

This thesis is presented as a Thesis by Compilation. This thesis comprises both chapters and published articles. The chapters include an introduction, two literature reviews, methodology, result chapters, and a discussion including limitations and future research directions.

The structure of the thesis is as follows:

Chapter 1	Provides background knowledge relating to recurrent pregnancy loss, the recommendations regarding folic acid, folate metabolism, and the key genetic polymorphisms that may affect metabolism and how this relates to infertility. This chapter also provides an overview of the thesis structure, including the relevant research questions and research aims.
Chapter 2	Provides two literature reviews, the first pertaining to the amount of folic acid women in preconception and pregnancy are exposed to in countries with folic acid fortification and a second literature review on the effect of excess folic acid on pregnancy and offspring outcomes.
Chapter 3	Presents the research design and methodology for study elements.
Chapter 4	Details the findings of the randomised clinical trial pilot study including the sub-analysis of the participant intake interviews
Chapter 5	Presents the results of the sub-study analysis of participant interviews from the RCT trial

Chapter 6	Presents the results of the practitioner survey
Chapter 7	Presents the discussion
Chapter 8	Presents the conclusion
Chapter 9	Presents the appendices

List of Publications

The peer-reviewed publications contained within this thesis are outlined below.

Paper #1	
Title:	*Women Taking a Folic Acid Supplement in Countries with Mandatory Food Fortification Programs May Be Exceeding the Upper Tolerable Limit of Folic Acid: A Systematic Review*
Authors:	*Ledowsky C, Mahimbo A, Scarf V, Steel A*
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Paper # 2	
Title:	*Health Effects Of Excess Folic Acid Use And High Blood Folate During Preconception And Pregnancy: A Systematic Review.*
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Paper # 3	
Title:	*Health Professionals Require More Defined Protocols, Better Funding And Patient Resources To Support Couples With Recurrent Pregnancy Loss (RPL)*
Authors:	*Ledowsky C, Scarf V, Steel A*
Journal:	*Scientific Reports. 2025 August; 15(1)*
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Paper # 4	
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Authors:	*Ledowsky C, Scarf V, Rogers K, Steel A*
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Peer-reviewed publications not incorporated into the thesis

Paper #1	
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Paper # 2	
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Published conference abstracts and presentations based on the thesis results

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Indicate: Oral / Poster / Other	*Oral*
# 2	
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# 3	
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# 10	
Title:	*Women Taking a Folic Acid Supplement in Countries with Mandatory Food Fortification*

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Author/s:	*Ledowsky C, Steel A, Schloss J*
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Title:	*The Different Forms and Dosages of Folate that Practitioners Specialising in Infertility Support Prescribe to Patients with the MTHFR Genetic Polymorphisms: a Case Study*
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Statement of Contribution of Authors Contained in the Thesis

This thesis contains three results chapters, one has been published in a relevant peer-reviewed journal, and one has undergone review and is with the editorial team for a final decision, with the final paper currently under review, as displayed in the appendices. For each of these articles, I have been primarily responsible for the development of the research questions, drafting the methodology protocols, performing analysis, drafting the full manuscripts, submitting the manuscripts for publication, and responding to peer-review comments.

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List of Abbreviations

5-MTHF	5-methyltetrahydrofolate
ACTRN	Australian New Zealand Clinical Trials Registry
AE	Adverse Event
AHPRA	Australian Health Practitioner Regulation Agency
ART	Assisted Reproductive Technologies
CAM	Complementary Alternative Medicine
CONSORT	Consolidated Standards of Reporting Trials
CTCAE	Common Terminology Criteria for Adverse Events
CRF	Case Report Form
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
ESHRE	European Society of Human Reproduction and Embryology
FA	Folic Acid
FA-B	Folic acid group in the RCT trial
GP	General Practitioner
HPs	Health Professionals
IVF	In vitro fertilisation
IVF-ICSI	IVF with intracytoplasmic sperm injection
MTHF-A	5-MTHF group in the RCT trial

MTHFR	Methylenetetrahydrofolate Reductase
NHMRC	National Health and Medical Research Council
NTD	Neural Tube Defects
PHI	Protected Health Information
PIF	Participant Information Form
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
REDCap	Research Electronic Data Capture
RBCF	Red Blood Cell Folate
RPL	Recurrent Pregnancy Loss
SD	Standard Deviation
THF	Tetrahydrofolate
TS	Thymidylate Synthase
TTC	Trying to Conceive
UK	United Kingdom
UL	Upper Tolerable Limit of FA
UMFA	Unmetabolised Folic Acid
WHO	World Health Organisation

Abstract

Background

Women with recurrent pregnancy loss (RPL) frequently go on to have more pregnancy losses. Folic acid has traditionally been recommended by Governments worldwide, although emerging research suggests that high cumulative exposure of folic acid may have biochemical and metabolic implications in some individuals. Alternative forms of folate, such 5-methyltetrahydrofolate (5-MTHF) are increasingly available but are not widely prescribed or understood and evidence regarding its use in RPL is limited. In addition, common genetic polymorphisms affecting folate metabolism, including those with the *MTHFR* gene, may influence folate handling and response to supplementation.

Methods

A two-phase research design was employed. Phase 1 was a double-blind controlled feasibility trial of twenty-two couples experiencing pregnancy loss, comparing a prenatal multivitamin containing 2,500 mcg folic acid with an identical formulation containing 2.500 mcg 5-MTHF. The study assessed recruitment feasibility, protocol acceptability, adherence and biochemical marker response without being powered to evaluate clinical efficacy. A qualitative sub-study explored women's experiences of care following pregnancy loss. Phase 2 comprised a national cross-sectional survey of Australian health professionals to describe their prescribing and referral practices, including folate prescriptions for couples with RPL.

Results

Phase 1 identified that couples with recurrent pregnancy loss are willing to follow protocols to support their fertility, including taking 5-methyltetrahydrofolate at higher doses, avoiding foods with folic acid, and abstaining from trying to conceive for at least two reproductive cycles to support increased folate biomarkers. All participating couples carried *MTHFR* polymorphisms, with a higher prevalence of homozygous variants observed among male partners. In the 5-MTHF group, UMFA decreased from baseline of 0.61 nmol/L (SD 1.17) to 0.39 nmol/L (SD 0.22). In contrast, the FA group experienced an increase in UMFA, rising from 0.41 nmol/L (SD 0.20) to 2.78 nmol/L (SD 5.27). These findings describe biochemical responsiveness rather than clinical effect. Qualitative interviews highlighted that women frequently perceived gaps in continuity of care, access to information, and emotional support following pregnancy loss.

Phase 2 surveyed 175 health professionals across Australia. Most respondents routinely recommended folic acid, while fewer than 11% reported prescribing any form of folate to male partners. Almost all practitioners prescribed folic acid to their female patients. Male reproductive health was infrequently addressed within preconception care. Respondents identified substantial variability in practice and expressed strong demand for clinical guidance and protocols (95.7%), dosing information for different folate forms (62.8%), educational resources for patients (87.2%), improved referral pathways (55.9%) and a need for improved funding to allow for more specialised appointments and midwifery care (51.6%). The health care professionals acknowledge that RPL is of increasing importance and are

keen to learn more.

Conclusion

This thesis provides novel feasibility data and contextual insights into the use of different folate forms in couples experiencing recurrent pregnancy loss. The findings highlight variable biochemical responses to folate form, a high frequency of genetic polymorphisms affecting folate metabolism, and significant gaps in current models of care, particularly with respect to male partner inclusion and practitioner guidance. While no conclusions regarding clinical effectiveness can be drawn, the results support the need for adequately powered trials to evaluate folate form, dosage, and metabolic context in RPL.

Chapter 1 Introduction

1.2 Topic Introduction

1.2.1 Recurrent Pregnancy Loss – A Persistent Clinical Challenge

Recurrent pregnancy loss (RPL) remains one of the most emotionally and clinically challenging conditions in reproductive medicine. Defined as the loss of two or more pregnancies [6], RPL affects up to five percent of couples worldwide, yet up to 75% of cases are classified as 'unexplained' [7]. Despite advances in assisted reproductive technologies (ART), 50—75% of those experiencing repeated losses without a clear diagnosis or effective treatment pathway [7]. The emotional toll of RPL is profound, often characterised by disenfranchised grief, anxiety, and frustration with fragmented care systems [8, 9]. Couples frequently report feeling unsupported and compelled to self-advocate, navigating a complex landscape of medical advice, online communities, and complementary therapies [10-12].

Clinically, RPL is associated with a wide range of potential causes, including chromosomal abnormalities (such as aneuploidy of which trisomy's represent 60% of all abnormalities), maternal thrombophilic disorders, structural anomalies, immune and endocrine dysfunction, and obesity [8, 9, 13-19]. However, the lack of consistent diagnostic protocols and the absence of targeted interventions for idiopathic RPL underscore the need for new approaches [8]. Assisted reproductive technologies remains a common pathway for couples with RPL, yet success rates are modest with only 18.1% of ART cycles resulting in live births in Australia [20]. Out of pocket costs often exceed $10,000 AUD [21] and the financial burden is shared by the government, which spent approximately $AUD 250 million in 2009 for ART

services [22] . Beyond these direct costs, there are indirect economic and social costs arising from the emotional and physical toll on couples and their families. This includes lost productivity due to time off work, increased demand for mental health services, and strain on relationships and family dynamics. A 2020 Dutch study estimated the indirect costs related to each IVF cycle (i.e. absence from work and lost productivity) was around $4,000 AUD and with each couple completing around 4 cycles, the indirect cost for four cycles would be $16,000 AUD [23]. This is considerable considering there were 112,000 IVF cycles in Australia in 2023 [24] equating to $1.792 billion per year in indirect costs. Research has shown that infertility is a chronic stressor due to the inability to have a child and the pressure they felt constantly from family and friends who often commented on their childlessness, with long-term consequences, including anxiety, frustration and isolation [22].

1.2.2 Folate Metabolism and the Emerging Role of *MTHFR*

Folate, a critical nutrient for DNA synthesis and methylation, has long been recognised for its role in preventing neural tube defects (NTDs) [25-29]. Governments worldwide have implemented folic acid (FA) fortification programmes and/or recommend supplementation for women of reproductive age [30, 31]. However, emerging evidence challenges the assumption that ‘more is better’ [32-43]. In fortified populations, amongst individuals who also take folic acid based supplements, the upper tolerable intake (UL) of 1,000 mcg/day is exceeded in as much as 18% of the general population 13 years and older [44-47]. We found in our first review paper that almost all women in pregnancy that take a prenatal multivitamin with folic acid are exceeding the UL [48]. Unmetabolised folic acid is detected in >90% of individuals in

populations with mandatory fortification [49], with substantially lower prevalence (18-21%) in pre-fortification era samples [50]. Associations between unmetabolised folic acid (UMFA) levels and adverse health outcomes are highly context-dependent and modified by vitamin B12 status, genetic polymorphisms, race/ethnicity, and folic acid dose. In individuals with low vitamin B12 status (<148 pmol/L), detectable UMFA is associated with lower cognitive test scores and altered hematologic parameters [51], while no such associations exist in those with adequate B12 [51]. Cord blood UMFA shows strong positive associations with food allergy (OR 8.50, 95% CI: 1.7-42.8) [52] and autism spectrum disorder risk specifically in children of Black ethnicity (adjusted OR 9.85, 95% CI: 2.53-38.31) [53], but late-pregnancy maternal UMFA shows no association with infant allergic outcomes [54] and no ASD association in predominantly European cohorts [55]. UMFA is positively associated with nonalcoholic fatty liver disease prevalence [56] and shows mixed associations with immune function, including reduced natural killer cell cytotoxicity (23% lower with detectable UMFA) [57] but lower proinflammatory cytokines [58]. Cancer outcomes show largely null associations with UMFA in both pre- and post-fortification populations [50, 59], though subgroup analyses suggest positive associations in men and those with specific *MTHFR* genotypes [50]. Threshold effects emerge above 400 mcg/d supplemental folic acid intake, particularly in individuals with *dihydrofolate reductase* (*DHFR)* genetic variants [60] and where UMFA accumulation increases substantially [61]. Evidence does not support consistent adverse effects of typical UMFA levels in individuals with adequate B12 status consuming ≤400 mcg/d supplemental folic acid but suggests potential risks in specific vulnerable populations

including those with B12 deficiency, certain genetic polymorphisms, or specific racial/ethnic backgrounds. This is best summarised by Maruvada et al. (2020), following the NIH workshop, that although some epidemiologic and experimental studies raise questions, current evidence is insufficient to infer causality and substantial research gaps remain [44] . While the presence of UMFA has not been shown to be linked to adverse health outcomes, elevated serum folate biomarkers show complex, context-dependent associations with adverse health outcomes in adults, critically modified by vitamin B12 status. Among individuals with vitamin B12 deficiency, high serum folate >59 nmol/L is associated with substantially increased risks of cognitive impairment cognitive impairment (OR: 2.5; 95% CI: 1.6, 3.8).[62], anemia [odds ratio (OR): 2.7; 95% CI: 1.7, 4.2], and more pronounced metabolic dysfunction [63], while among those with normal B12 status, high serum folate appears protective for cognitive outcomes (OR 0.4-0.5) [62]. Multiple studies demonstrate U-shaped associations for mortality outcomes where both low and high folate increase cardiovascular mortality [64] and all-cause mortality [65] compared to moderate levels. Elevated serum folate is associated with increased risk of prostate cancer [66]. The evidence indicates that elevated folate biomarkers are associated with adverse outcomes primarily when vitamin B12 is deficient, when levels are extremely high rather than moderately elevated in the presence of pre-existing disease or genetic susceptibility. For example, genetic susceptibility modified cancer risk. High plasma folate increased breast cancer risk only in women carrying the *MTHFR* C677 allele (P-trend=0.03, P-interaction=0.002), while having no significant effect in those with wild-type genotype [67].

Central to this issue is the role of genetic variability in folate metabolism,

particularly polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) gene [68-75]. *MTHFR* is essential for converting FA into its biologically-active form, 5-MTHF [76]. Individuals with *MTHFR* polymorphisms, especially the C677T and A1298C variants, exhibit reduced enzymatic activity [77], impairing folate metabolism [78] and potentially contributing to RPL [79-85]. Despite this, routine screening for the *MTHFR* C677T and A1298C variants is not widely adopted, and FA supplementation remains largely uniform, with little consideration for genetic differences.

1.2.3 Rethinking Folate Supplementation and the Case for Personalisation

This thesis explores the theory that personalised folate supplementation, particularly the use of 5-MTHF in individuals with *MTHFR* polymorphisms, may provide a safer and more effective approach to managing RPL. Unlike FA, 5-MTHF bypasses the *DHFR* dependent conversion pathway enzyme [86, 87] and is immediately available for cellular uptake and methylation [79]. Clinical and biochemical data suggest that 5-MTHF improves folate biomarkers without causing UMFA accumulation [86, 88], especially in individuals carrying *MTHFR* gene variants such as C677T and A1298C [86, 89-94].

The research presented in this thesis builds on these insights through a mixed method investigation comprising a pilot randomised controlled trial (RCT), a sub-study of the RCT which included qualitative interviews with couples affected by RPL and a practitioner survey to explore the prescribing practices of health professionals that support couples with RPL. Together, these studies explore the feasibility, acceptability, and clinical relevance of using 5-MTHF as an alternative to FA, with a focus on improving reproductive

outcomes through a personalised approach.

1.3 Thesis Aim and Structure

1.3.1 Thesis Aim

The overarching aim of this thesis is to evaluate the feasibility and clinical relevance of using 5-MTHF as an alternative to FA in couples experiencing RPL, particularly those with *MTHFR* C677T and A1298C polymorphisms. The thesis is structured around six key research objectives, each addressed through distinct methodological phases.

1.3.2 Research Objectives

The objectives are explored through a mixed method approach comprising systematic reviews, a pilot RCT, practitioner survey, and qualitative interviews with participants as follows:

1. To examine the feasibility of an RCT comparing 5-MTHF and FA treatment to prevent pregnancy loss in couples with RPL.
2. To explore the impact of both FA and 5-MTHF and its effect on UMFA concentrations
3. To observe the occurrence of *MTHFR C677T and A1298C* polymorphisms among couples with RPL and consider if 5-MTHF is a more appropriate folate choice than FA for individuals with these genetic variants.
4. To explore couples' experiences of current RPL treatments and identify their views on the need for new and more acceptable treatment options.
5. To explore the current practices of health professionals who commonly treat couples with RPL with regard to RPL management

for both reproductive partners, and with a particular focus on folate prescription.

6. To explore the needs of health professionals in facilitating behavioural change around folate supplementation (folic acid versus 5-MTHF) for couples experiencing RPL.

1.3.3 Significance of the Thesis to the Evidence Base

This thesis is grounded in the need to strengthen the evidence base surrounding folate supplementation and its role in reproductive health, particularly in the context of RPL. While 500 mcg FA supplemental intake is a globally endorsed recommendation to prevent NTDs, emerging concerns about excessive intake, especially in populations exposed to mandatory FA fortification of food (which range from 140 mcg FA per 100g enriched cereal grain in the United States to 250 mcg per 100g in Australia [95]), highlight gaps in our understanding of its long-term safety and optimal dosing.

Despite the existence of an upper tolerable intake, current guidelines do not adequately account for individual differences in folate metabolism, such as those caused by *MTHFR* polymorphisms. Moreover, clinical protocols for RPL rarely include male partners or consider biochemical individuality, leaving a significant proportion of pregnancy losses ‘unexplained’.

By aiming to investigate the biochemical, genetic, and clinical dimensions of folate metabolism in RPL, this thesis seeks to address these critical gaps. Its mixed method design, including a randomised control trial, patient interviews and a practitioner survey, was chosen to generate foundational evidence that can inform future research, clinical guidelines, and public health policy. The overarching goal is to contribute a more nuanced and personalised

understanding of folate supplementation in reproductive care, and especially in RPL, to inform more targeted and effective clinical interventions particularly for individuals with impaired folate metabolism due to *MTHFR* polymorphisms.

1.3.4 Structure of the Thesis

This thesis is a compilation of eight chapters, each addressing a distinct aspect of the research aim and objectives through a mixed method approach. Three are published manuscripts, one in preprint and one under peer review.

Chapter 1: Research Aim, Objectives, and Overarching Research Questions

This chapter introduces the overarching aim of the thesis, to evaluate the feasibility and clinical relevance of using 5-MTHF as an alternative to FA in couples experiencing RPL, particularly those with *MTHFR* polymorphisms. It outlines six research objectives and presents the key research questions that guide the investigation.

Chapter 2: Systematic Literature Reviews

Two systematic reviews are presented. The first examines folic acid exposure in preconception and pregnancy, highlighting widespread intake above the upper tolerable limit. The second explores the biological risks of excess folic acid, folate biomarkers and UMFA identifying associations with gestational diabetes and neurodevelopmental disorders. These reviews establish the rationale for investigating alternative folate forms and personalised supplementation strategies.

Chapter 3: Methodology

This chapter details the mixed method design of the thesis, including the rationale for each study type, participant recruitment strategies, data

collection protocols, and analytical approaches. It explains how the research phases align with the objectives and questions and describes the integration of qualitative and quantitative methods.

Chapter 4: Pilot Study Results

This chapter presents findings from a pilot randomised controlled trial comparing 5-MTHF and FA supplementation in couples experiencing RPL. It describes the biochemical outcomes, including changes in folate biomarkers and unmetabolised folic acid (UMFA), along with participant adherence and acceptability rates for those who participated in the trial. The chapter also evaluates the feasibility and acceptability of scaling the trial.

Chapter 5: Sub-study with Participant Interviews

Qualitative interviews with trial participants explore their lived experiences of RPL, perceptions of current treatment options, and views on personalised folate supplementation. Themes include emotional burden, systemic gaps in care, and the desire for informed choice and validation of *MTHFR* polymorphism related concerns.

Chapter 6: Practitioner Survey

Findings from a national survey of health professionals reveal current prescribing practices and highlight variability in folate recommendations for couples experiencing RPL. The survey showed limited inclusion of male partners in preconception care and inconsistent consideration of folate metabolism. Practitioners expressed openness to changing clinical protocols if supported by a range of evidence.

Chapter 7: Integrated Discussion

This chapter synthesises findings across all research phases. It discusses the implications for clinical practice, public health policy, and future research. Key themes include redefining FA safety thresholds, integrating genetic screening into preconception care, promoting male-inclusive care models, and developing personalised folate supplementation protocols for those with RPL and particularly *MTHFR* polymorphisms.

Chapter 8: Thesis Conclusion

The final chapter summarises the thesis contributions, emphasising the need to explore a paradigm shift from generic FA supplementation to personalised approaches. It outlines recommendations for clinical guidelines, education, and policy reform, and proposes future research directions to advance reproductive care, reduce unexplained infertility and pregnancy loss.

1.4 Background and Context

Having outlined the structure and scope of this thesis, the following sections provide a detailed overview of the clinical, biochemical and genetic context that informs the research. These foundational concepts are essential for understanding the rationale behind the thesis aim, the selection of research methods and the relevance of personalised folate supplementation in the management of RPL.

1.4.1 Folate and Human Health

1.4.1.1 Folate

Folate-dependent biochemical processes are influenced by the form and bioavailability of folate [78, 96]. Folate is a generic term for vitamin B9 that includes several forms, including those that are naturally found in food. While

all folate forms share a common structure, consisting of a pteridine ring linked to p-aminobenzoic acid and a polyglutamate chain, and they differ in the oxidation state of the pteridine ring and the number of glutamate residues attached [89, 96]. Naturally occurring food folate is found in foods such as leafy green vegetables and is in a reduced form [78]. In food, folate exists predominantly as polyglutamates, which must be hydrolysed to a monoglutamate form in the intestinal lumen before absorption [86]. The intestinal absorption of polyglutamate derivatives of tetrahydrofolate (THF) into monoglutamates is done by the brush border enzyme glutamate carboxypeptidase II [44] and does not require the activity of the *DHFR* enzyme [86, 87]. All forms of folate must be converted to 5-MTHF which is the main form of folate found in plasma [89].

Serum folate concentrations above 45.3 nmol/L (20 ng/mL) are often labelled as elevated, [97], but according to the World Health Organisation (WHO), this value reflects the upper measurement limits of early folate assays, which lacked the dynamic range to accurately quantify higher folate concentrations, rather than from evidence of biological risk [98, 99].

Red blood cell folate (RBCF), a more reliable long-term marker, is recommended to exceed 906 nmol/L for NTD prevention [100], a threshold derived from a 1995 study by Daly et al [101]. However, this evidence lacks robust validation according to the GRADE working group [102]. A closer examination of Daly's findings reveals that while the lower limit of normal RBCF was 317 nmol/L, the authors speculated that despite there being too few cases to confirm their analysis, the risk reduction continued beyond 1292 nmol/ [101] .

Subsequent studies have questioned the reliability of these thresholds and a

follow-up study by the same group noted that most mothers with NTD cases were not folate deficient “(8.6% had a plasma folate level below 1.5mg/L and 14.3% had erythrocyte folate levels below 150 mcg/L, the usual cut offs for deficiency”, suggesting that plasma folate alone may not fully explain NTD risk [103]. Crider et al (2014) estimated that RBCF concentrations around 1000 nmol/L were optimal for NTD prevention [104], but this too was based on statistical modelling and considered low-quality evidence by the GRADE working group (low quality defined as**:** Our confidence in the effect estimate is limited. The true effect may be substantially different from the estimate of the effect) [100, 102]. Importantly, these thresholds were developed solely in relation to NTD prevention and do not account for broader biological risks associated with excess FA intake. Evidence from animal studies indicates several broader biological risks associated with excess FA intake. High-folic-acid diets have been shown to increase DNA mutation rates and impair DNA repair capacity through hypermethylation of DNA repair genes in mice [105], disrupt prenatal cortical neurodevelopment and alter offspring behaviour [106], modify cortical gene expression networks and neuronal activity [107], and exacerbate weight gain, adipose inflammation and glucose intolerance in rodent metabolic models [108]. These findings suggest that excess FA intake may influence genomic stability, neurodevelopmental processes and metabolic regulation, highlighting the need to consider biological risks beyond NTD prevention.

However, not all individuals metabolise FA efficiently. Genetic polymorphisms in the *MTHFR* gene, particularly C677T and A1298C, can significantly reduce enzymatic activity, impairing the conversion of 5-MTHF and may disrupt one-carbon metabolism, with potential consequences for

DNA methylation [109].

1.4.1.2 MTHFR Polymorphisms

MTHFR is a key enzyme required to activate all forms of folate into 5-MTHF, the biologically active form of folate [110, 111]. Specifically, *MTHFR* catalyses the conversion of 5,10 methylenetetrahydrofolate to 5-MTHF [109] which serves as the methyl donor for the remethylation of homocysteine to methionine and is the precursor to S-adenosylmethionine (SAM) [112], the universal methyl donor involved in DNA methylation and other methylation dependent processes [79-85]. Genetic variations in the *MTHFR* gene, particularly the C677T and A1298C polymorphisms can significantly reduce enzymatic activity [77]. These polymorphisms result in amino acid substitutions that destabilise the enzyme [77], impairing the conversion of 5,10-methylenetetrahydrofolate to 5-MTHF [80, 113]. This enzymatic pathway is the only route by which cells generate 5-MTHF in the cell [114], and disruptions in folate status due to *MTHFR* polymorphisms can compromise one-carbon metabolism [78], directly affecting SAM production and DNA methylation [79].

Homozygous C677T variant genotypes are present in approximately 10-14% of Caucasians, 21% of Hispanics, 1% to 7% of individuals of African descent, and approximately 11% of Asians [115]. Homozygous C677T polymorphisms may reduce the enzyme activity by up to a 70% [77, 109], while twenty to twenty-five percent of the general population exhibit heterozygous polymorphisms [114] which reduce the activity of the enzyme by 30% [77]. Homozygous A1298C genotypes experience a 40% reduction in enzyme activity [77]. Emerging evidence suggests that disruptions in folate metabolism, due to *MTHFR* polymorphisms, may contribute to infertility and

RPL.

The C677T polymorphism substantially impairs homocysteine metabolism and methylation capacity compared to wild-type *MTHFR*. Across 25 studies encompassing over 36,000 individuals, homozygous 677TT individuals exhibited 8-45% higher homocysteine levels [116], 13-22% lower plasma folate [117], and approximately 48% lower DNA methylation [118] compared to 677CC wild-type, with heterozygotes showing intermediate effects [116], researchers have investigated how such genotype-related reductions in methyl-group supply may influence the epigenetic programming of gametes and embryos [114]. This thesis explores whether personalised folate supplementation can mitigate the effect of *MTHFR* polymorphisms.

As such, researchers have explored the use of 5-MTHF as a therapeutic alternative for FA. Unlike FA, 5-MTHF does not require the initial *MTHFR*-dependent reduction step to enter the folate cycle, as it is already the end-product of the *MTHFR* reaction. This allows supplemental 5-MTHF to directly participate in homocysteine remethylation and methylation reactions upon absorption [86, 89-94]. Following methyl donation, however, 5-MTHF is converted to tetrahydrofolate (THF), and regeneration of 5-MTHF from the folate pool remains dependent on *MTHFR* activity. *MTHFR* therefore functions as a rate-limiting and tightly regulated metabolic gatekeeper directing one-carbon units toward methionine and S-adenosylmethionine synthesis, rather than being bypassed entirely [119, 120]. In this context, supplemental 5-MTHF may reduce reliance on impaired endogenous conversion in individuals with reduced *MTHFR* activity, without eliminating the requirement for *MTHFR* in ongoing folate cycle recycling.

Studies have shown that 5-MTHF increases folate concentrations more

effectively than FA at equivalent doses (e.g.; 400 mcg) [121, 122], and can rapidly replete folate stores in deficient individuals [123]. Compared to FA, 5-MTHF has demonstrated superior efficacy in raising plasma folate concentrations [88]. Safety data for 5-MTHF is robust. Animal studies report no adverse effects at high doses [124] and human trials have shown no side effects at doses up to 17mg daily for 12 weeks [125]. In pregnancy, doses of 1.13mg have been used without side effects [126] and higher doses of 7.5mg and 15mg have been administered without side effects in patients with depression [127, 128]. High-dose folic acid supplementation (most commonly defined as ≥5 mg/day) demonstrates population-specific and dose-dependent adverse effects. In children of mothers with epilepsy taking antiseizure medications, prenatal exposure to high-dose folic acid (≥1 mg/day) was associated with increased childhood cancer risk (adjusted HR 2.7, 95% CI 1.2-6.3) [129], though this finding lacks replication and the absolute risk increase was small (0.8%) [130]. High-dose maternal supplementation (>72,000 mcg defined as 400 µg/day for 6 months or 800 µg/day for 3 months, taken every day) increased infant asthma risk (OR 3.16, 95% CI 1.15-8.71) [131], while doses ≥1000 mcg/day in the periconceptional period increased risk of small for gestational age (OR 2.21-2.32) [132]. Biochemical effects include near-universal UMFA accumulation [133], development of pseudo-*MTHFR* syndrome with elevated homocysteine despite supplementation [134], increased oxidative stress markers [135] , and reduced natural killer cell number and cytotoxicity [136]. In men with infertility, 6 months of FA 5 mg/day caused global sperm methylation loss, particularly pronounced in individuals with the *MTHFR* C677T genotype [137]. However, the largest randomised trial found no

significant adverse effects from 4 mg/day in 2,464 high-risk pregnant women [138] , and extended follow-up of women receiving 5 mg/day in pregnancy showed no excess cancer or cardiovascular mortality over 33 years [139]. The heterogeneity in findings reflects population differences (genetic polymorphisms, concomitant medications, baseline folate status), dose-response relationships showing potential threshold effects, and outcome specificity. These findings may support the use of 5-MTHF, particularly for individuals with *MTHFR* genotypes as doses of 800 mcg have been shown to bypass the *MTHFR* enzyme and restore folate status [86, 90-94, 140]. As yet no clinical trials investigating RPL have compared both 5-MTHF and UMFA. This thesis investigates its potential to improve folate status and reproductive outcomes in couples experiencing infertility and RPL.

1.4.1.3 Folic Acid Dose and the Need to Define Excess

Dosages of 400-500 mcg of folic acid are recommended for preconception and pregnancy, however, many governments advise supplementation in addition to dietary folate intake. In Canada typical prenatal multivitamins contain at least 1000 mcg of FA [141-143], while in the United States 800 mcg is standard [5]. Women considered at high risk of pregnancy loss are routinely prescribed FA doses of 4-5mg [28, 92]. FA is widely used in supplements and food fortification due to its stability, low cost, and availability [26, 144-146]. Murto et al found that elevated FA intake did not improve pregnancy outcomes in women undergoing fertility treatment who carried *MTHFR* polymorphisms [147]. The presence of UMFA has frequently been reported in populations exposed to high FA intake from supplements and food fortification [148]. However, UMFA is an imperfect biomarker of chronic FA excess, as its detection varies substantially across studies due to

differences in analytical sensitivity, limits of quantification, fasting duration, and timing of recent intake [148]. While UMFA commonly appears in the postprandial state following FA ingestion, population based studies have demonstrated that detectable UMFA can persist after prolonged fasting in a substantial proportion of individuals [148, 149]. Importantly, total FA intake correlates only weakly with circulating UMFA concentrations, suggesting that interindividual differences in FA metabolism, such as dose frequency, *DHFR* activity, and genetic variability, contribute to UMFA persistence [148, 150]. Accordingly, UMFA should not be interpreted as a definitive marker of toxicity, but rather as an indicator of folic acid metabolic saturation and altered handling, particularly in the context of chronic high intake and susceptible subpopulations [39].

1.4.1.4 Limitations of Translating Animal Research to Human Outcomes

Animal studies have provided valuable insights into FA toxicity allowing researchers to isolate variables such as diet, genetics, and environmental exposures [106, 151]. Animal studies have revealed that high-dose FA induces multi-system toxicity through distinct molecular mechanisms. Genotoxic and cytotoxic effects manifest through lipid peroxidation-mediated DNA damage in liver, blood, and plasma [152], while nephrotoxicity occurs via inflammatory cascades involving IL-6, MCP1, and TNF-α that progress to fibrosis [153]. Mechanistically, FA exposure upregulates fatty acid-binding protein 4 (Fabp4) in kidney tubular epithelial cells, driving injury through p65/STAT3 signalling pathways, and inhibition of Fabp4 mitigates renal dysfunction and fibrosis [153]. Additional mechanisms include oxidative stress, mitochondrial dysfunction, ferroptosis, and pyroptosis [154]. Critically, developmental exposure studies demonstrate that prenatal and postnatal FA

supplementation cause persistent metabolic dysfunction in offspring, characterised by insulin resistance and dyslipidaemia mediated through adiponectin suppression [155].

However, translating these findings to human populations is challenging due to genetic variability, inconsistent dosing, exposure durations and ethical constraints. Despite these limitations, animal studies have uniquely enabled elucidation of organ-specific vulnerabilities, developmental programming effects, and therapeutic targets (Fabp4, PPARγ pathway) that collectively establish high-dose FA as a multi-mechanistic toxicant affecting genotoxic, inflammatory, and metabolic pathways, findings that inform safe upper limits for human supplementation but require corroboration through human biomarker studies. As a result, human studies on FA excess rely heavily on observational designs, which are inherently vulnerable to confounding and bias [156]. Since 1998, researchers have raised concerns about the potential risks of excessive FA intake, yet there remains a lack of definitive human data to guide clinical practice. The continued absence of biologically defined thresholds for FA excess, both in terms of intake and circulating biomarkers, represents a critical gap in reproductive and public health research.

1.4.2 Fertility and Infertility

Fertility and infertility are multifactorial conditions influenced by genetic, biochemical, environmental, and lifestyle factors. This section provides an overview of key contributors to reproductive challenges, including both male and female infertility, pregnancy loss and the underlying mechanisms that may affect conception and gestational success. The subsections below explore these topics in greater detail.

1.4.2.1 Female Infertility

1.4.2.1.1 Pregnancy Loss

Miscarriage is defined as the spontaneous loss of a clinically recognised intrauterine pregnancy before fetal viability, typically before 20-24 weeks gestation [157]. Approximately ten to twenty-five percent of clinically recognised pregnancies end in miscarriage [158]. Chromosomal abnormalities are considered to be the major underlying cause [158]. Recurrent pregnancy loss (RPL) is defined as the loss of two or more clinically recognised pregnancies before twenty to twenty-four weeks gestation [157]. Although professional bodies such as the National Institute for Health and Care Excellence (NICE) [159], European Society of Human Reproduction and Embryology (ESHRE) [157], and the Australasian Certificate in Reproductive Endocrinology and Infertility *(*CREI) consensus panel [160, 161] have published extensive guidelines for the investigation and management of recurrent miscarriage, clinical practice remains highly variable and often inconsistent [162].

1.4.2.1.2 Causes of Pregnancy Loss

Chromosomal aneuploidy (abnormal number of chromosomes) is the leading cause of miscarriage, particularly in early pregnancy [161]. Aneuploidy can arise from either maternal or paternal genetic contributions and typically arise from errors during meiosis and are often sporadic [163] and are believed to affect up to sixty percent of miscarriages [164]. Sperm DNA fragmentation is increasingly recognised as a contributor to miscarriage [165, 166].

DNA methylation is a key epigenetic mechanism regulating gene expression and genomic stability [110, 167, 168]. Abnormal methylation affects genome stability and can lead to aneuploidy [169]. This process is essential for oogenesis [167], spermatogenesis [110, 111] and genomic imprinting [170].

Aberrant DNA methylation patterns in spermatozoa have been identified in couples experiencing RPL [171, 172]. *MTHFR* polymorphisms can significantly reduce enzymatic activity, impairing the conversion of homocysteine to methionine and may disrupt one-carbon metabolism, with potential consequences for DNA methylation [79], fertility and pregnancy outcomes [79-85]. The *MTHFR* gene is therefore essential for reproductive health, and its polymorphisms have been linked to infertility in both men and women [79-85]. Unfortunately for at least 50 percent of those that miscarry, no obvious cause can be found [173].

1.4.2.1.3 Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL) or recurrent miscarriage has been defined as the loss of two or more pregnancies [157, 174]. The causes of multiple miscarriages have been researched extensively and range from maternal age, chromosomal abnormalities, maternal thrombophilic disorders, structural anomalies, immune and endocrine dysfunction, obesity and more [8, 9, 13-19], yet causal underlying factors remain classified largely as idiopathic [18]. This lack of defined aetiology can create a great deal of emotional and financial burden on couples leading to isolation, anxiety and depression, therefore there is worldwide interest in reducing the incidence of pregnancy loss [8]. Most couples are referred by their GP to fertility clinics where they undergo assisted reproductive technology (ART) and in 2010 in Australia and New Zealand there were 61,774 ART cycles performed leading to 18.1% live births [20]. This low success rate often leaves women powerless, frustrated and isolated [22] and together with the financial burden of $10,275 per IVF cycle [21] we can understand the toll this takes on the couple.

1.4.2.2 Male Infertility

Globally, sperm quality has declined over recent decades, coinciding with the introduction of folic acid-fortification [175]. While the reason for loss of sperm quality remains unclear, this trend raises questions about environmental and nutritional exposures, particularly in genetically susceptible individuals. Despite contributing 50% of the DNA of the genetic material to the offspring, men are rarely encouraged to take prenatal multivitamins containing folate [176]. It is well established that a lack of folate typically defined as serum folate less than 7nmol/L or erythrocyte folate less than 150 mcg/L [98], is associated with impaired sperm concentration, reduced morphology, increased DNA damage and poorer pregnancy outcomes [177-180]. Conversely sperm concentration, quality and pregnancy rates improve with physiological folate [181, 182] and micronutrient supplementation [183-185]. These findings highlight the epigenetic vulnerability of sperm and the importance of testicular methylation in male fertility [171]. One study, combining a human clinical cohort with controlled mouse experiments, showed that high dose folic acid supplementation (5 mg/day in men; 10× and 20× dietary equivalents in mice) induced sperm DNA hypomethylation and that the effect was magnified when *MTHFR* function was reduced [34]. A previous review found strong associations between *MTHFR* genotypes and male infertility, including azoospermia and oligozoospermia [85]. The *MTHFR* A1298C polymorphism demonstrates consistent associations with male infertility across diverse populations, particularly for azoospermia and severe oligozoospermia. A meta-analysis of 7 case-control studies found significant associations between the 1298C allele and male infertility susceptibility (OR=1.12), with stronger effects specific to azoospermia in both homozygote comparison (OR=1.66) and recessive models (OR=1.67) [186]. Individual

studies reported odds ratios ranging from 1.64 to 3.45 for the CC genotype [187] in severe oligozoospermia and azoospermia [188, 189]. The C677T polymorphism shows more population-specific effects, with consistent positive associations in East Asian (OR=1.72-2.68) [190] , South Asian [191], and Brazilian populations (OR=1.95-2.68) [82], but null results in Western European studies [192]. These associations operate through altered folate metabolism affecting DNA synthesis and methylation during spermatogenesis [187], with evidence that adequate dietary folate intake can modify genetic risk [191]. The strongest evidence supports an association between both *MTHFR* polymorphisms and severe male infertility phenotypes, though the magnitude and consistency of effects vary by population ancestry and nutritional context. These findings highlight the need to include male partners in folate-related interventions and support the rationale for male-inclusive preconception strategies and personalised supplementation for males in couples affected by RPL.

1.4.2.3 Systemic Gaps in RPL Care

RPL is not only a clinical condition but also a deeply personal and often traumatic experience [141-143]. Despite its prevalence, couples experiencing RPL frequently report feeling unsupported, dismissed, and emotionally isolated [141-143]. The absence of a clear diagnostic pathway, particularly in cases deemed “unexplained”, can lead to disenfranchised grief and frustration with fragmented care systems [193].

Although the existing national framework guiding the management of RPL is recent, it does not outline clear pathways for where couples should seek care [26, 144-146]. Couples often encounter uncertainty about where to seek care, whether through general practitioners, fertility specialists, emergency

departments, or early pregnancy assessment services (EPAS) [194]. This lack of clarity reflects broader systemic issues, including poor coordination between primary and hospital-based care, limited access to dedicated RPL appointments, and inconsistent inclusion of both reproductive partners in treatment planning [44, 57, 61, 87, 195, 196].

Clinical protocols tend to focus on diagnostic testing and exclude psychosocial support, nutritional counselling, and genetic screening [44, 57, 61, 87, 195, 196]. Male partners are rarely considered [106, 151], despite contributing half of the genetic material to the offspring and being equally affected by the emotional toll of pregnancy loss [197, 198]. The lack of trauma-informed, patient-centred care models further compounds the distress experienced by couples navigating RPL [79, 92, 94, 199-201].

These systemic gaps underscore the urgent need for integrated, multidisciplinary approaches to RPL management, ones that recognise the emotional, genetic, and biochemical complexity of the condition. This thesis responds to that need by exploring personalised folate supplementation strategies and advocating for inclusive care frameworks that support both partners.

1.5 Research Gap

Despite widespread FA supplementation policies and the success of fortification programmes in reducing NTDs, emerging evidence suggests that excess intake may pose risks such as increased asthma risk, small for gestational age, oxidative stress, reduce natural killer cell activity and male infertility, particularly in individuals with impaired folate metabolism due to *MTHFR* polymorphisms. Current guidelines do not account for genetic variability, nor do they address the accumulation of UMFA or its potential

impact on fertility and pregnancy outcomes. Additionally, male partners are often excluded from preconception care and couples experiencing RPL face fragmented, emotionally unsupported care pathways. These gaps highlight the need for a more personalised, inclusive approach to folate supplementation. This thesis hypothesises that personalised folate supplementation, particularly the use of 5-MTHF in individuals with *MTHFR* polymorphisms, may offer a safer and more effective approach to managing RPL and improving reproductive outcomes.

1.6 Chapter Conclusion

This chapter has established the clinical and scientific rationale for investigating personalised folate supplementation in couples experiencing RPL. It has highlighted the limitations of current FA dosing practices, the potential consequences of UMFA accumulation, *MTHFR* polymorphisms and the underrepresentation of male partners in preconception care. It has examined current FA dosing practices and the limitations of biomarker thresholds used to guide supplementation. In response to these gaps, the chapter introduces 5-MTHF as a metabolically active form of folate that may offer advantages for individuals with impaired folate metabolism, such as those with *MTHFR* polymorphisms.

This chapter also introduced the thesis structure and research design, which progresses through a series of interlinked phases. The systematic reviews in Chapter 2 provide foundational evidence regarding FA exposure and its potential risks. Chapter 3 outlines the methodology for the pilot RCT and embedded qualitative study, which assesses the feasibility and acceptability of 5-MTHF supplementation. Chapter 4 presents the RCT results, while Chapter 5 explores patient narratives to contextualise the feasibility findings.

Chapter 6 investigates practitioner perspectives to understand current prescribing practices and implementation barriers. Chapter 7 integrates findings across all phases, and Chapter 8 concludes with recommendations for clinical practice, policy reform, and future research.

Together, these chapters form a cohesive investigation into how personalised folate supplementation may improve outcomes for couples with RPL. The following chapter begins this journey by reviewing the evidence base for FA exposure and its potential health implications.

Chapter 2 Literature Review

2.1 Chapter Introduction

This chapter presents two systematic literature reviews that form the foundational evidence base for the thesis. These reviews were designed not only to explore existing knowledge gaps but also to justify the structure and sequence of the subsequent research phases. As such, this chapter plays a critical role in linking the broader thesis aim, evaluating the feasibility and clinical relevance of personalised folate supplementation in couples with RPL, to the specific research questions and selection of research methods that follow.

The first review investigates FA exposure among women of reproductive age, particularly in countries with mandatory FA food fortification of flour and grain products. It assesses whether women in preconception and pregnancy are exceeding the upper tolerable intake (UL) of 1,000 mcg/day and highlights the potential for overexposure when FA based supplements are used alongside fortified foods. These findings raise important questions about current public health recommendations and the safety of widespread FA use, especially in populations with genetic polymorphisms affecting folate metabolism.

Building on this, the second review examines the biological risks associated with excessive FA intake and elevated blood folate concentrations. It explores associations with gestational diabetes, neurodevelopmental disorders, and other adverse outcomes, providing further justification for investigating alternative folate forms such as 5-MTHF.

2.2 Women Taking a Folic Acid Supplement in Countries with Mandatory Food Fortification Programs May Be Exceeding the Upper Tolerable Limit of Folic Acid: A Systematic Review

2.2.1 Publication Details

This paper was accepted for publication in Nutrients and published in June 2022 (Appendix 1)

Ledowsky C, Mahimbo A, Scarf V, Steel A. Women Taking a Folic Acid Supplement in Countries with Mandatory Food Fortification Programs May Be Exceeding the Upper Tolerable Limit of Folic Acid: A Systematic Review. *Nutrients*. 2022 Jun 29;14(13):2715. doi: 10.3390/nu14132715.

2.2.2 Introduction

Governments worldwide encourage women of childbearing age to take folic acid (FA) prior to pregnancy to avoid neural tube defects (NTDs) [1-5, 25-28, 202] and some governments (e.g., Australia, Canada and the United States) have implemented a FA food fortification programme to ensure that women who unintentionally conceive or are not taking a folic acid supplement have sufficient folate to prevent neural tube defects [30, 31]. Since the mandatory folic acid fortification programme began in 2009, Australian policy makers have recommended 400 mcg of FA in addition to fortification for individuals considering conception [25].

While the fortification policy has prevented NTDs, emerging research has documented the presence of unmetabolised folic acid (UMFA) in the serum in people in countries that have a folic acid-fortification programme and/or take a FA supplement [44-46]. While the effect of this is unclear, some have raised concerns regarding UMFA and the potential for adverse health effects as stated previously [87, 196, 203-206]. While folic acid fortification has

delivered substantial public health benefits, particularly in reducing neural tube defects, ongoing debate remains regarding optimal dosing, form, and long-term metabolic effects in the context of chronic exposure, particularly given emerging evidence of dose dependent accumulation of unmetabolised folic acid and the absence of clear causal outcome data [207].

2.2.2.1 Folate Metabolism

Folate-dependent biochemical processes are influenced by the form and bioavailability of folate [78]. Folate is a generic term for vitamin B9 that includes several forms, including that naturally found in food (natural folate). All folate forms have a common structure but differ based on the pteridine ring and whether it is reduced or oxidised [89]. Folate derived from food such as leafy green vegetables are polyglutamates (Figure 1), whereas synthetic FA is a monoglutamate (Figure 2) [87].

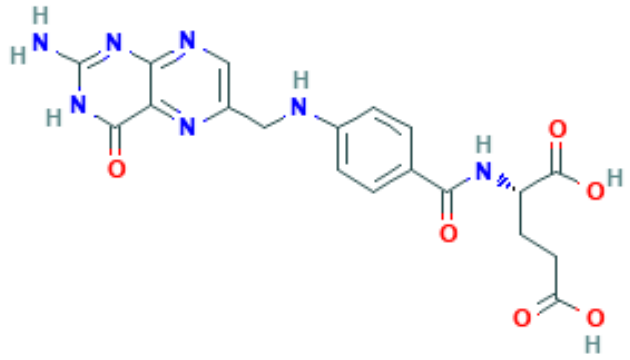

Figure 1- Folic acid Structure

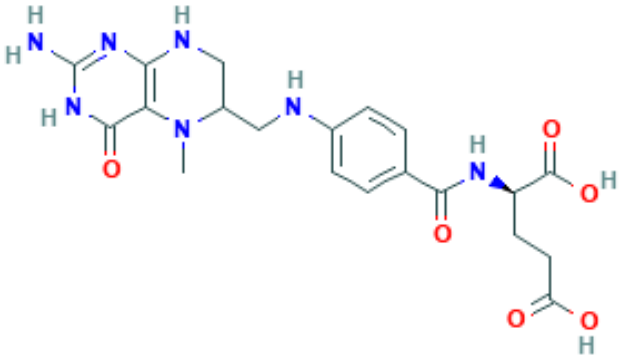

Figure 2 - 5-MTHF structure

Food folate is naturally present in foods such as leafy green vegetables and is in a reduced form [44]. This natural folate exists in a polyglutamate form and needs to be further hydrolysed to a monoglutamate form by the intestinal lumen to be transported [86]. The intestinal absorption of polyglutamate derivatives of tetrahydrofolate (THF) into monoglutamates is carried out by the brush border enzyme glutamate carboxypeptidase II (GPCII) [94]. This crossing of the apical brush border is carried out via two transporters, the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC) [208, 209]. Most of the absorbed natural folate is metabolised to 5-methyltetrahydrofolate (5-MTHF) in the intestine and/or liver because the RFC has a higher affinity for reduced folates such as 5-MTHF compared to FA [208]. The fate of both natural folate and FA is to be metabolised to 5-MTHF [210]. The RFC transporter is found throughout the intestinal tract and is highly expressed in the liver and placenta [208]. The PCFT's key role is as the major transporter of dietary folate in the proximal small intestine, specifically in the brush border of the duodenum and jejunum and has an optimal function with an acidic PH but has a higher affinity to FA than it does to reduced folates such as 5-MTHF [196, 208-211]. Once absorbed by the RFC or PCFT, the folate is transported into the hepatic portal vein by

multidrug resistance-associated protein 3 (MRP3) and then to the liver, where it is taken up via hepatocytes [208]. The intestine can convert reduced folates to 5-MTHF quite well; however, it is the first-pass metabolism and intestinal absorption where the difference between the metabolism of FA and the reduced folate forms exists as its reduction and methylation are dose dependent [45, 210].

While natural folates do not need to be converted to 5-MTHF to cross the mucosal cell (figure 3) and enter the portal vein to the blood, they do need to be metabolised to 5-MTHF in the liver or released into the blood or bile [209]. One small study showed that the majority of FA (at a physiologic dose) passes into the portal vein in an unmodified form, whilst monoglutamates are almost all converted to 5-MTHF [212]. All forms of folate must be converted to 5-methyltetrahydrofolate (5-MTHF), which is the main form of folate found in plasma, accounting for approximately 98% of total serum folate [89, 196]. Where natural folates can be converted directly to 5-MTHF directly, FA relies on the *DHFR* enzyme to be reduced [86, 87]. The most extensively studied *DHFR* polymorphism is the 19bp deletion variant [213] with a prevalence of around 23% [214]. The activity of *DHFR* is slow and easily saturated in the first step conversion to dihydrofolate and therefore when the dose exceeds the capacity of the enzyme or there is a *DHFR* polymorphism, an increase in free FA in plasma may be seen [209]. The main circulating form of folate in the blood, 5-MTHF, is in high demand during fetal development as DNA synthesis and cell division are increased [208]. The folate receptor-α (encoded by the folate receptor 1 (FOLR1) gene) is expressed on epithelial cells and ensures uptake of 5-MTHF by the placenta and the brain during development. This receptor is upregulated in pregnancy due to the rapid

increase in folate-dependent activities such as tissue growth, placental development, and enlargement of the uterus [208, 215]. 5-MTHF donates its methyl group to support DNA methylation via DNA methyltransferase enzymes and 5-MTHF is directly linked to the viability of the embryo and the ability to maintain DNA methylation patterns in replicating cells [208, 216]. A lack of 5-MTHF may therefore compromise DNA methylation, cause uracil misincorporation, chromosomal breaks, impaired ovarian follicle development, and increased risk of pregnancy loss [215]. Folate metabolism can be impaired in people who carry the methylenetetrahydrofolate gene (MTHFR) polymorphisms, resulting in reduced enzyme activity and therefore a reduction in 5-MTHF [115, 200, 201]. The *MTHFR* C677T and A1298C polymorphisms have been linked to infertility in both men and women [79-85]. This is particularly pertinent given that studies in mice show that excess FA reduces MTHFR activity irrespective of MTHFR polymorphisms thereby reducing methylation capacity. Research has identified 5-MTHF as a possible alternative for FA in those with *MTHFR* polymorphisms as doses of 800 mcg have been shown to bypass the *MTHFR* enzyme [86, 89-94].

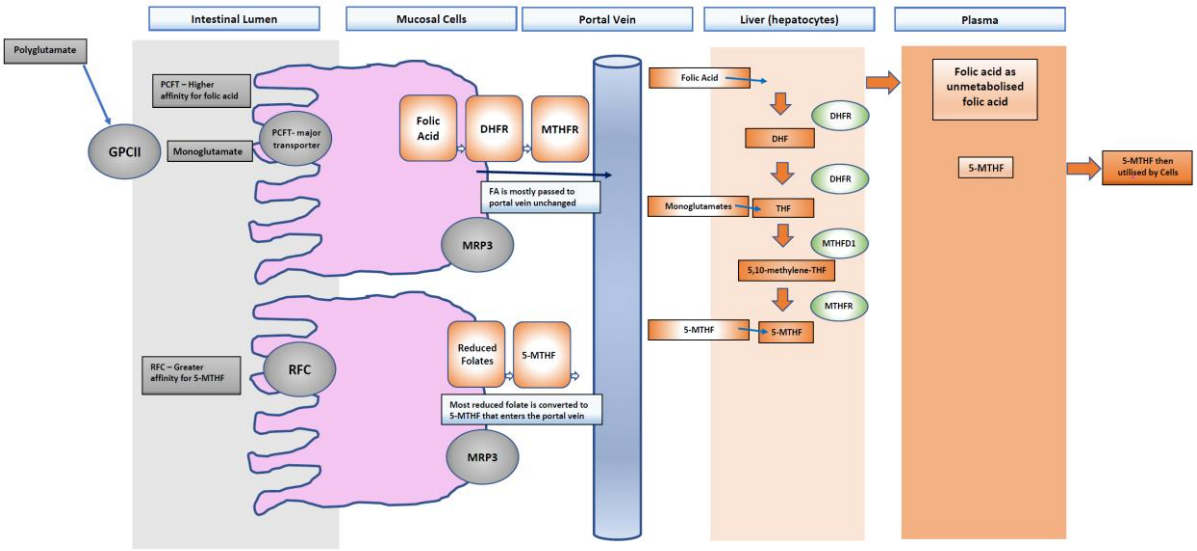

Figure 3 - Polyglutamate conversion and absorption

Polyglutamates are converted to monoglutamates by glutamate carboxypeptidase II GPCII and absorbed by the reduced folate carrier (RFC) or the proton-coupled folate transporter (PCFT). Almost all the reduced folates are converted into 5-MTHF and transported to the portal vein by multidrug resistance-associated protein 3 (MRP3) to the liver and blood. Folic acid is mostly passed unchanged to the portal vein, where it needs to be metabolised in the liver to dihydrofolate (DHF) by the enzyme dihydrofolatereductase (DHFR) and to tetrahydrofolate (THF) also by DHFR and finally to 5-methyltetrahdyrofolate (5-MTHF). What is not converted will pass into the plasma.

2.2.2.2 Folic Acid Dose

Although mandatory fortification exists in some countries, the fortification levels per 100 mg of food differs between countries, with no upper limit on the number of foods that may be fortified voluntarily, easily leading to an intake level above the original estimated level of 100 mcg per day [30, 31, 143, 202, 204, 217-220].

FA is widely used in supplements and food fortification as it is relatively inexpensive, stable, and freely available [26, 144-146]. Women are encouraged by governments to take FA supplements during preconception and pregnancy in addition to any folate they may consume through natural folate and fortified foods. Doses of 400–500 mcg are recommended and included in prenatal multivitamins by the Department of Health in Australia [221], whereas in the United States, the Centers for Disease Control and Prevention recommends 800 mcg per day [5, 25, 26] and in Canada at least 1000 mcg of FA are typically found in a prenatal multivitamin [141-143].

Women at high risk of pregnancy loss or who had a previous pregnancy affected by a NTD, are routinely prescribed FA doses of 4–5 mg in Australia, Canada, and the United States [25, 26, 28, 92]. FA is considered to be better

absorbed and therefore a measurement, known as *total food folate daily folate equivalent (DFE)* (see Box 1), has been devised by the Institute of Medicine and is the recommended method to provide comparability between synthetic FA and natural food folates whereby the folate from fortified food is weighted 1.7-fold greater than naturally occurring folate [1, 222]. For example, a serving of food from leafy greens may provide 100 mcg of folate, which equals 100 mcg DFE; however, 100 mcg of folic acid in a fortified food is estimated to be 170 mcg DFE.

Box 1. Daily folate equivalent (DFE).

Total food folate DFE = mcg natural food folate mcg + synthetic folic acid from fortified foods and supplements times a bioavailability factor of 1.7 = mcg DFE/day (natural food folate) + (folic acid mcg × 1.7) [1, 2] or 1 DFE equals 1 mcg food folate, 0.6 mcg synthetic folic acid consumed with food or 0.5 mcg consumed on an empty stomach with fortified food [3-5].

2.2.2.3 Upper Tolerable Limit of Folic Acid

Unlike 5-MTHF (and natural folate), FA has an upper tolerable limit (UL), which the US Institute of Medicine sets at 1000 mcg due to the potential for high FA intake to mask a vitamin B12 deficiency in the user [223]. The UL does not include naturally occurring folate from food or 5-MTHF as there is no recognised UL for these forms [210]. The studies used to determine this upper limit do not consider the potential impact of elevated FA intake on folate metabolism [196]. Apart from masking a vitamin B12 deficiency, the only documented risk factor for excessive FA intake is the presence of unmetabolised folic acid (UMFA) [44, 57, 87, 196, 206, 224]. UMFA is the FA that cannot be metabolised to 5-MTHF due to limited *DHFR* activity and, therefore, starts to appear in the circulation even with 200 mcg of FA acid intake per day [225, 226]. This UMFA has been linked to adverse health effects including reduced brain function and motor and somatosensory

processing in offspring [227], cleft lip [228], asthma [219, 229], autism spectrum disorder [230] anaemia [51], natural killer cell cytotoxicity [57], adverse cardiac events and cancer [87] and cognitive impairment [51].

There is a risk that women may be exposed to unintended adverse health outcomes through excess FA intake; for this reason, it is imperative that preconception and pregnancy folate intake from all sources for women of childbearing age is more clearly understood.

2.2.3 Methods

This review aimed to assess the total all-source intake of folate in women of childbearing age and in pregnancy in high-income countries with folate food fortification programs.

This systematic review was designed in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines.

We conducted a detailed search of the following electronic databases: MEDLINE (OVID), Embase, CINAHL (EBSCO), Scopus, and BiblioMAP. The search strategy is attached (Appendix 3, Search Strategy) and was conducted on the 7th and 8th of October 2021. It included countries identified by the World Bank as high-income countries, pregnant women or women in preconception, folate exposure and source of folate.

Studies published from 1998 until the search date of 8 October 2021 were included. We wanted to include only studies that reflected total folate intake and therefore the start date of 1998 was selected as the date that mandatory folic acid-fortification began in the U.S. and other countries around the world [202, 225, 231]. Scientific posters and conference abstracts were not

included. There were no language limits. A PICO(T) format was used to formulate the search strategy and help design the research question. The population of interest (P) was identified as women of childbearing age either in preconception or pregnancy period from high-income countries (as identified by the World Bank [231] that have a mandatory fortification policy of fortifying foods with FA. The intervention/exposure (I) was folate intake of any form, that is dietary folate from non-fortified foods, dietary intake from fortified foods and/or supplements. A comparator (C) was not included. We excluded control studies and intervention studies except where the control group was representative of the study group and nutritional/supplemental data were available. The outcome (O) measured was intake of folate in any form in women of childbearing age, whether in preconception or pregnancy. This included folate from natural food sources such as leafy green vegetables, FA from fortified foods, and all folate forms in supplementary dietary products and multivitamins.

Titles and abstracts were reviewed by one reviewer (CL). Manuscripts were included if they were from epidemiological observational studies that explicitly reported on folic acid intake in women of reproductive age, specifically in preconception and pregnancy. There were no language restrictions. Studies were excluded if they did not report on folate intake (rather than folate status), included women who were breastfeeding, and were not from countries with a mandatory fortification policy.

Full texts were reviewed and assessed based on the inclusion and exclusion criteria above. If full texts could not be accessed, we contacted the authors and/or publisher and if the full text could not be located, they were excluded. Any uncertainty about the inclusion of a study was discussed by the other

authors (Amie Steel, Vanessa Scarf and Abela Mahimbo), and consensus was gained.

Figure 4 presents the flowchart of study selection and inclusion. A total of 1885 studies were identified. After removing duplicates (*n* = 780) and excluding citations by title and abstract (*n* = 648), 457 full-text studies were assessed for eligibility. Of those, 421 were excluded because they were either not measuring folate intake, not from a country that had a mandatory fortification policy, not an academic paper, or had an incorrect study design or date range. This left a total of 36 studies for inclusion.

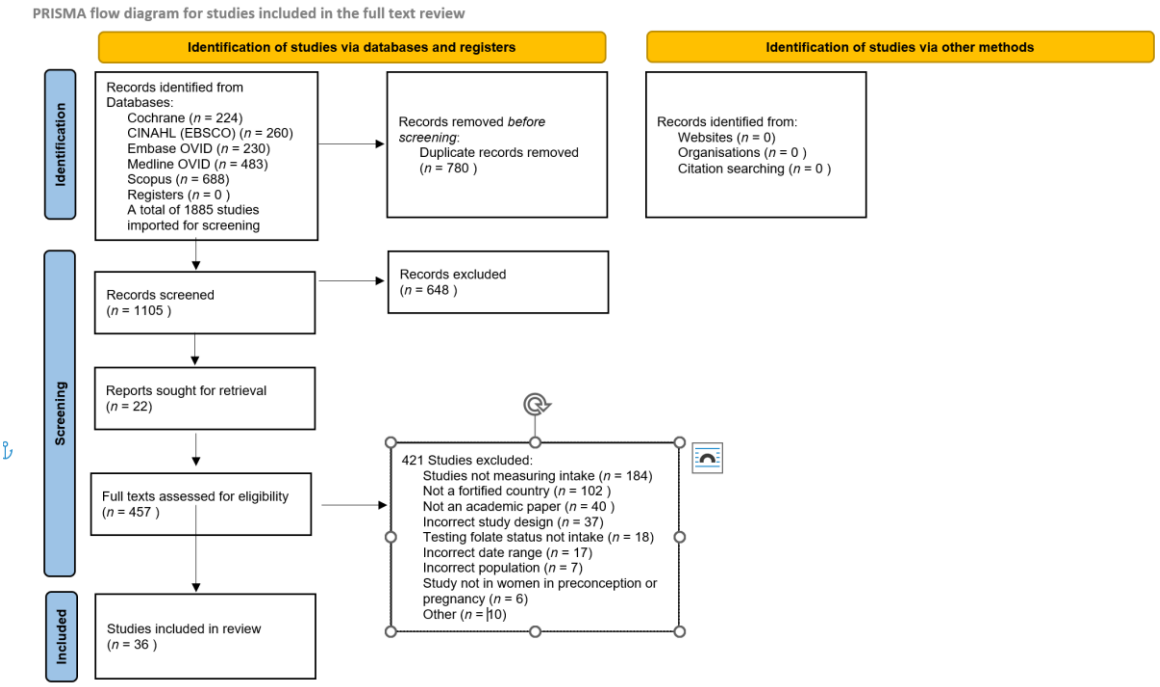

Figure 4 - PRISMA-P flowchart of study selection [232].

2.2.4 Quality Assessment of Study

Critical appraisal of the studies was conducted using the Axis tool for cross-sectional studies (Appendix 3). The studies were assessed for key issues such as suitability of the study to answer the research question, and possibility of bias being introduced into the study. All studies except five [2, 3, 229, 233, 234] detailed funding sources and sources of conflict. All studies stated clear aims and objectives, and the study design was appropriate for the aim.

The few areas where data were lacking was in the sample size justification with twenty [2, 3, 141, 142, 216, 220, 222, 226, 234-245] of the thirty-six studies giving data on sample size justification. Another question that was not answered by many studies was the area of non-responders; few studies (*n* = 9) [3, 142, 216, 226, 229, 233, 239, 242, 245] gave information about non-responders. Twenty-one of the thirty-six studies addressed and categorised non-responders. Clarity around statistical significance and/or precision estimates was also lacking.

Table 1- Search string for Literature review 1

Databases	Search string
MEDLINE (OVID) Embase (OVID) CINAHL (EBSCO) Scopus BiblioMAP	Search strategy: Andorra OR "Antigua and Barbuda" OR Aruba OR Australia OR Austria OR Bahamas OR Bahrain OR Barbados OR Belgium OR Bermuda OR "British Virgin Islands" OR Brunei OR Canada OR "Cayman Islands" OR "Channel Islands" OR Chile OR Croatia OR Curacao OR Cyprus OR "Czech Republic" OR Denmark OR Estonia OR "Faroe Islands" OR Finland OR France OR "French Polynesia" OR Germany OR Gibraltar OR Greece OR Greenland OR Guam OR "Hong Kong" OR Hungary OR Iceland OR Ireland OR "Isle of Man" OR Israel OR Italy OR Japan OR Korea OR Kuwait OR Latvia OR Liechtenstein OR Lithuania OR Luxembourg OR Macao OR Malta OR Monaco OR Nauru OR Netherlands OR "New Caledonia" OR "New Zealand" OR "Northern Mariana Islands" OR Norway OR Oman OR Palau OR Poland OR Portugal OR "Puerto Rico" OR Qatar OR "San Marino" OR "Saudi Arabia" OR Seychelles OR Singapore OR "Sint Maarten" OR "Slovak Republic" OR Slovenia OR Spain OR "St. Kitts and Nevis" OR "St. Martin" OR Sweden OR Switzerland OR "Trinidad and Tobago" OR "Turks and Caicos Islands" OR "United Arab Emirates" OR "United Kingdom" OR "United States" OR Uruguay OR "Virgin Islands" AND pregnancy OR preconception care OR periconceptional OR pre-pregnancy OR prenatal care OR perinatal Care OR pregnant woman AND Folic acid OR folvite OR pteroglutamic acid OR vitamin B9 OR vitamin M OR unmetabolised folic acid OR unmetabolized folic acid OR folate AND Food, fortified OR food supplemented OR dietary supplements OR biofortification OR food supplements OR food assistance OR nutrition surveys OR health surveys OR prenatal supplement

2.2.5 Results

The 36 studies included in this review were separated into two categories, those sampling women of childbearing age (*n* = 18) and those sampling pregnant women (*n* = 21). Three studies were included in both categories. The study designs were predominantly cross-sectional or cohort and provided data about the folate intake from all sources in the sample populations. The analysis of these studies identified four key areas of discussion or themes: natural folate intake, food fortification intake,

supplement intake, and the rate of women exceeding the upper tolerable limit of 1000 mcg FA.

2.2.5.1 Study Characteristics

2.2.5.2 Women of Childbearing Age

Table 2 shows summarised data from the 18 studies sampling non-pregnant women of childbearing age [1-5, 216, 222, 234, 239, 240, 243, 244, 246-250]. The age of women included in the studies was 17–49 years of age and sample sizes ranged from 51 participants to 9707, with a total of 52,580 women being sampled across all studies. All studies were from the United States (n = 15), Canada (n = 2) and Australia (n = 1).

2.2.5.3 Pregnant Women

Table 3 shows summarised data from 21 studies sampling pregnant women [141-143, 217, 219, 226, 229, 233, 235-238, 240-242, 245, 249, 251-254]. Two studies included women in their first trimester [233, 237], nine studies included women in either their first or second trimester [142, 217, 226, 229, 236, 238, 242, 253, 254], one study included women with less than 26 weeks' gestation [219], one study less than 27 weeks' gestation [141], three studies in their second or third trimester [143, 249, 252] and five studies at any stage of gestation [235, 240, 241, 245, 251]. The sample size ranged from 51 participants to 2146, with a total of 16,314 sampled across all studies. All studies were from Canada (n = 8), United States (n = 7) or Australia (n = 7). One study was conducted before the mandatory folic acid fortification programme commenced [238].

2.2.5.4 Natural Food Folate and Fortification

In women of childbearing age, the recommended intake of 400 mcg folic acid per day was not met by any study from natural folate sources (see Table 2).

One U.S study found that fortified cereals and grains contribute approximately 70.8% of dietary folate [5]. The studies showed that after mandatory folic acid fortification, the mean intake of folate from natural food folate ranged from 228.5 to 324.3 mcg/day [234], and serum folate concentrations increased by approximately 50% and RBCF concentrations increased by approximately 59% compared with pre-fortification levels [220]. Another study showed that 43% of folate came from both natural and fortified foods [247]; however, food alone did not achieve the recommended 400 mcg/day for women in childbearing years. One study that investigated only food sources of folate found that 80% of women of reproductive age did not meet the recommended daily allowance (RDA) of 400 mcg per day [249], while another study showed that only 23% of women achieved the RDA of 400 mcg per day [245].

In Australia, two studies by Dorise and Beringer (Table 3) found that only 20% [236] and 55% [251] of pregnant women met the folic acid recommendations from diet alone (including natural food folate and fortified foods), respectively. In another study, folate was consistently below the estimated average requirement (EAR) of 520 mcg [240] particularly in the periconceptional period [242]. In Canada, 70% of women did not meet the EAR of 520 mcg of folate in the first trimester of pregnancy [237] nor was the dietary intake from food or fortification sufficient to achieve the minimum 400 mcg/day FA recommendation [217].

Table 2- Included studies sampling women of childbearing age, not pregnant.

Author/ Year	Location	Study Design	Study Period	Population Description	Total Number of Participants	Objective of the Study	Summary of Key Findings in Relation to Folate
Cena 2008[1]	United States	Cross-sectional study	Prior to 2008	Women 18–45 years	157	To assess folate intake among low-income, non-pregnant women of childbearing age	* ~85% met RDA for folate * 4 exceeded UL of 1000 mcg FA/day food, fortified foods and supplements
Crider 2018[216]	United States	Cross-sectional study	2007–2012	Women 12–49 years	4783	To estimate the usual daily FA and RBCF to prevent NTDs	* If women only have fortified foods > risk of NTDs. * Require additional FA intake to achieve 400 mcg/day
Dietrich 2005[3]	United States	Cross-sectional study	1999–2000	Women 20–39 years	2260 NHANES III 356 NHANES 1999–2000	To explore the changes in serum and erythrocyte folate following FA-fortification	* Fortification increased the serum folate to acceptable levels * <10% reach RBCF to reduce NTD risk
French 2003[4]	Canada	Cross-sectional study	2001–2001	Women 18–45 years	148	To estimate folate intake and knowledge in women of childbearing age, in relation to risk of NTDs	* 7% exceeded the UL * Fortification does not cause >UL, supplements do
Gaskins 2012[5]	United States	Cohort study	2005–2007	Women 18–44 years	259	To evaluate the association between dietary FA intake and hormones in healthy women	* Mean dietary intake of 500 mcg achieved without supplementation (FA 50.8%/49.2% natural folate * 29.1% of dietary folate came from fortified cereals 41.1% from fortified grains * 18.1% from vegetables and 11.7% from beans.
Gaskins 2014[248]	United States	Cohort study	2006–2013	Women 18–46 years	316	To evaluate the association of folate with ART outcomes	* ~57% women's folate came from supplements * 78% took supplements with 400 mcg FA, 19% 1000 mcg * 43% from foods (both natural and fortified)
Gaskins 2019[247]	United States	Cohort study	2004–2017	Women 18–46 years	513 ART cycles from 304 women.	To evaluate folate intake, air pollution and livebirth in women using ART	* 20% of women consumed 1000 mcg of supplemental FA
Hamner 2013[239]	United States	Cohort study	2001–2008	Women 15–44 years	5369	FA-fortification to increase folate concentrations in Mexican women with lower acculturation	* 24.0% women had FA intake of 400 mcg
Hure 2009[240]	Australia	Cross-sectional study	2003–2003	Women 25–30 years	9076 (606 pregnant)	To investigate and report the diet quality of young Australian women by pregnancy status	* Folate < EAR * Dietary deficit * No supplements recorded
Jun 2020[241]	United States	Cross-sectional study	1999–2014	Women 20–44 years	8096	To estimate dietary supplements, use and prevalence	* 44.8% used dietary supplements * Only supplements measured

Marchetta 2016[243]	United States	Cross-sectional study	2001–2010	Women 15–44 years	4985 NHW and MA non-pregnant women	To assess the differences in serum and RBCFRBCF concentrations by acculturation factors	* Supplements with FA impact blood folate status * Acculturation factors impact folate concentrations * RBCFRBCF concentrations indicate long-term status
Mojtabai 2004[234]	United States	Cohort study	1999–2000	Women 17–49 years	1351	To discover the impact of BMI on serum folate	* Fortification increased FA levels from mean 228.5 to 324.3 mcg/day * Serum folate increased due to FDA mandate of fortification.
Pick 2005[249]	Canada	Case control study	Prior to 2004	Women 20–40 years	112	To examine the diets of healthy women	* FA + natural folate in food does not achieve recommended 400 mcg/day * 80% of women did not meet the RDA for folate * Supplements not included
Rai 2015[244]	United States	Cohort study	2003–2008	Women 19–50 years	3641	To evaluate nutritional status in women of childbearing age and ethnicity	* RBCF cut off for deficiency is 200 nmol/L * Most women achieved minimum in relation to folate deficiency but few reached 906 nmol/l for prevention of NTDs * RDA of 400 mcg folate 90% did not achieve RBCF concentrations to prevent NTDs * At 1000 mcg/d, less than 25% of women had RBCF concentrations that prevent NTDs
Sotres-Alvarez 2013[250]	United States	Case control study	1997–2005	Women childbearing age	1047 cases with NTDs 6641 CHD 6123 nonmalformed controls	Dietary intake and NTDs and CHDs	* Non-users of FA/multivitamin supplements who ate more fruits and vegetables significantly less likely to have NTDs * Supplements not included
Tinker 2012[2]	United States	Cohort study	2003–2008	Women 15 to 44 years	4272	BMI supplemental FA intake and folate status	* Women 25–44 years age more likely to use FA supplements than 15–24-year-olds * Non-users and users of dietary supplements similar FA intakes of DFEs * BMI may affect body distribution of folate
Tinker 2012[222]	United States	Cross-sectional study	2003–2004, 2005–2006, 2007–2008 NHANES-2008	Women childbearing age	4272	We sought to model FA intake under various fortification and supplementation scenarios	* UL influenced by supplements * Median intake influenced by enriched cereal grain products * 23% of women achieve RDA of FA * 2.4% exceed the UL
Yang 2007[220]	United States	Cohort study	2001–2002	Women 14–49 years	1685	To examine FA intake in women of childbearing age in the United States	* Average serum folate concentrations show 50% increase since fortification * RBCF concentrations show 59% increase since fortification

	* 47.5% of FA intake from supplements * 5.7% women consuming supplements and fortified foods exceeded the UL * Underreporting of FA from fortified foods

Legend 1- ART = assisted reproductive cycles, BMI = body mass index, CHD = congenital heart defects, DFE = daily folate equivalent, EAR = estimated average requirement, FA= folic acid, NTDs = neural tube defects, RBCF = red blood cell folate, RDA = recommended daily intake, UL = upper tolerable limit of folate (1000 mcg).

Food fortification and natural folates contributed approximately 303 mcg/day preconceptionally [253] and in pregnancy ranged from 282.2 mcg dietary folate equivalents (DFE)/day [245], 483 mcg DFE/day in early pregnancy [226] to 331 mcg/day in women 20–38 weeks' gestation [249], and 465 mcg DFE/day in late pregnancy [226].

Table 4 outlines women's folate intake by folate source or type. Of those studies that only measured natural food folate intake, women of childbearing age consumed between 170.92 and 259 mcg per day, and pregnant women consumed between 140 and 483 mcg DFE. The average intake of folate from natural food folate was 205.48 mcg [1, 4, 5, 216, 250] in these studies.

Synthetic folic acids from fortified foods contributed 181.7 to 239 mcg per day in non-pregnant women and 96 to 768 mcg per day in pregnant women.

The combined food folate of natural and synthetic folic acid based on the conversion of folates to daily folate equivalents was as high as 500.5 mcg DFE (369.9 mcg) in women of preconception age [5] and 627.6 mcg DFE per day in pregnant women [245].

Table 3- Included studies sampling pregnant women

Author/ Year	Location	Study Design	Study Period	Population Description	Total Number of Participants	Objective of the Study	Summary of Key Findings in Relation to Folate
Bailey 2019[235]	United States	Cross-sectional study	2001–2014	Pregnant women 20–40 years at all stages of gestation	1003	To estimate nutrient intakes (from foods and dietary supplements) and prevalence of meeting or exceeding RDA among pregnant U.S. women	* Supplements responsible for UL being exceeded * Supplements required for pregnant women to meet RDA * 33.4% exceed UL FA
Beringer 2021[251]	Australia	Cross-sectional study	2009–2019	Pregnant Indigenous women in all stages of gestation	152	To determine sources of key nutrients contributing towards nutritional adequacy during pregnancy	* 75% met EAR * 55% met folate EAR from diet alone * ~51% of women took a supplement containing folate
Boeke 2013[233]	United States	Cohort study	1999–2006	Pregnant women in T1/T2	1896	To examine maternal T1/T2 dietary intake of methyl donor nutrients during pregnancy in relation to child visual memory	* Second trimester folate intake average was 1268 mcg/day
Dorise 2020[236]	Australia	Retrospective study	2015–2015	10–18 weeks' gestation	231	To evaluate the effectiveness of a group-based outpatient dietary intervention in pregnancy to reduce excessive gestational weight gain	* Supplement required to meet EAR * 20% met with folate EAR from diet alone * Mean folate intake 533 mcg/day NB: measuring food (adjusted for supplementation)
Dubois 2017[237]	Canada	Cohort study	2010–2012	Pregnant women T1	1533	To assess nutritional intakes during pregnancy by examining dietary sources and supplements and comparing them to RDI	* 70% did not meet EAR with diet * With supplements 87% exceeded the UL (diet and supplement sources)
Furness 2013[238]	Australia	Prospective observational study	Prior to 2009	Pregnant women < 20 weeks' gestation	46 low risk and 91 high risk women = total of 137	To determine if methyl donor nutrients < 18–20 weeks' gestation are associated with subsequent adverse pregnancy outcomes. To investigate maternal B vitamin concentrations with DNA damage markers	* Older women had increased RBCF, serum folate * FA supplementation >1000 mg/day resulted in the highest RBCF * 100% of the high-risk women exceeded the UL of FA (2116 mcg mean) range 1615–2617 mcg NB: study conducted before mandatory FA-fortification from 18 September 2009 in Australia
Gómez 2015[141]	Canada	Cohort study	2009–2010	Pregnant women < 27 weeks' gestation	599	To describe the use of natural health products (NHP) by pregnant women in each	* Average FA intake was 200% above RDA in each trimester * 25% pregnant women exceeded UL in each

						trimester of pregnancy	trimester * IOM guidelines met by 97% in first trimester, decreasing to 91% in third trimester NB: supplement use only recorded
Hromi-Fiedler 2012[252]	United States	Cross-sectional study	2004–2006	Pregnant Latinas between 16 and 32 weeks' gestation	241	To document nutrient and food intakes from food sources among Latina subgroups living in the same geographical area	* Mean FA intake 768 mcg from diet alone NB supplement use not recorded
Hure 2009[240]	Australia	Cross-sectional study	2003–2003	Women aged 25–30 years at any stage of pregnancy	606	To investigate and report the diet quality of young Australian women by pregnancy status	* Folate was consistently below EAR * Only food reported/no supplements
Jun 2020[241]	United States	Cross-sectional study	1999–2014	1314 women 20–44 years of age at all stages of pregnancy	1314	To estimate the prevalence of use and the micronutrient contribution of dietary supplements among pregnant, lactating, and non-pregnant and non-lactating women	* 77% used dietary supplements * More than 60% of pregnant women used supplements with FA * Mean intakes of FA from supplements alone were at or above the RDI * Dietary supplements contributed a mean daily intake of 787 mcg of FA * >40% supplement users exceeded the UL of FA
Livock 2017[242]	Australia	Cohort study	2011–2012	Women < 19 weeks' gestation	2146	To examine overall micronutrient intake periconceptionally and throughout pregnancy	* Many women failed to meet RDI for folate in periconceptional period (fortified food not included) * UL exceeded through diet and supplemental sources esp. late in T1 * 80% of folate for over consumers coming from supplements NB: food fortification not included
Martinussen 2012[253]	United States	Cohort study	1997–2000	Pregnant women < 24 weeks' gestational age	1499	To assess whether FA intake during T1 of pregnancy is related to asthma in the offspring by the age of 6 years	* Mean intake FA ↑ from 303 mcg preconceptionally, to 404 mcg in 1st, 605 mcg in 2nd and 676 mcg in the 3rd month of pregnancy * Mean supplementation in T1 was 497 mcg * 51% women used FA supplements before pregnancy * 61% used FA supplements in first month of pregnancy * 81% in second and 88% in third month of pregnancy 92% women used FA in first trimester >800 mcg FA NB: only supplements measured. No food or fortification

Masih 2015[142]	Canada	Cohort study	2010–2012	pregnant women at <16 weeks' gestation	353	To determine dietary and supplemental intakes and major dietary sources of one-carbon nutrients	* 85% women exceeded UL FA through supplements alone * Typical dose FA in supplements 1000 mcg * Pregnant women exposed to FA 2.5-fold RDI of 400 mcg/day
Murphy 2021[217]	Canada	Case control study	2013–2015	Pregnant women 24–26 weeks' gestational age 18–44 years of age	51	This was an ancillary study within the Folic Acid Clinical Trial (FACT), a randomised, double-blinded, placebo-controlled, phase III trial designed to assess the efficacy of high-dose FA to prevent preeclampsia	* All women exceeded the WHO RBC total folate 906 nmol/L cut off for NTDs * ~80% of women were above the 97th percentile for RBCFRBCF concentrations * Folate status for all women > WHO cut off for NTD risk reduction * UMFA measurable in all women and some at high levels * High-dose FA is unwarranted for this clinical population
Murphy 2021[143]	Canada	Case control study	2011–2015	Pregnant women 8–16 weeks' gestation	1198	To evaluate the dietary and supplemental intakes of FA and to determine the proportions of pregnant women exceeding the estimated average requirement (EAR) and tolerable upper intake (UL)	* FA intake from diet (food and fortification) insufficient to achieve 400 mcg FA/d * Median food/fortification 333 mcg DFE/d * If FA supplements are added mean folate 2167 mcg DFE/day * 89.2% of participants > EAR if supplements taken * 96% exceeded UL 1000 mcg/day, 0.4% below RDA (400 mcg)
Pick 2005[249]	Canada	Case control study	Prior 2004	Women 20–38 weeks' gestation aged 20–40 years	112	The objectives of this pilot study were to examine the diets of pregnant women and healthy women of child-bearing age	* Daily dietary folate intake for pregnant women was 331 mcg/day * 98% of pregnant women did not meet minimum RDI for folate from food Note: Supplements not included in study
Plumptre 2015[226]	Canada	Cohort study	2010–2012	Pregnant women aged 18–45 years between 10 and 22 weeks' gestation	368	Determine maternal and cord blood concentrations of folate and unmetabolised folic acid (UMFA) and examine effect of maternal intakes of folate and FA and fetal genetic variants in folate metabolism on folate status	* Folate intake (natural folate and fortified foods) mean 483 mcg DFE/day early T1 and 465 mcg DFE/d late pregnancy * 83% > UL FA * Median maternal folic acid intake was 1000 mcg early T1 * Early T1, maternal plasma UMFA detected in 97% of women * Maternal serum folate concentrations significantly decreased during pregnancy, whereas RBCFRBCF significantly ↑ * UMFA detectable in 93% of cord blood samples

Roy 2012[254]	Canada	Cohort study	2002–2005	Pregnant women between 10 and 22 weeks' gestation	2019	Examine dietary intake of iron, zinc and folate, from food and supplement sources	* Mean food intake of FA 473 mcg DFE/day * Mean FA supplement intake 1338 mcg DFE/day, mean total folate 1811 mcg DFE/day * Only 16% did not reach RDA of 600 mcg DFE/day * Supplement intake > UL 1000 mcg
Shin 2016[245]	United States	Cross-sectional study	2003–2012	795 pregnant women at all stages of gestation	856	To examine relationship between pre-pregnancy weight status/diet quality and nutritional status	* Normal-weight women had a mean dietary intake of 282.2 mcg DFE/day, and dietary supplemental intake 1329 mcg DFE/day
Trivedi 2018[219]	United States	Cohort study	1999–2002	Pregnant women 1–26 weeks' gestation—T1 and T2	1279 mother-child pairs	To examine this association in the United States, where the food supply is generally fortified with FA	* Mean intake of FA first trimester 930 mcg, second trimester 1238 mcg * ~75% exceeded 400 mcg * 94% women took FA supplement
Whitrow 2009[229]	Australia	Cohort study	1998–2005	Pregnant women <16 weeks' gestation	557	To investigate the effect of the timing, dose, and source of folate during pregnancy on childhood asthma	* Median intake of FA from supplements was 2948 mcg/day * Supplements contributed to 84% of FA in early pregnancy and 63% in late pregnancy NB: Study conducted before fortification in Australia implemented

Legend 2- DFE = daily folate equivalent, EAR = estimated average requirement, FA = folic acid, NTDs = neural tube defects, IOM = Institute of Medicine, RBC = red blood cell, RDA = recommended daily allowance, RDI = recommended dietary intake, T1 = Trimester 1, T2 = Trimester 2, T3 = Trimester 3, UL = upper tolerable limit of folate 1000 mcg/day, UMFA = unmetabolised folic acid, and WHO = World Health Organisation.

Table 4- Folate intake per day by natural food intake, fortified food intake, supplements, and total intake.

Author/ Year	Natural Food Folate Intake Dietary	Food Folate and FA-Fortified Foods Combined Intake	Synthetic FA Intake—from Fortified Foods	Synthetic FA Intake from Supplements	Folate Intake Total (Food and Supplements)	Supplement Use during Study	Stage of Pregnancy/ Weeks' Gestation
Women of childbearing age/Not pregnant							
Cena 2008[1]	180.7 mcg	n/a	253.4 mcg	148.6 mcg	402.0 mcg, 864.0 mcg DFE	Yes	NIL
Crider 2018[216]	236 mcg	n/a	239 mcg, 582 mcg DFE	461 mcg	661 mcg, 1341 mcg DFE	Yes	NIL
Dietrich 2005[3]	n/a	294 mcg DFE	n/a	n/a	n/a	No	NIL
French 2003[4]	259 mcg DFE	n/a	470 mcg DFE	n/a	812 mcg DFE	Yes	NIL
Gaskins 2012[5]	181.7 mcg	500.5 mcg, 368.9 mcg DFE	181.7 mcg	n/a	n/a	No	NIL
Gaskins 2014[248]	n/a	764.54 mcg DFE	n/a	1013.46 mcg	1778 mcg DFE	Yes	NIL
Gaskins 2019[247]	n/a	459.1 mcg	n/a	338.4 mcg	797.5 mcg	Yes	NIL
Hamner 2013[239]	n/a	244 mcg	n/a	380 mcg	n/a	Yes	NIL
Hure 2009[240]	n/a	265.9 mcg	n/a	n/a	n/a	No	NIL
Jun 2020[241]	n/a	n/a	n/a	375 mcg	n/a	Yes, no food	NIL
Marchetta 2016[243]	n/a	n/a	n/a	381 mcg	n/a	Yes	NIL
Mojtabai 2004[234]	n/a	332.1 mcg	n/a	n/a	n/a	No	NIL
Pick 2005[249]	n/a	300 mcg	n/a	n/a	n/a	No	NIL
Rai 2015[244]	n/a	456.4 mcg DFE	n/a	n/a	n/a	No	NIL
Sotres-Alvarez 2013[250]	170.92 mcg, 430.70 mcg DFE	430.70 mcg DFE	130.76 mcg DFE	n/a	n/a	No	NIL
Tinker 2012[2]	n/a	434 mcg DFE	n/a	>400 mcg, 475 mcg DFE	n/a	Yes	NIL
Tinker 2012[222]	n/a	n/a	n/a	n/a	n/a	No	NIL
Yang 2007[220]	151 mcg	221 mcg	128 mcg	n/a	n/a	No	NIL
Pregnancy Studies							
Bailey 2019[235]	n/a	630 mcg DFE	n/a	n/a	1451 mcg DFE	Yes	All
Beringer 2021[251]	n/a	502.6 mcg DFE	n/a	833.4 mcg DFE	996.6 mcg DFE	Yes	All
Boeke 2013[233]	n/a	n/a	n/a	n/a	T1 972 mcg T2 1268 mcg	Yes	T1
Dorise 2020[236]	n/a	533 mcg	n/a	n/a	n/a	No	10–22 weeks
Dubois 2017[237]	n/a	463 mcg	n/a	n/a	2181 mcg DFE	No	T1

Furness 2013[238]	283 mcg	n/a	n/a	668–2116 mcg	n/a	Yes	18–20
Gómez 2015[141]	n/a	n/a	n/a	T1: 1225 mcg T2: 1353 mcg T3: 1228 mcg	n/a	Yes, no food	<27
Hromi-Fiedler 2012[252]	284.9 mcg	610 mcg	768 mcg	n/a	n/a	No	16–32
Hure 2009[240]	n/a	284.4 mcg	n/a	n/a	n/a	No	All
Jun 2020[241]	n/a	n/a	n/a	787 mcg DFE	n/a	Yes, no food.	All
Livock 2017[242]	n/a	n/a	T1: 247 mcg T2: 245 mcg T3: 251 mcg	T1: 522 mcg T2: 527 mcg T3: 518 mcg	T1: 840 mcg T2: 760 mcg T3: 690 mcg	Yes	<19
Martinussen 2012[253]	n/a	n/a	n/a	month 1 402 mcg month 2 605 mcg month 3 676 mcg	n/a	Yes, no Food	<24
Masih 2015[142]	T1: 313 ± 140 mcg DFE T3: 297 ± 131 mcg DFE	T1: 483 mcg DFE T3: 465 mcg DFE	T1: 96 ± 54 mcg T3: 96 ± 50 mcg	1000 mcg	n/a	Yes	<16
Murphy 2021[217]	148.2 mcg DFE	346.7 mcg 512.5 mcg DFE	226.6 mcg	1100 mcg	n/a	Yes	24–26
Murphy 2021[143]	140 mcg DFE	485 mcg DFE	333 mcg DFE	1000 mcg	2167 mcg DFE	Yes	8–16 weeks
Pick 2005[249]	-	331 mcg	n/a	n/a	n/a	No	20–38
Plumptre 2015[226]	T1: 483 ± 203 mcg DFE T3: 465 ± 186 mcg DFE	n/a	n/a	1000 mcg	n/a	Yes	10–22 weeks
Roy 2012[254]	n/a	473 mcg DFE	n/a	1338 mcg	1811 mcg DFE	Yes	10–22 weeks
Shin 2016[245]	n/a	627.6 mcg DFE	n/a	781.8, 1329 mcg DFE	n/a	Yes	All
Trivedi 2018[219]	n/a	n/a	n/a	n/a	T1: 930 mcg T2: 1238 mcg	Yes	<25 weeks
Whitrow 2009[229]	T1: 224.7 mcg T3: 208.4 mcg	n/a	n/a	T1: 658.3 mcg T3: 300 mcg	n/a	Yes	<16

Legend 3- DFE = dietary folate equivalents; n/a = not applicable, did not measure; WD = Western diet; mcg = micrograms; T1: Trimester 1; T2: Trimester 2; T3: Trimester

2.2.5.5 Supplementation and Upper Tolerable Limit

Across studies, supplements contributed substantially to total folic acid intake in women of childbearing age, accounting for 47.5% intake in one study [220] and 57% in another [246]. Supplemental FA intake typically ranged between 400-1000 mcg/day, depending on the study design and population. The proportion of women consuming 400 mcg/day of FA varied widely, from 24% in one cohort [239] to approximately 78% in another [246]. Intake at the 1000 mcg/day supplement level was also reported, representing approximately 20% of the sample population in some studies [246, 247]. When intakes exceeded the UL of 1000 mcg/day, supplements were consistently identified as the source [4, 222, 255]. However, the prevalence of women exceeding the UL within this age group was low compared with pregnant women and ranged between 2.4-7% across studies [4, 222, 255].

Pregnant women demonstrated substantially higher FA intake than non-pregnant women due to a higher frequency of supplement use. Supplements contributed between 77% [241] and 92% [253] of FA intake and accounted for an estimated 84% of total FA intake in early pregnancy and 63% in late pregnancy [229]. In contrast, supplement use in the preconception period was notably lower, with 63% of women not taking a prenatal FA supplement in the three months before conception [242].

Across all studies examining pregnant women, those who exceeded the UL did so due to FA supplementation [141, 142, 217, 219, 229, 235, 237, 238, 241, 242, 254]. The percentage of women exceeding this UL ranged from 25% [141], 33.4% [235], 40% [241], 83–85% [142, 226], 87% [237], 96% [217] to 100% [238]. It was estimated that supplements contributed to a mean daily intake of 878 mcg FA per day [241], 1000 mcg/day [142] to over 2000

mcg/day [217, 229]. In one study, this overconsumption was more likely to occur in the first trimester [242].

It was difficult to evaluate a consistent method of overall intake of folate (food folate, fortified food, and supplements) as not all studies provided intake in DFEs and therefore only DFE ranges will be reported. In women of childbearing age, the total intake ranged from 864 mcg DFE [1] to 1778 mcg DFE [247]. In pregnant women, total intake was much higher, ranging from 1451 mcg DFE [235] to 2181 mcg DFE [237]. The total DFE intake in pregnant women was recorded predominantly in Trimester 1 of pregnancy [217, 226].

The overall intake of FA throughout pregnancy including supplemental FA, resulted in higher RBCF concentrations compared with pre-supplementation baseline levels as shown in trials of high dose FA supplementation [143, 238]. This contributed to total folate intakes reaching up to 200% above the RDA [141], with some women consuming as much as 2948 mcg/day [229]. Studies assessing UMFA demonstrated that UMFA was detectable in all pregnant women measured [143] and that early first-trimester maternal plasma UMFA was detected in 97% of women and 93% of cord blood samples [226].

2.2.6 Discussion

2.2.6.1 Natural Food Intake and Fortification

There is a general consensus that women of childbearing age require a minimum of 400 mcg of FA per day in addition to their normal dietary intake to reduce the risk of neural tube defects [202] and, based on studies such as the ones included in this review, it is clear that women of childbearing age are not receiving sufficient dietary folate to protect against neural tube defects, but rather they are receiving on average, half the recommended

dose of folate from food. In response to studies that supported this finding of low folate intake from the diet, mandatory fortification of food products was introduced in the United States in 1998, at a level of 140 mcg per 100 gms in all cereal grain products [30]. In Australia, mandatory fortification in wheat flour commenced in 2009 with 200 mcg per 100 gms of wheat flour [256]. This regulation was based on modelling studies that showed that the increase in FA was expected to be approximately 100 mcg of additional FA per day; however, it is clear from the studies in this review that the intake of FA from foods alone is closer to 239 mcg or DFE 582, with the combined folate intake from natural food folate and folic acid-fortification reaching close to the required intake of 400 mcg in women of childbearing age 500 mcg DFE. The fortification of folic acid was introduced to prevent neural tube defects in populations that were vulnerable to low levels of folate and by all accounts FA has been successful in achieving this [257-262]. The fortification program, however, did not cap the number of foods being fortified, nor did it consider populations that may be vulnerable to large amounts of FA like children and older adults [87, 263, 264] or tailor the intake levels via supplementation suggested for women in preconception and pregnancy.

2.2.6.2 Folate Measurement

The assessment of folate intake from food (natural folate and fortified foods) in the studies is based on the long-standing assumptions that synthetic FA is more bioavailable than natural food folates, which is why intake is often expressed in dietary folate equivalents. These assumptions originated from early metabolic-unit experiments and were reinforced by earlier work suggesting that natural folate has approximately half the bioavailability of synthetic FA fortified foods [223]. This assumption, however traces back

largely to a single small study of ten women [265] and its limitations have since been debated [202, 265]. Researchers have since argued that this 50% estimate may not accurately reflect real-word bioavailability [263]. Indeed later controlled investigations show natural folate bioavailability values ranging from 78% of FA in some foods [266], to as high as 98% in others, highlighting the complexity of assessing food folate bioavailability [267]. A controlled human study adds important clarity. In a three month randomised intervention comparing the functional effectiveness of increasing natural food folate versus synthetic FA in women of reproduction age, it was demonstrated that increasing natural food folate intake by 400 mcg/day did not significantly raise RBCF concentrations, whereas an equivalent 400 mcg/day provided as FA fortified foods or as supplements produced a significant increase in RBCF [268]. This finding demonstrates that functional bioavailability, that is, the degree to which increased folate intake results in measurable improvement in biological folate status, is substantially higher for FA than for natural dietary folate under real world conditions.

A very detailed report on folate chemistry and metabolism highlights that, in principle, natural food folates (predominantly reduced polyglutamate forms) are not inherently less bioavailable than monoglutamates (synthetic folic acid) because their absorption depends on intestinal conjugation carrier-mediated transport, and intracellular reduction pathways rather than intrinsic chemical limitation [209]. This biochemical framework suggests that, once liberated from the food matrix and full deconjugated, natural folates could theoretically achieve similar availability to FA. However, the real world findings from Cuskelly et a. (1996) [268] make clear that biochemical potential does not equate to physiological effectiveness.

The measurement of food folate is difficult as confounding factors like loss of folate due to cooking, brush border enzyme activity, pH of the intestinal lumen, certain nutrients affecting absorption, and poor compliance in studies where participants are faced with difficult interventions [209, 267, 269, 270], detract from the accuracy of the study. It is important, therefore, that we gain a better understanding of folate metabolism because if we are underestimating the amount of natural folate that is bioavailable, this may have a bearing on FA-fortification rates currently and future directions for many governments around the world. Further, ongoing bioavailability research comparing FA with 5-MTHF is critical. Several controlled studies suggest that the bioavailability of folinic acid and 5-MTHF may be equivalent to that of FA when measured by changes in plasma folate concentrations [263, 271]. Given the accumulating evidence that FA and natural folate differ substantially in practical bioavailability, further work comparing FA with the biologically active form 5-MTHF will be important for refining both clinical and public health recommendations.

2.2.6.3 Folate Metabolism

This review has highlighted the extent to which women of childbearing age, particularly in pregnancy, are exceeding the UL of FA, and this may be impacting on their folate metabolism. Bioavailability is very different to metabolism; although FA may be more bioavailable, it is not metabolised the same way as natural folate. Natural folate uptake by the brush border enzymes does depend on the type of food, the pH of the intestinal tract and quality of the mucosa; however, there is no solid evidence that it is 50% less absorbed than FA and the studies show huge variation in percentage uptake of natural folates versus FA. Bioavailability is key because if FA is not

metabolised sufficiently to 5-MTHF, which is the main circulating form of folate used by the placenta and brain, then the increased amount may be inhibiting the production of 5-MTHF and its re-uptake. It has been found that synthetic FA has no coenzyme activity until it can be reduced by *DHFR* [210] and that the *DHFR* enzyme is easily saturated which means that above a dose of 200–300 mcg the normal intestinal absorption is saturated and rather than being converted by *DHFR* to dihydrofolate (DHF) or tetrahydrofolate (THF), we begin to see an accumulation of unmetabolised folic acid in the serum [45, 272-274]. This then becomes an important question regarding both fortification and supplementation. If, as studies in this review suggest, the level of FA from fortification alone is reaching this saturation point for FA metabolism, and women, particularly in pregnancy, are almost always exceeding this threshold, should the recommendation of further FA in preconception and pregnancy be changed to 5-MTHF to achieve the recommended folate level? 5-MTHF cannot accumulate [208, 209] and is considered to be as good as FA, if not better in raising folate concentrations and preventing neural tube defects [86, 88, 89, 92, 94, 123]. The studies in this review confirm that food-based folate is not sufficient to achieve recommended levels of folate in preconception and pregnancy; however, they do highlight that although FA may be considered to be absorbed better, it is not metabolised into 5-MTHF like natural folates and is accumulating instead.

Studies that have investigated 5-MTHF as a possible substitute for FA found that 5-MTHF at a dose of 7.5 mg every 12 hours over four days rapidly increased serum total folate and has an advantage over FA as it can replete body stores in folate-insufficient women within a few days [123]. 5-MTHF

compared to folic acid was found to increase plasma folate concentrations more effectively [88]. Safety studies of high-dose 5-MTHF in rats have shown no adverse events [124] and doses as high as 17 mg/day for 12 weeks have been reported as well tolerated in non-pregnant adults [125]. In pregnancy, direct evidence on isolated 5-MTHF is limited. One retrospective study of a multi nutrient prenatal medical food which contained 5-MTHF combined with multiple other active ingredients including 500 mcg of vitamin B12, reported no supplement related adverse events at a dose that delivered 1.13mg 5-MTHF [126]. However this study did not assess UMFA and did not isolate the effects of 5-MTHF alone and its B12 content is a critical confounder because of the interdependence of folate and vitamin B12 in one carbon metabolism [275]. Additional clinical reports show that 7.5 mg and 15 mg 5-MTHF have been used without adverse effects in patients with depression [127, 128]. As yet, only one retrospective series compared 5-MTHF to folic acid supplementation in hypo-fertile couples with *MTHFR* polymorphisms, however safety data for the mothers was not reported and in the folic acid group, pathologies were detected in offspring including two children with autism spectrum disorder, one child with significantly elevated circulating homocysteine and one child affected by oculo-auriculo-vertebral spectrum [276]. No pathology was detected in children of the 5-MTHF treatment group suggesting a potential safety advantage of 5-MTHF over FA in those with *MTHFR* polymorphisms. Overall, while 5-MTHF is effective at improving folate status and may offer advantages in infertility and recurrent pregnancy loss [86, 208] there is a need in fertility studies to establish long-term safety data to both mother and offspring.

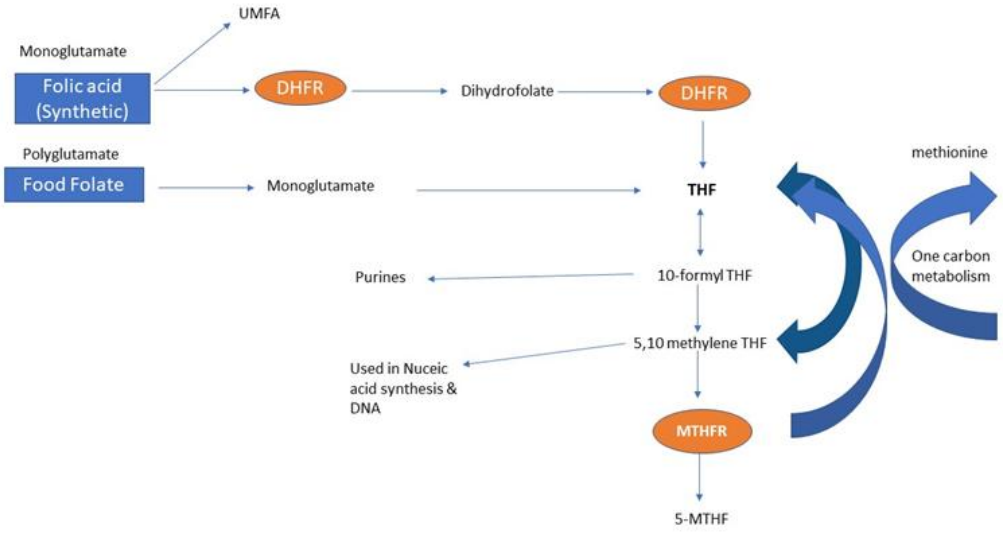

Figure 5 - Folate metabolism leading to one-carbon metabolism - the end goal is for folates to be metabolised to 5-MTHF, where it is used by methionine synthase reductase (MTR) for one-carbon metabolism and methylation reactions: 5-MTHF = 5-methyltetrahydrofolate reductase, DHFR = dihydrofolate reductase, THF = tetrahydrofolate, MTHFR: methylenetetrahydrofolate reductase, and UMFA: unmetabolised folic acid.

2.2.6.4 Unmetabolised Folic Acid

Previous studies demonstrate that individuals consuming ≤ 200 mcg/day of FA are unlikely to have unmetabolised folic acid in serum, whereas intakes of 300 to 400 mcg/day commonly lead to its appearance in serum [196, 273]. Because virtually all women in preconception and pregnancy exceed this intake through fortified foods and supplements, UMFA would be expected. Murphy et al. in their study, showed that all the women had the presence of UMFA irrespective of the dose, even in the 'low dose' group, whose intake was < 1.1 mg/day, a level already well above the threshold at which UMFA typically appears [143]. Thus, the presence of UMFA in that group is unsurprising and reflects the dose-dependent saturation of *DHFR* rather than a unique effect of high dose supplementation. Likewise, Plumptre et al. found that 93% of cord blood samples had UMFA detected [274], further

demonstrating that most pregnant women exhibit circulating UMFA due to total folic acid exposure during gestation. UMFA has been implicated in reduced natural killer cell activity in post-menopausal women [57], various forms of cancer, cardiovascular disease [87], autism spectrum disorder [277] and orofacial clefts [278, 279]. This has serious implications, given the studies in this review show that many women in preconception and almost all women in pregnancy have the presence of UMFA and are exceeding 400 mcg of FA per day. UMFA has also been found to cause a pseudo-*MTHFR* deficiency in rats [280] and reduce 5-MTHF. This effect has concerning implications such as low folate being associated with uracil misincorporation [281], reduced DNA methylation [280] and changes in epigenetic patterns [282]. So too, the presence of UMFA has been linked to aberrant DNA methylation [283]. Methylation and the one-carbon metabolism cycle (Figure 5) is a critical pathway required for oogenesis [167] and spermatogenesis [110, 111] and is required for epigenesis and imprinting [170] and folate is required for the synthesis of purines and thymidylate for RNA and DNA synthesis [210], thus having implications for preconception, pregnancy, birth defects and the outcome of the offspring. The folic acid UL of 1000 mcg has been set based on the potential for high folic acid intake to mask a vitamin B12 deficiency [196, 210, 274] and not the level at which serum UMFA appears. Based on the studies in this review, every single woman may exceed the level of FA intake where UMFA would appear. This is not the case with 5-MTHF which cannot accumulate and has a feedback loop to ensure that there is a homeostatic mechanism that prevents excessive concentrations of folate in tissues [210].

Previous research has shown that high-dose folic acid results in a global loss

of methylation across the sperm methylome and leads to altered sperm epigenome and sperm DNA methylation, lower pregnancy rates, infertility rates and abnormal embryos [33, 284].

A lack of 5-MTHF contributes to an elevation in follicular levels of homocysteine [200, 285] which correlates to adverse pregnancy outcomes as folate receptor-α has a high affinity for 5-MTHF and ensures cellular uptake where the folate receptor is expressed in the ovary, oocytes and human embryonic stem cells [215]. These biochemical mechanisms may underpin the reported links between *MTHFR* C677T and A1298C polymorphisms and infertility [79, 80, 82, 83, 113], implantation failure [78] and recurrent pregnancy loss [86, 92, 201, 285, 286]. Such mechanisms may also explain preliminary research that has found replacing FA supplements with folinic acid or MTHF supplements may increase pregnancy success in women with *MTHFR* polymorphisms who have experienced recurrent pregnancy loss [287].

2.2.6.4.1 Upper Tolerable Limit

The studies in this review confirm that many women preconceptionally and almost all women at some stage in pregnancy are exceeding the UL of 1000 mcg/day. The upper limit seems to be almost inconsequential as it is simply a consideration of vitamin B12 masking and does not take into account the consideration of metabolism. If metabolism is a concern, then all women in pregnancy are exceeding the threshold and most women taking a prenatal multivitamin most certainly are as well. Given the documented concerns regarding the presence of UMFA, this upper tolerable limit needs to be re-evaluated.

2.2.7 Conclusions

Natural folates may not deliver the optimal amount of folate to reduce the risk of NTDs or methylate DNA sufficiently to prevent miscarriage. Women may be at risk of producing UMFA due to their intake of FA from food fortification. Any supplementation of FA over and above has the potential to cause adverse health outcomes, reduce *MTHFR* activity, mask vitamin B12 deficiency, reduce methylation of DNA, and therefore contribute to adverse pregnancy outcomes such as pregnancy loss. The results of this review warrant close attention from public health researchers and policy makers and may justify a review of the current approach to ensure folate sufficiency in the general population and avoidance of UMFA that may influence fertility and pregnancy outcomes.

2.3 Section Summary

In summary, this literature review examined the challenge of achieving adequate folate intake during the preconception period and pregnancy, while avoiding excessive exposure to synthetic FA. The evidence consistently shows that women rarely meet recommended folate intakes from food alone making supplementation necessary when considering pregnancy. However, in countries with mandatory FA fortification, the combined intake from fortified foods and FA based prenatal supplements frequently leads women to exceed the tolerable UL. In these circumstances, circulating UMFA becomes common, raising concerns about potential downstream effects, including altered one-carbon metabolism, masked vitamin B12 deficiency, perturbations in DNA methylation and possible associations with adverse reproductive outcomes. The literature also introduced 5-MTHF as a potential alternative to FA, however the evidence supporting 5-MTHF as a universally

safe or superior substitute remains limited. While 5-MTHF does not appear to produce UMFA, there are no randomised controlled trials in pregnant women demonstrating that 5-MTHF is safer or more effective than FA, nor that it prevents neural tube defects. Existing human data are mostly pharmacokinetic or involve specific clinical subgroups and therefore cannot be generalised to population level recommendations.

Furthermore, although 5-MTHF may directly participate in methylation pathways, the biological consequences of increasing the rate or volume of one-carbon flux, particularly in early embryonic development, are not fully understood. The interaction between folate and vitamin B12 is especially important. Both nutrients converge in the methionine synthase reaction and balance between them may alter methylation capacity or mask B12 deficiency, the existing evidence remains incomplete and often difficult to interpret. Likewise, although 5-MTHF offers theoretical advantages and does not lead to UMFA, the current human data is insufficient to confirm its safety or effectiveness as a universal substitute for FA especially during pregnancy. For these reasons, the following chapter systematically examines the literature on the effects of excess FA intake on pregnancy outcomes and offspring health, with the aim of clarifying the nature and strength of the evidence and identifying areas requiring further research.

2.4 The Adverse Health Effects of Excessive Folic Acid Use and High Blood Concentrations of Folate During Preconception and Pregnancy on Mother and Offspring. A Systematic Review.

2.4.1 Publication Details

This paper was accepted for publication in Reproductive Biomedicine Online in September 2025 and is available as preproof at this time. This paper was a collaboration between UTS and St Thomas' Hospital and Kings College London.

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2.4.2 Introduction

Increased folic acid (FA) intake is one of the most significant public health achievements for improved maternal and child health [288] as FA supplementation during peri-conception is widely recognised as having a critical role in preventing neural tube defects (NTDs) in newborns [31, 289]. However, many women of child bearing age do not meet the recommended dose of 500 mcg of folate daily [48]. While dietary folate comes from foods such as legumes, fruits and leafy greens [44, 290], many women do not consume enough of these foods. This is one of the reasons why countries like the United States (in 1998), Canada (in 2000) [30, 291], Australia (in 2009) and Chile (in 2000) implemented fortification programmes, fortifying enriched grains and flours to ensure women have adequate folate levels to prevent NTDs [30, 31, 292]. In addition to mandatory fortification present in

many countries already, governments worldwide have also recommended women of childbearing age take FA supplements prior to conception and first trimester of pregnancy [25-29].

Folate (vitamin B9) is essential for DNA synthesis and plays a crucial role in embryonic and fetal development, particularly in early pregnancy. Different forms of folate exist in nature; FA is a synthetic form of folate and unlike in natural folates, the pteridine ring of folic acid is not reduced making this form resistant to chemical oxidation [89, 293]. All forms of folate are converted to 5-methyltetrahydrofolate (5-MTHF), the main circulating form of folate , to allow uptake into cells and introduction into metabolic processes [140, 196]. 5-MTHF donates one carbon group (methyl) into the methionine cycle to convert homocysteine to methionine and generate tetrahydrofolate. These reactions are required for the synthesis of the universal methyl donor S-adenosylmethionine (SAM) which is critical for methylation reactions [294] including DNA methylation. Other nutrients involved in one carbon metabolism for nucleotide synthesis include vitamin B12, vitamin B2, vitamin B6, methionine, betaine and choline [295]. Impairment of methylation reactions leads to epigenetic modifications of DNA, affecting gene expression and potentially the epigenome of developing germ cells [296, 297]. Polymorphisms in enzymes involved in folate and homocysteine metabolism such as methylenetetrahydrofolate reductase (*MTHFR*) can also impact the function of these pathways resulting in a reduction in enzyme activity, affecting folate status [298]. *MTHFR* polymorphisms have been as-8sociated with methylation disturbances which affect spermatogenesis [85, 192, 299, 300], oocyte maturation [300, 301], endometriosis [302], infertility [81] and recurrent pregnancy loss [303].

In 1998, the Institute of Medicine in US set the upper tolerable limit (UL) of FA at 1,000 mcg [202]. The current exposure to FA is significantly higher than the original expectation by Government agencies of 100 mcg per day, for all age groups, particularly in countries where both voluntary and mandatory FA fortification are in place [27, 44-46, 304-306]. Furthermore, many women taking FA-based multivitamins during pregnancy often exceed the upper limit of 1,000 mcg per day [27, 44, 45, 48, 225, 306, 307]. In Australia, prenatal multivitamins typically contain 400-500 mcg of FA [221] however the leading prenatal multivitamin contains 800 mcg [308], while in the United States and Canada, doses are higher ranging from 800 mcg [5, 25, 26] to 1,000 mcg per unit [141-143]. Women at high risk of pregnancy loss, diabetes or with a history of NTDs, may be prescribed doses of FA as high as 4 to 5 mg per day [25, 26, 28, 92]. While fortification programmes have successfully reduced NTD rates, concerns are mounting about the potential risks of excessive FA intake [87, 196, 309-312]. Recent research suggests that higher levels of folate, beyond what is required to prevent NTDs, may not offer additional benefits but could actually pose health risks to both the mother and offspring [37, 81, 87, 106, 196, 226, 280, 294, 313-328]. Excessive FA intake has been associated with adverse outcomes, including impaired placental development [329], metabolic and hormonal disturbances during pregnancy [330] and neurodevelopmental issues in the offspring [37, 326, 329, 331, 332]. One key issue is that the enzyme *DHFR*, responsible for converting FA into its active forms, dihydrofolate and tetrahydrofolate, can become easily saturated at FA doses as low as 280 mcg [226]. When this occurs, unmetabolised FA (UMFA) may accumulate in the bloodstream causing a pseudo-*MTHFR* deficiency [280, 320] and other

harmful effects such as [333] inflammation, weight gain, and fat accumulation [108].

The ability to determine whether an individual has adequate or excess folate status relies on a range of assessment methods, the most common of which are plasma/serum or RBC folate concentrations. Each of these tests comes with analytical caveats, which complicates obtaining standardised and consistent results across different studies [293, 334]. Availability of UMFA testing which could be indicative of excessive FA intake or impaired metabolism is poor. UMFA is measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which has a detection limit (LOD) of around 0.1-0.2 nmol/L (method specific) [335]. A recent US population study measured serum UMFA concentrations ranging from <0.14 nmol/L (LOD) to 282 nmol/L, with an overall geometric mean (95% confidence interval) of 1.21 nmol/L (1.15-1.28 nmol/L) [336]. Although it offers high sensitivity and specificity, the method is costly, difficult to perform, and not widely available, limiting the routine measurement of UMFA in clinical practice [226, 337, 338].

The most common marker of folate status assessment is the total folate in serum/plasma [293]. Serum/plasma folate is an indicator of recent folate intake [293]. Fasting samples are preferred for accuracy [293] with normal serum/plasma folate levels ranging from 13.5 – 45.3 nmol/L (6-20 ng/mL) [100]. Serum folate concentrations above 45.3 nmol/L (20 ng/mL) are considered elevated, [97], although according to a 1968 World Health Organisation (WHO) report [98, 99], this threshold was based on assay limitations rather than specific biological risks. Serum/plasma folate is an indicator of short-term intake and, while useful, does not capture long term folate levels in the body [339] . In contrast, RBC folate is a more reliable

indicator of long-term folate status, reflecting levels over the approximate 120-day lifespan of RBCs [223, 293] and is thus a valuable marker for assessing chronic folate status, especially in women planning pregnancy or in early gestation. However, the reliability of RBC folate as a biomarker is highly dependent on the analytical method used [340] As demonstrated by Hunt et al., 2020), the chemiluminescence immunoassays substantially overestimate RBC folate concentrations due to methodological limitations and poor agreement with reference assays. In contrast, microbiological assays, and more recently liquid chromatography–tandem mass spectrometry (LC-MS/MS), provide analytically robust, reproducible, and biologically meaningful measurements of RBC folate. Consequently, interpretation of population-level RBC folate data and derived thresholds must account for assay methodology, as reliance on immunoassay-based estimates may misrepresent true folate status and obscure biologically relevant risk relationships.

The WHO recommends RBC folate concentrations above 906 nmol/L for women of reproductive age to reduce the risk of NTDs in offspring [100].This recommendation is based on a 1995 study by Daly et al [101] that stated that NTDs were due to low folate, however this study is considered to be of low quality evidence by WHO [100]. The same authors published a follow up paper in the same year explaining that “the vast majority of women who go on to have an NTD-affected embryo in the same pregnancy are not folate deficient, and that the role of folic acid in prevention is that of overcoming a metabolic block in folate metabolism”. They go on to say: “Our results suggest that direct or indirect malfunctioning of the enzyme (methionine synthase) is responsible for the majority of NTD” [103]. Crider et al in 2014

tried to assess the potential association between RBC folate and NTD and assumed the optimal range of RBC folate concentrations was approximately 1000 nmol/L to 1300nmol/L for the prevention of NTD [104]. Crider also considered the effect of *MTHFR* polymorphisms (TT and CT) to be associated with increased risk of NTD. However, this paper is also considered to be low quality of evidence as per the Grading of recommendations, Assessment, Development and Evaluations (GRADE) methodology which is a transparent framework for developing and presenting summaries of evidence and provides a systematic approach for making clinical practice recommendations [102].

While FA has already, and continues to, contribute unquestionable population health benefits for maternal and child health, all individuals involved in supporting healthy pregnancy and birth such as clinicians, policymakers, public health practitioners and expectant parents, need a clear understanding about the safe limits of FA intake and/or blood concentrations to ensure its ongoing benefits to population health are not undermined by unexpected adverse outcomes. Despite the complexities in assessing folate status, growing concerns regarding the impacts of excess FA alongside continued efforts to implement FA fortification programs around the world call for a closer examination of the health implications of excess FA. In response, this review systematically evaluated studies from 1998 to the present and sought to answer the research question: What is the implication of excess folic acid and blood folate concentrations on preconception, pregnancy and birth outcomes in human studies?

2.4.3 Methods

2.4.3.1 Aim

This systematic review evaluates the potential adverse health outcomes associated with excessive FA intake and elevated blood folate concentrations during preconception and pregnancy.

2.4.3.2 Study Design and Setting

A protocol for this systematic review was registered with PROSPERO (CRD42023458047) that encompassed both human and animal studies. This review focused specifically on the outcomes of the human studies and was designed in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [232]. Data Sources, Search Strategy and Eligibility Criteria

We conducted a detailed search of the following electronic databases: MEDLINE (OVID), Embase, CINAHL (EBSCO), Scopus, TROPHI, Cochrane, Maternity and Infant Care, clinical trials database, and hand searches. The search terms used for the literature search are presented in Appendix 5.

2.4.3.3 Eligibility Criteria

The review included randomised controlled trials (RCT's), cohort studies, experimental studies, case-control studies, and cross-sectional studies. The target population consisted of women in the pre- or periconception periods or during pregnancy, and offspring. The key independent variable was excess exposure to FA supplementation and high blood concentrations of folate. Where multiple ingredients were included in a trial compared to folic acid as a control, the folic acid only results were considered. FA supplementation

excess was defined as intake equal to or greater than upper tolerable limit (UL) of 1,000 mcg/day as defined by the Institute of Medicine [202]. Excess folate concentrations in blood (serum/plasma and/or RBC and/or UMFA) were defined based on available international (or national) guidelines as follows: serum/plasma folate concentration greater than 45.3 nmol/L (6-20 ng/mL) as defined by the World Health Organisation (WHO) [100], RBCF greater than 1360 nmol/L was chosen as the cut off for high RBCF [341] and UMFA greater than 1.2 nmol/L. However, given the WHO-specified level of excess serum/plasma folate was described as “not based on biological implications for health” [98], and in the absence of scientific certainty regarding excess folate blood concentrations, we also included results from studies that reported concentrations lower than those defined above, in which the authors identified adverse biological implications on health in pregnancy or offspring and concluded those concentrations indicated excess folate.

Exposure to FA was assessed through dietary intake (using food frequency questionnaires, interviews and surveys), blood tests and/or supplement use reporting. The dependent variables were any outcomes that could affect pregnancy or birth outcomes in offspring.

The review included studies published between 1998 and September 7, 2023, as 1998 marked the start of mandatory folic acid-fortification in the US [225, 231, 342]. No language restrictions were applied. Excluded from the review were systematic reviews, meta-analyses, narrative commentaries and studies that did not report excess FA intake, folate blood or UMFA concentrations. Posters and conference abstracts were not included.

2.4.3.4 Study Selection and Data Extraction Process

After conducting the database search, references were organised using EndNote X9 bibliographic software and then imported into the Covidence systematic review platform (Veritas Health Innovation, 2020) for citation filtering and critical appraisal. The study selection process is illustrated in Figure 6 using the PRISMA flow diagram. Initially, the search strategy included animal and human studies where 1,590 studies were identified. All animal studies (n=) were excluded (to be reported elsewhere); only the human studies (n=) underwent the PRISMA selection process for the current review After removing duplicates (n=310), one reviewer screened 1,024 citations based on title and abstract, excluding those that did not meet the criteria. Next, a team of three reviewers independently assessed 256 full-text studies for eligibility, ensuring that each study was reviewed by at least two reviewers. Any disagreements were discussed until consensus was reached. Of these, 170 were excluded for reasons such as not reporting FA intake, not measuring folate blood concentrations, involving a non-eligible patient population, or using an ineligible study design. Data extraction was performed by one reviewer and cross verified by one of the other authors. Any discrepancies were subsequently resolved by two designated reviewers. 45 studies were ultimately included in the systematic review.

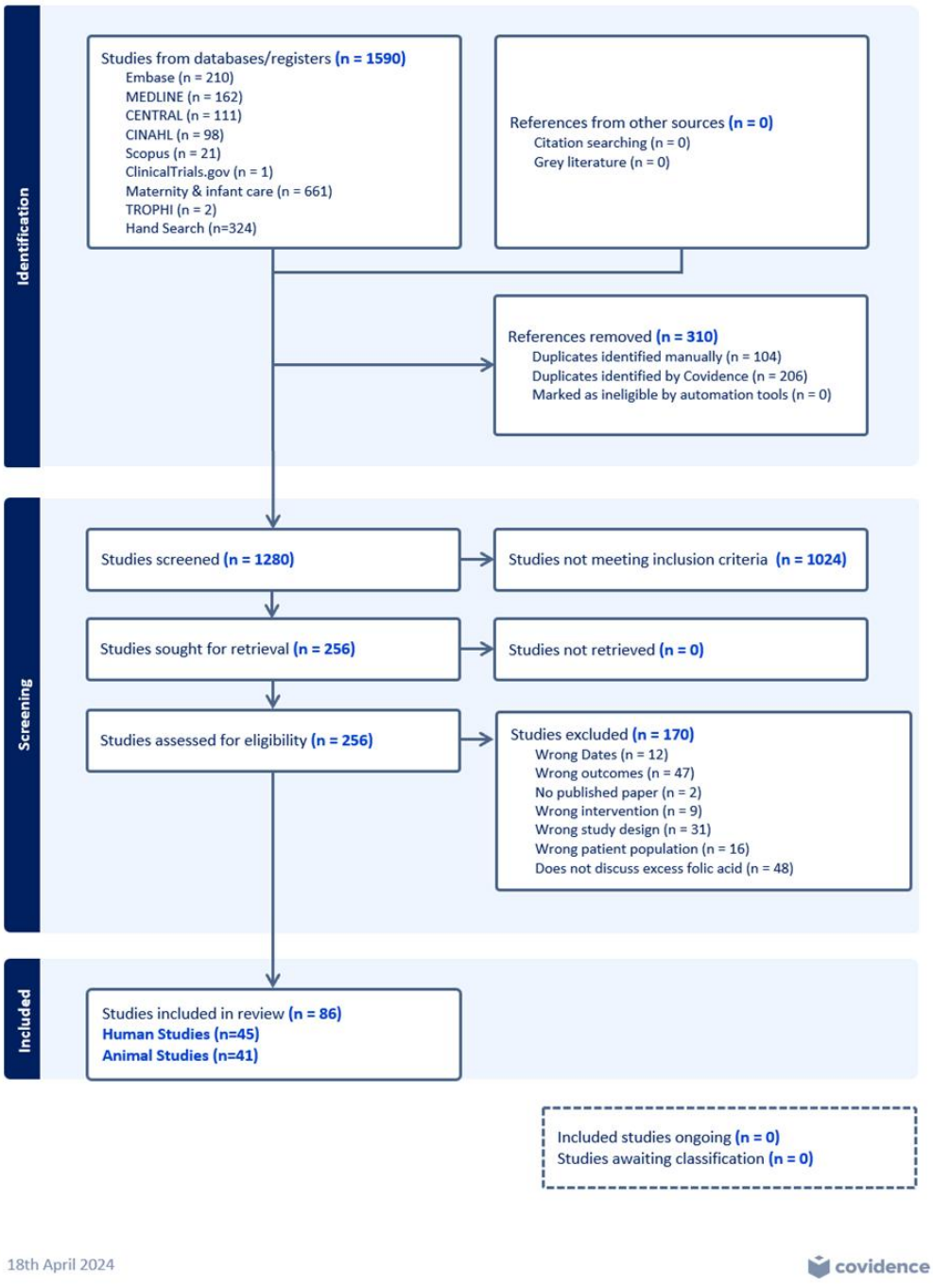

Figure 6 - Prisma Flow diagram of included studies

2.4.3.5 Data Synthesis and Analysis

The studies were divided into two main categories based on the reported outcomes: those assessing effects on offspring and those examining fertility, fetal development, or pregnancy outcomes. Animal studies will be reported at a later date. The human studies are presented in Appendix 6 & 7 that

summarise the characteristics of the studies with health outcomes related to the offspring and those relating to preconception and pregnancy, including the interventions, FA dose, the comparison/control groups and the offspring's metabolic outcomes. Due to the heterogeneity of the included studies, a meta-analysis was not feasible.

2.4.3.6 Quality Assessment

The quality of the included human studies was evaluated using the ErasmusAGE quality assessment tools for systematic reviews [343]. This tool rates interventional and observational studies based on several criteria, including selection bias, study design, confounders, blinding, data collection methods, withdrawals and dropouts and intervention integrity. Studies were rated as strong, moderate, or weak according to these criteria [343, 344]. Quality assessment was conducted by one reviewer and independently verified by two other reviewers. Most studies were rated as strong n= 27 or moderate n=17, with only one study rated as weak [345] (see Appendices 8 and 9).

2.4.4 Results

Forty-five studies were included in this review. They predominantly employed cohort study designs n=30, randomised controlled trials (n=8), case control studies (n=6), cross-sectional study (n=1), and were published from 2011 to 2023. No studies were published between 1998 and 2011 that meet the criteria. The studies were conducted in the following countries: China (n=11), United States (n=6), Spain (n=5), Australia (n=4), Netherlands (n=4), Canada (n=2), Iran (n=2), Singapore (n=2), Brazil (n=1), Israel (n=1), Italy (n=1), Japan (n=1), Norway (n=1), Russia (n=1), Sweden (n=1), United Kingdom (n=1), Vietnam (n=1). One study [346] was in Russian language and was

Table 5 - ***Outcomes of studies (n=20) which have evaluated the effects of high levels of folic acid supplementation or measured blood folate levels on pregnancy-related outcomes***

Study	Did an increase in FA increase risk of adverse outcome?	Outcomes of interest	Results
Fertility and Pregnancy			
Bortulus et al., 2021	No	Composite adverse pregnancy outcome (spontaneous abortion, SGA, preterm delivery, major CMs)	Higher dose FA associated with reduced risk of composite outcome, spontaneous abortion, SGA and preterm delivery. Composite (no) - reference Composite (yes): aRR = 0.52 (95% CI : 0.38–0.72); p = 0.0001 Spontaneous abortion : aRR = 0.56 (95% CI : 0.34–0.93) ; p = 0.02 SGA : aRR = 0.43 (95% CI: 0.19–0.97) ; p = 0.04 Preterm delivery: aRR = 0.44 (95% CI: 0.20–0.95); p = 0.04
Murto et al., 2017	Yes	Unexplained Infertility	Increased likelihood of unexplained infertility with FA supplement use Total (any use): OR = 3.314 (95%CI: 2,158,5.087) P <0.001 Consistent daily use: OR = 5.902 (95% CI:3.462,10.060) p < 0.001 FA only intake: OR =1.002 (95% CI: 1001,1.002) P= 0.001 Median FA intake (mcg/d) [range]: 500 (0–1400) vs 0 (0–1600); p<0.001 Additional findings Homocysteine lower at folate ≥22.5 nmol/L: p=0.003 Correlations: folate vs FA intake r=0.444; p<0.001; homocysteine vs folate r=–0.188; p<0.001

	No	Pregnancy rate = positive urinary hCG test after embryo transfer / number of women undergoing infertility treatment cycle.	Pregnancy outcomes (questionnaire-based FA use vs. no FA use) Pregnancy rate : 34.3% vs 38.1%; OR=1.01; 95% CI: 0.52–1.95; NS Outcomes by plasma folate cut-off (≥22.5 nmol/L vs <22.5 nmol/L) Pregnancy rate: 37.2% vs 36.0% ; OR=0.94 ; 95% CI : 0.50–1.78 ; NS
	No	Clinical pregnancy rate = presence of a gestational sac at 7 weeks / number of women undergoing infertility treatment cycle.	Pregnancy outcomes (questionnaire-based FA use vs. no FA use) Clinical pregnancy rate: 32.8% vs 35.7%; OR=1.00; 95% CI: 0.52–1.95; NS Outcomes by plasma folate cut-off (≥22.5 nmol/L vs <22.5 nmol/L) Clinical pregnancy rate: 35.9% vs 34.8% ; OR=0.95 ; 95% CI: 0.50-1.80; NS
	No	Miscarriage rate = pregnancy loss after verified gestational sac but before 20 weeks / number of clinical pregnancies.	Pregnancy outcomes (questionnaire-based FA use vs. no FA use) Miscarriage rate: 8.8% vs 4.8%; trend only; NS
	No	Live birth rate = delivery of a child after infertility treatment / number of women undergoing infertility treatment cycle.	Pregnancy outcomes (questionnaire-based FA use vs. no FA use) Live birth rate: 24.1% vs 31.0%; OR=1.36; 95% CI: 0.67–2.75; NS Outcomes by plasma folate cut-off (≥22.5 nmol/L vs <22.5 nmol/L) Live birth rate: 28.2% vs 27.0%; OR=0.94; 95% CI: 0.48–1.86; NS
Polzikov, et al., 2019	Yes	IVF outcomes Number of fertilised oocytes	Increased association with having less fertilised oocytes if folate level in Q4 (pairwise Mann–Whitney U comparison between Q1 and Q4, P=0.049) Data: mean ± standard deviation; median (minimum-maximum) Q1: 9.16±5.62 ng/ml; 8.0 (0.0-24.0) Q2: 7.04±3.49 ng/ml; 7.0 (0.00-13.0) Q3: 7.42±7.50 ng/ml; 7.5 (1.0-17.0) Q4: 7.74±7.49 ng/ml; 5.0 (0.00-34.0)

		Number of aspirated oocytes	Association with having significantly lower aspirated oocytes if folate level in Q4. Pairwise Mann–Whitney U Q1 vs Q4 (p-value not reported) Number of oocytes aspirated in each quartile: mean ± standard deviation; median (min-max): Q1: 13.0 ±7.12; 12 (4.0-31.0) Q2: 11.0 ±3.45; 11.5 (2.0-17.0) Q3: 9.83±4.20; 9.0 (2.0-18.0) Q4: 10.2 ±7.08; 8.0 (3.035.0)
		Number of MII oocytes	Association with having reduced number of MII oocytes if folate level in Q4. Pairwise Mann–Whitney U Q1 vs Q4 (p-value not reported) Number of MII oocytes in each quartile: mean ± standard deviation; median (min-max): Q1: 10.7 ±5.89, 10.0 (1.0-25.0) Q2: 8.12-3.10,7.0 (2.0-17.0) Q3: 8.63± 3.99, 8.0 (2.0-17.0) Q4: 9.04± 7.16, 6.0 (2.0-34.0)
		Live births	Increased association with reduced live birth rate if folate level in Q4. Pairwise Mann–Whitney U Q1 vs Q4 (P=0.047) Live birth rate in each quartile: median (min-max): Q1: 32% (15.0% -53.5%) Q2:30.8% (14.4% -51.8%) Q3:29.2% (12.6% -51.9%) Q4: 8.7% (1.07% -28.0%)
		Clinical pregnancies	Increased association with lower clinical pregnancy rate if folate level in Q4. pairwise Mann–Whitney U comparison of Q1 vs Q4 (P=0.047) Clinical pregnancy rate in each quartile: median (min-max) Q1: 32.0 (15.0-53.5) % Q2: 34.8(20.2-59.4) % Q3: 33.3 (18.8-59.4) % Q4: 8.7 (1.07-28.0) %

Van Uitert et al., 2014	Yes	RBC folate & embryonic growth (measurements at 6 weeks and 12+6 weeks) Parameter: Crown-rump length (CRL) at 6- and 12-weeks gestation	Increased likelihood of reduced embryonic size with folate level in Q4 6 weeks: • Q4 CRL: 4.1 (95% CI: 3.5–4.7) mm • Q3 CRL: 5.4 (95% CI: 4.8–6.1) mm • Difference: 24.2% smaller in Q4 12 weeks: • Q4 CRL: 55.1 (95% CI: 52.9–57.3) mm • Q3 CRL: 59.6 (95% CI: 57.4–62.0) mm • Difference: 7.6% smaller in Q4 Effect estimates (fully adjusted linear mixed model; Q3 = reference): • Q1: β = –0.24 (95% CI: –0.46 to –0.02); P = 0.03 • Q2: β = –0.24 (95% CI: –0.45 to –0.03); P = 0.02 • Q3: Reference (β = 0) • Q4: β = –0.30 (95% CI: –0.50 to –0.10); P < 0.01
Vila-Nova et al., 2013	No	Miscarriage risk with high FA (self-reported spontaneous abortion ≤20 weeks gestation	No significant difference in miscarriage risk between high-dose (4 mg) and low-dose (0.4 mg) FA groups (P=0.4877)
Pre-eclampsia / Gestational Hypertension			
Corsi et al., 2022	No	High FA for prevention of pre-eclampsia in twin pregnancy (pre-eclampsia diagnosed if diastolic BP ≥ 90 mmHg on 2 occasions at least 4 hours apart & significant proteinuria (> 2+ on dipstick or urinary protein > 300 mg /mmol) in women ≥ 20 weeks gestation).	No significant difference in the incidence of severe pre-eclampsia between the high-dose FA group and placebo : 2.3% vs 0.9%; RR= 1.98, 95% CI 0.40–9.78; P = 0.40

Hashemi et al.,1999	No	FMD measured in brachial artery before and after cuff using high-resolution B-mode ultrasound to ass nitric-oxide–dependent vasodilatory response, i.e., endothelial function. Pre-eclampsia criteria: new-onset hypertension (SBP ≥ 140 mmHg or DBP ≥ 90 mmHg) after 20 weeks' gestation together with new-onset proteinuria (≥300 mg/24 h, or ≥1–2+ on dipstick, or protein:creatinine ratio ≥0.3).	Reduced association of endothelial dysfunction with 5 mg FA Baseline FMD (pre-intervention) vs Placebo: FMD Baseline FMD (mean ± SD): FA group: 9.64 ± 5.57% Post-intervention FMD (mean ± SD): FA group: 13.72 ± 7.89% (P=0.013) Baseline FMD (mean ± SD): Placebo group: 9.30 ± 4.25% (P = 0.64) Post-intervention FMD (mean ± SD): Placebo group: 10.02 ± 4.81% (P = 0.397) The difference between groups post-intervention (P=0.002)
Li et al., 2020	Yes	GH Chronic hypertension diagnosed if BP elevated (120–129/<80 mmHg) ≤20 weeks of gestation. GH diagnosed if BP ≥130/80 mmHg >20 weeks of gestation. GH stage 1, BP 130–139/80–89 mmHg; GH stage 2, BP ≥140/90 mmHg	Increased risk of GH if: ≥800 mcg/d FA supplement from pre-pregnancy through mid-pregnancy compared to non-users RR = 1.33 (95%CI: 1.08,1.65); aOR =1.32 (95%CI: 1.06,1.64)
	No	Pre-eclampsia Pre-eclampsia diagnosed if GH plus proteinuria (2+ on dipstick or >300 mg/24 h) after 20 weeks of gestation	No increased risk of GH for ≥800 µg/day FA with short duration compared with non-users.

Manizheh et al., 2009	No	Effect of FA & homocysteine on pre-eclampsia Severe pre-eclampsia diagnosed if BP ≥160/110 mmHg, proteinuria ≥2.0 g/24 h or ≥2+ dipstick, serum creatinine >1.2 mg/dl)	Neither 5 mg nor 0.5 mg FA groups developed pre-eclampsia or severe pre-eclampsia.
Wen et al., 2018	No	Prevention of pre-eclampsia in women with risk factors (pre-eclampsia diagnosed if diastolic BP ≥90 mm Hg on two occasions ≥4 hours apart and proteinuria (> ++ on dipstick, or urinary protein ≥300 mg/ 24, or random protein: creatinine ratio ≥30 mg protein/mmol) ≥ 20 weeks gestation	FA did not prevent pre-eclampsia in those with high risk FA vs placebo : RR=1.10 (95% CI : 0.90 ,1.34) ; P=0.37
Gestational Diabetes			
Chen et al., 2021	Yes	GDM 75 g OGTT performed during 24–28 weeks gestation. GDM diagnosed if FPG ≥5.1 mmol/L, 1 h glucose ≥10.0 mmol/L, or 2 h glucose ≥8.5 mmol/L.	RBC folate concentrations and GDM risk: RBC <400 ng/mL = reference. 400–600 ng/mL: aOR =1.39 (95% CI 0.94–2.04), p=0.09 ≥600 ng/mL: aOR =1.58 (95% CI 1.03–2.41), p=0.033 Mean RBC folate: GDM 426.44 ng/mL vs controls 380.95 ng/mL, p=0.045 B. FAS and GDM risk: Regular intake of FA tablets or compound vitamin supplements after the last menstrual period and during pregnancy: aOR =1.73 (95% CI 1.19–2.53), p=0.004 Continuous RBC folate: OR =1.08 (95% CI 1.00–1.17), p=0.033
Cheng et al., 2022	Yes	GDM 75 g OGTT performed during 24−28 weeks gestation. GDM diagnosed if FPG >5.1 mmol/L,	Increased likelihood of GDM with high serum FA Q4 vs Q1 : aOR=1.84 (95% CI :1.07-3.16), p=0.012 High serum FA: Q4 vs Q3: OR = 1.84 (95% CI: 1.07–3.16), p = 0.012 L Among women with pre-pregnancy BMI ≥24 kg/m²: • High FA Q4 vs. Q3: OR = 2.09 (95% CI: 1.11–3.93), p = 0.022

		and/or 1 h glucose >10.0 mmol/L, and/or 2-hour glucose >8.5 mmol/L.	
Lai et al., 2018	Yes	GDM GDM diagnosed after a 75g OGTT if FPG ≥7.0 mmol/L, and 2 h glucose ≥7.8 mmol/L	Increased likelihood of GDM with high folate (T3) & low B12: OR 1.97 (95%CI: 1.05, 3.68) Association of folate and vitamin B12 with GDM: High folate and low B12 (T3 vs T1 within B12 deficient women): OR = 1.97 (95% CI: 1.05–3.68), p = 0.034. Per 1 SD increment in folate: OR = 1.40 (95% CI: 1.17–1.68), p = 0.001. Model 1:aOR = 1.30 (95% CI: 1.07–1.59), p = 0.009. Model 2:aOR = 1.29 (95% CI: 1.01–1.60), p = 0.039. Associations with plasma glucose: Higher 2 h glucose per 1 SD increment in folate: β = 0.15 (95% CI: 0.04–0.25), p = 0.014. Higher odds of GDM per 1 SD folate: OR = 1.29 (95% CI: 1.01–1.60). Associations strongest among Indian mothers (ethnicity stratified regression subanalysis): High folate associated with GDM: OR = 2.28 (95% CI: 1.24–4.18), p = 0.008. Model 1: Adjusted for maternal age, ethnicity, education, income, smoking, alcohol, physical activity, pre pregnancy BMI, parity, family history of diabetes, previous GDM. Model 2: As Model 1 plus mutual adjustment for the other B vitamins, because folate/B6/B12 function within shared metabolic pathways
Li et al., 2019a	Yes	GDM Screening 75 g OGTT at 24 to 28 weeks gestation. GDM diagnosed if FPG ≥5.1 mM, 1 h glucose ≥10.0 mM, or 2 h glucose ≥8.5 mM.	Increased risk of GDM and higher glucose concentrations with elevated maternal folate levels. Highest vs. lowest folate tertile: aOR = 1.98 (95% CI: 1.00,3.90; p = 0.049) Increased risk of GDM with high folate: B12 ratio especially in older mothers (≥30 years). . Highest vs. lowest ratio tertile: aOR = 3.08 (95% CI: 1.63,5.83; p = 0.001) Logistic regression analysis of GDM in relation to tertiles of maternal folate, < 6.9 Reference 6.9-12.2 aOR =1.00 (95% CI : 0.53,1.89) P= 0.999 ≥ 12.2 aOR= 1.53 (95% CI : 0.80,2.93) P= 0.194 Combined effect of a high folate:vitamin B12 ratio and advanced maternal age (≥30 years) significantly increased GDM risk (aOR = 2.13; 95% CI: 1.09–4.15; p = 0.027) compared with younger women with a low ratio

Li et al., 2022a	Yes	GDM & higher folate levels Screening 75 g OGTT during 24–28 gestational weeks. GDM diagnosis if FPG ≥ 5.1 mmol/L, 1 h glucose ≥ 10.0 mmol/L, and/or 2 h glucose ≥ 8.5 mmol/L.	Increased likelihood of GDM with higher folate quartiles Q1 (ref) Q2 : OR = 1.30 (95% CI : 0.87–1.93), p = 0.199 Q3 : OR = 1.43 (95% CI : 0.96–2.12), p = 0.075 Q4: OR = 1.76 (95% CI: 1.20–2.58), p = 0.004, aOR = 2.28 (95% CI: 1.49–3.61), p < 0.001 p for trend < 0.001 Per IQR increase in folate: OR = 1.36 (95% CI: 1.07–1.72), p = 0.011, aOR = 1.59 (95% CI: 1.22–2.13), p = 0.002 Higher B12 reduced GDM risk (aOR = 0.45; 95% CI: 0.30–0.69); p < 0.001
	Yes	GDM & *MTHFR* polymorphisms	Increased likelihood of GDM with high folate with *MTHFR TT* genotype • Folate per IQR: OR = 1.66 (95% CI: 1.20–2.30), p = 0.002 • Serum B12 per IQR: OR = 0.80 (95% CI: 0.65–0.98), p = 0.033 *MTHFR rs1801131* (TT, TG, GG) TT genotype = high-risk group; TG/GG = protective genotypes. Genotype associations with GDM: • TT (reference) • TG: OR = 0.68 (95% CI: 0.49–0.96), p = 0.027 • GG: OR = 0.30 (95% CI: 0.07–1.33), p = 0.113 • Dominant model (GG/TG vs TT): OR = 0.65 (95% CI: 0.47–0.91), p = 0.012 • Additive model: OR = 0.66 (95% CI: 0.48–0.89), p = 0.008
Li et al., 2019b	Yes	GDM GDM diagnosed following 75 g OGTT at 24–28 gestational weeks if any one of 3 plasma glucose values was ≥threshold value: FPG, 5.1 mmol/L, post-prandial, 1 h, 10.0 mmol/L or 2h, 8.5 mmol/L (IADPSG 2010 criteria)	Increased likelihood of GDM with FA800-L Compared with non-users: FA400–800 mcg/d: Short duration: OR= 1.23(95% CI: 0.84,1.80); P>0.05 Long duration: OR= 1.23 (95% CI: 0.78,1.94); P>0.05 FA≥800 mcg/d Short duration: OR = 1.37 (95% CI 0.93,2.01); P>0.05; Long duration: OR = 2.36 (95% CI : 1.51,3.69), aOR 2.09 (95% CI : 1.30,3.36); P<0.05 FA800-L had higher OGTT glucose values than non-users: 1h: 0.34 mmol/L increase (95% CI: 0.14–0.54; p < 0.05) 2h: 0.21 mmol/L increase (95% CI: 0.06–0.36; p < 0.05) No significant difference in fasting glucose values.

Liu et al., 2020	Yes	GDM in those with *MTHFR* polymorphisms *MTHFR* C677T (rs1801133) — genotypes CC, CT, TT GDM diagnosed following 75 g OGTT at 24–28 gestational weeks if any one of 3 plasma glucose values was ≥threshold value: fasting plasma glucose ≥ 5.1 mmol/L, 1-hour glucose ≥ 10.0 mmol/L, or 2-hour glucose ≥ 8.5 mmol/L. (IADPSG 2010 criteria)	Increased likelihood of GDM with higher RBC folate level quartiles: Q1: reference aOR =1.00 Q2: aOR= 1.354 (95% CI: 0.530-3.456) P= 0.526 Q3: aOR= 1.374 (0.546-3.455) P= 0.500 Q4: aOR= 2.47 (1.013-6.037) P= 0.047 No increased risk with *MTHFR* polymorphisms
Liu et al., 2023	Yes	GDM GDM diagnosed following 75 g OGTT at 24–28 gestational weeks if any one of 3 plasma glucose values was ≥threshold value: fasting plasma glucose ≥ 5.1 mmol/L, 1-hour glucose ≥ 10.0 mmol/L, or 2-hour glucose ≥ 8.5 mmol/L. (IADPSG 2010 criteria)	Increased RR of GDM across serum quartiles in relation to maternal serum folate Q1 : reference Q2 : RR=1.15 (95% CI : 1.04, 1.26) ;p <0·001 Q3 : RR= 1.40 (95% CI : 1.27, 1.54) ; p <0·001 Q4 : RR=1.54 (95% CI : 1.40, 1.69) ; p <0·001 P for trend < 0.001 Continuous: per SD serum folate increment: median 44.79 (IQR 32.46-54.03) Adjusted model: RR=1.16 (95% CI 1.13-1.19); p <0·001 Normal vs excessive serum folate for GDM: GDM with normal (<=45.3nmol/L) vs excessive serum folate (>45.3 nmol/L) Normal folate group: mean ± SD: 31.73 ± 8.92 nmol/L, Excessive folate group: mean ± SD: 53.10 ± 3.64 nmol/L, p < 0.001 (group comparison of serum folate). Association with plasma glucose (continuous analysis, linear regression): Increase in plasma glucose per SD increase in serum folate: β = 0.01 mmol/L (95% CI: 0.00–0.01), p < 0.001

Saravanan et al., 2021	Yes	GDM. Diagnoses based on NICE criteria (FPG ≥5.6 mmol/l or 2 h post-load plasma glucose ≥7.8 mmol/l) and IADPSG criteria (FPG ≥5.1 mmol/l or 2 h-PG ≥8.5 mmol/l)	Increased relative risk of GDM with excess folate. Each SD increase in serum folate (12.16 nmol/L in early pregnancy) was associated with an 11% higher risk of GDM, compared with women at the mean folate level (adjusted RR = 1.11, 95% CI: 1.036–1.182, p = 0.002). (Adjusted relative risk from log-binomial regression; this result reflects a continuous dose–response effect)
Xie et al., 2019	Yes	GDM GDM diagnosed following 75 g oral glucose tolerance test at 24–28 weeks gestation if fasting glucose ≥ 5.5 mmol/L or 2 h plasma glucose ≥ 8.0 mmol/L	Increased risk of GDM with increasing RBC folate concentrations RBC folate was analysed three ways: (1) Continuous variable: Per SD increase in log-transformed RBC folate: RR = 1.16 (95% CI: 1.03–1.30), p = 0.012. (2) Proposed clinical cut-offs; 906-1,292 nmol/l: OR=2.17, 95% CI, 1.20,3.95, P = 0.011 ≥1,292 nmol/l: OR 2.76; 95% CI 1.56,4.89, p<0.001; p trend <0.001 *Reference group:* <906 nmol/L (3) Quintiles based on the distribution in controls GDM risk according to maternal RBC folate by quintile ;QU1:reference QU2:OR =2.31 (95% CI : 1.52,3.50) aOR= 2.25 (1.47-3.43) QU3: OR =2.86 (95% CI : 1.91,4.29) aOR= 2.65 (1.75-4.02) QU4: OR= 2.08 (95% CI : 1.37,3.18) aOR= 1.78 (1.16-2.75) QU5: OR= 2.64 (95% CI : 1.75,3.97) aOR= 2.31 (1.51-3.52) P trend= 0.012

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Study Characteristics Related to Preconception and Pregnancy

The preconception and pregnancy outcome studies, n=19 (see Appendix 6 and 7) included a total of 68,280 women and assessed FA intake through dietary fortified foods only (n=9), supplements only (n=6), or a combination of diet and supplements (n=4). Nineteen studies evaluated the impact of excess FA on the following: gestational diabetes mellitus (GDM) (n=10) [317, 325, 347-353], preeclampsia (n=5) [354-358], and fertility and miscarriage risk (n=4) [346, 359-361]. These studies were conducted in Asia (China, Iran, Singapore) (n=12) [317, 325, 347-351, 353, 355-357, 362], Europe (Netherlands, Russia, Sweden, United Kingdom) (n=4) [346, 352, 359, 360], South America (Brazil) (n=1) [361], North America (Canada) (n=1) [354] and one multi-centre trial across five countries (Argentina, Australia, Canada, Jamaica and United Kingdom) [358].

2.4.4.1.1 Fertility and Miscarriage Risk

Three studies found an increased likelihood of infertility, reduced in-vitro fertilisation (IVF) outcomes and smaller embryonic size in women [346, 359, 360].

The first study, conducted in women diagnosed with unexplained infertility undergoing assisted reproduction (primarily IVF), investigated FA supplement use where plasma folate concentrations >22.5 nmol/L were used as a biomarker of FA intake versus non-use (≤22.5 nmol/L). Pregnancy was confirmed by ultrasound at 7 weeks, and miscarriage was defined as pregnancy loss after a verified gestational sac but before 20 weeks of gestation. Women with unexplained infertility were significantly more likely to use FA-containing supplements compared to fertile controls (OR 3.31; 95%

CI: 2.16–5.09; p<0.001), and daily FA use was strongly associated with supplement intake (OR 5.90; 95% CI: 3.46–10.06; p<0.001). Although there was a nonsignificant trend toward more miscarriages among FA users (8.8% vs 4.8%), no significant association was found between FA supplementation and clinical pregnancy or live birth outcomes [359].

Polzikov et al. observed that high serum folate in the highest quartile (Q4: ≥33.0 ng/mL; equivalent to ≥74.6 nmol/L) was associated with poorer IVF outcomes compared to the lowest quartile (Q1: 2.9–10.7 ng/mL; [346]. Women in Q4 had significantly fewer fertilised oocytes (median 5.0 [range 0–34]; mean ± SD: 7.74 ± 7.49) compared to Q1 (median 8.0 [range 0–24]; mean ± SD: 9.16 ± 5.62; p = 0.049). The number of mature oocytes was also lower in Q4 (median 6.0 [range 2–34]; mean ± SD: 9.04 ± 7.16) versus Q1 (median 10.0 [range 1–25]; mean ± SD: 10.7 ± 5.89), although the p-value for this comparison was not reported. Clinical pregnancy and live birth rates were markedly reduced in Q4 (8.7% for both outcomes) compared to Q1 (32% for both), with differences reaching statistical significance (p = 0.047 for both comparisons). These percentages represent the proportion of women achieving each outcome per quartile.

Van Uitert et al. showed that RBCF levels in the highest quartile (Q4: 1813–2696 nmol/L) were associated with smaller embryonic size, measured as crown–rump length (CRL). Compared with the third quartile (Q3: 1513–1812 nmol/L, reference group), embryos in Q4 were 24.2% smaller at 6 weeks (CRL: 4.1 mm; 95% CI: 3.5–4.7 vs 5.4 mm; 95% CI: 4.8–6.1) and 7.6% smaller at 12 weeks (CRL: 55.1 mm; 95% CI: 52.9–57.3 vs 59.6 mm; 95% CI: 57.4–62.0). Multivariable linear mixed model analysis confirmed this association (effect estimate for Q4 vs Q3: β = –0.30; 95% CI: –0.50 to –0.10;

$p < 0.01$), indicating that very high RBC folate may negatively influence early embryonic growth [360].

One study found that 4.0 mg per day of FA supplementation during the pre-conceptional period until the end of the first trimester, compared to a dose of 0.4 mg per day, did not increase the risk of miscarriage in women with non-syndromic orofacial clefts or who had a child with orofacial cleft. In contrast, a randomised controlled trial [363] reported that 4.0 mg/day FA supplementation versus 0.4 mg/day significantly reduced the risk of spontaneous abortion (adjusted relative risk [aRR] = 0.56; 95% CI: 0.34–0.93; $p = 0.02$) and improved other pregnancy outcomes. The composite outcome of one or more adverse events (including spontaneous abortion, intrauterine fetal death, preeclampsia, abruptio placentae, SGA, preterm delivery, and major congenital malformations) was lower in the high-dose group (aRR = 0.52; 95% CI: 0.38–0.72; $p = 0.0001$). Additional benefits included reduced risk of SGA (aRR = 0.43; 95% CI: 0.19–0.97; $p = 0.04$) and preterm delivery (aRR = 0.44; 95% CI: 0.20–0.95; $p = 0.04$). These findings suggest that higher FA intake may positively influence maternal and offspring outcomes.

2.4.4.1.2 Preeclampsia (PE)

Four studies (Table 5) investigated the relationship between excess FA intake and the incidence of preeclampsia (PE) or the incidence of gestational hypertension [354-358]. Corsi et al. (2022) defined preeclampsia as diastolic BP ≥90 mmHg on two occasions at least 4 hours apart plus significant proteinuria (>2+ on dipstick or >300 mg/mmol) after 20 weeks gestation and evaluated 4 mg/day FA supplementation [354]. Manizheh et al. (2009) used

the definition of severe preeclampsia as BP ≥160/110 mmHg with proteinuria ≥2 g/24 h or ≥2+ on dipstick and compared 5 mg/day vs 0.5 mg/day FA [357]. Hashemi et al. (2016) studied endothelial dysfunction in women already diagnosed with preeclampsia and assessed 5 mg/day FA vs placebo [355]. Wen et al. (2018) examined women at high risk for preeclampsia and tested 4 mg/day FA from 8 to 16 weeks gestation vs placebo [138]. Across these studies, high FA supplementation (≥800 mcg/day or 4–5 mg/day) was not associated with reduced risk of preeclampsia [354, 357, 358], although Hashemi reported that 5 mg/day FA improved endothelial function in preeclamptic patients, assessed by brachial artery flow-mediated dilation (FMD) using high resolution B-mode ultrasonography, where FMD increased from 9.64 ± 5.57% to 13.72 ± 7.89% after supplementation, compared to 9.30 ± 4.25% to 10.02 ± 4.81% in the placebo group (P = 0.002 between groups; P = 0.013 within intervention group).

A Chinese prospective cohort study of 4,853 participants [356] evaluated folic acid (FA) supplement use and dietary folate intake in relation to gestational hypertension (GH), defined as blood pressure ≥130/80 mmHg after 20 weeks' gestation. The study found that women consuming high-dose FA supplements (≥800 mcg/day) from pre-pregnancy through mid-pregnancy had an increased risk of GH compared to non-users (risk ratio [RR] = 1.33 (95%CI: 1.08-1.65); adjusted RR = 1.32 (95%CI: 1.06-1.64). Non-users were defined as women who never used FA supplements, or used FA at a daily dose <400 mcg, or for a duration of <4 weeks from pre-pregnancy to mid-pregnancy.

Importantly, dietary folate intake was also assessed using a validated food frequency questionnaire, and no significant differences were observed

across FA supplement groups, indicating that the elevated GH risk was associated with supplemental FA rather than dietary folate [356](Li et al., 2020).

2.4.4.1.3 Gestational Diabetes Mellitus (GDM)

Ten studies examined the relationship between supplemental FA intake or elevated blood concentrations of folate and GDM risk, consistently finding a positive association between higher FA supplementation (more than 400 mcg/day throughout pregnancy), serum folate (>27.5 nmol/L), RBCF (≥ 862.5nmol/L) and a risk of GDM [317, 325, 347-353, 364].

2.4.4.1.3.1 Supplemental FA Intake and GDM

A Chinese study reported that daily folic acid supplementation, being regular intake of folic acid tablets or compound vitamin supplements after the last menstrual period and during pregnancy (approximately 400 mcg/day) was associated with an increased risk of gestational diabetes mellitus (GDM) (adjusted odds ratio [aOR] = 1.73; 95% CI: 1.19–2.53; p = 0.004). The same study also found that women with high red blood cell (RBC) folate concentrations (≥600 ng/mL) had a higher GDM risk (aOR = 1.58; 95% CI: 1.03–2.41; p = 0.033) (Chen et al., 2021). RBCF concentration RBC folate for group with GDM =426.44 ng/mL and controls = 380.95ng/mL p=0.045 [347].

Another Chinese study of 4,353 pregnant women from the Tongji Maternal and Child Health Cohort found that taking a folic acid (FA) supplement daily for at least 4 weeks before pregnancy and continuing for 16 weeks during pregnancy (long duration) was associated with an increased risk of gestational diabetes mellitus (GDM), particularly at doses ≥800 mcg/day (adjusted OR = 2.09; 95% CI: 1.30–3.36; p < 0.05) compared with non-users.

The association remained consistent after stratification by gestational weight gain, family history of diabetes, and fetal sex. Women taking ≥800 mcg/day for the long duration also had significantly higher 1-hour and 2-hour OGTT glucose values than non-users by 0.34 mmol/L (95% CI: 0.14–0.54; $p < 0.05$) and 0.21 mmol/L (95% CI: 0.06–0.36; $p < 0.05$), respectively, while fasting glucose showed no significant difference [325]. Li et al. (2022a) strengthened this evidence, showing that women in the highest serum folate quartile (≥14.6 ng/mL) had a significantly increased risk of GDM (aOR 2.28; 95% CI: 1.49–3.61; $p<0.001$) [349]. The association was stronger in women with the *MTHFR* TT genotype, compared to women with the *MTHFR* TC or CC genotype, suggesting a gene–nutrient interaction. Liu et al. (2023) reported a clear dose-response relationship prior to 24 weeks gestation, with excessive serum folate (>45.3 nmol/L) associated with progressively higher relative risks across quartiles (Q4 RR 1.54; 95% CI: 1.40–1.69; p for trend <0.001) and significantly higher mean plasma glucose levels ($p<0.001$) [351].

2.4.4.1.3.2 RBC Folate Concentrations and GDM

Higher RBCF concentrations were also strongly linked to an elevated risk of GDM. In early pregnancy (9–13 weeks), the mean RBC folate concentration was significantly higher in women who later developed GDM compared to those who did not (426.44 ng/mL vs 380.95 ng/mL, $p = 0.045$) [347]. Furthermore, women with RBC folate concentrations ≥600 ng/mL had a 1.58-fold higher risk of GDM even after adjustment for age, periconceptional BMI, family history of diabetes, smoking exposure, and drinking status (aOR = 1.58; 95% CI: 1.03–2.41; $p = 0.033$) [347]. These findings are consistent with the study by Xie et al. who also found a clear dose-response relationship between RBC folate levels and GDM risk [353]. This study, which recruited

pregnant women between nineteen and twenty-four weeks gestation found that maternal RBC folate concentrations between 906 and 1,292 nmol/L were associated with a more than twofold increased risk of GDM (OR = 2.17; 95% CI 1.20-3.95, p=0.011) compared with those with folate concentrations less than 906 nmol/L after adjusting for maternal age, parity, BMI at enrolment. Women with the highest concentrations (≥1,292 nmol/L) had an even higher risk (OR 2.76; 95% CI 1.56-4.89 p<0.01) in the same study [347]. Li et al. (2022a) [349] reported that higher serum folate concentrations in mid-pregnancy were associated with increased GDM risk, while higher vitamin B12 was protective. Women in the highest folate quartile (>14.6 ng/mL) had more than twice the odds of GDM compared with those in the lowest quartile (<6.2 ng/mL) (aOR = 2.28; 95% CI: 1.49–3.61; p < 0.001). Conversely, higher B12 reduced GDM risk (aOR = 0.45; 95% CI: 0.30–0.69; p < 0.001). Importantly, the *MTHFR* rs1801131 TT genotype conferred greater susceptibility, with gene–nutrient interaction analyses showing that the association between folate and GDM was stronger among TT carriers (OR = 1.66; 95% CI: 1.20–2.30, p=0.002) [349]. These findings complement Liu et al. (2020), who observed that high RBC folate in early pregnancy (Q4 ≥862.5 nmol/L, vs. Q1) was associated with increased GDM risk (aOR = 2.47; 95% CI: 1.01–6.04; p = 0.047),) after adjustment for age, BMI, physical activity, family history of diabetes, parity, FA supplement use, homocysteine levels, C-Reactive Protein (CRP), haemoglobin, serum B12 [350]. This association was attenuated after adjusting for *MTHFR C677T* genotype. Together, these studies suggest that both folate status and genetic variants in folate metabolism pathways may influence GDM risk.

2.4.4.1.3.3 Plasma/Serum Folate Concentrations and GDM

High plasma/serum folate concentrations were also found to be associated with a higher risk of GDM. A study by Cheng et al. conducted in China in a cohort of 744 pregnant women reported that plasma folate concentrations in the highest quartile (≥19.1ng/mL) were linked to a significantly increased risk of GDM compared to the lower quartile, (Q1<10.77 ng/mL (aOR= 1.84 (95% CI:1.07,3.16), p=0.012)[317]. The authors found that the optimal level of serum folate to reduce GDM risk was < 42.9 nmol/L and there was a significant increased risk of GDM if folate was in Q4 (>19.1ng/mL) [317]. Li et al., (2019a) reported that an imbalance between folate and vitamin B12, expressed as a high folate to vitamin B12 ratio, was strongly associated with GDM risk. Women in the highest ratio tertile had over threefold higher odds of GDM compared with those in the lowest tertile (aOR = 3.08; 95% CI: 1.63–5.83; p = 0.001). Furthermore, the combined effect of a high folate to vitamin B12 ratio and advanced maternal age (≥30 years) significantly increased GDM risk (aOR = 2.13; 95% CI: 1.09–4.15; p = 0.027) compared with younger women with a low ratio [364]. Another study reported that higher plasma folate concentrations were associated with higher plasma glucose concentrations 2-hours post oral glucose tolerance test (OGTT) and a greater risk of developing GDM (OR = 1.29, 95% CI: 1.01–1.60, p = 0.039), particularly in certain ethnic groups, such as Indian mothers (sub-analysis: OR = 2.28, 95% CI: 1.24–4.18, p = 0.008) [348]. Saravan et al. observed that the risk of GDM increased by 11% for women with plasma folate concentrations exceeding 45 nmol/L (aRR = 1.11, (95% CI:1.04-1.18, p=0.002) [352].

2.4.4.2 Study Characteristics Related to Outcomes in the Offspring

Studies that reported outcomes in offspring included ages from birth to 10 years (n=26) (see Table 6). They predominantly assessed exposure of FA intake in the mother in combined fortified diet and supplement use (n=15), supplement intake only (n=8), and dietary fortified foods only (n=3). Included studies measured red blood cell folate (n=4), plasma folate (n=7), and UMFA (n=3). Three of these studies assessed both FA intake and their respective blood concentrations. The total number of participants in these studies was 376,410. This included 9383 mother-child dyads, 226,802 mothers and 140,225 children.

Table 6 – ***Characteristics of studies (n=26) which have evaluated the effects of higher maternal levels of folic acid supplementation or measured blood folate levels on offspring-related outcomes***

Study	Country	Study period	Study design	Population description	Participant numbers	Timing of exposure/ folate assessment	Type of folate exposure	Blood folate level measured *	Folic acid/blood folate level categories used for study analysis and considered excess where reported
Allergy									
Best et al., 2021	Australia	Nov 2011-Dec 2016	Cohort study	Pregnant women >18 years of age whose unborn infant had a first degree relative with a history of medically diagnosed allergic disease. Recruited women >36 wks gestation Infants at 1 year of age with high risk of allergy.	n=561 mother-infant dyads	Blood: 36 and 40 weeks of gestation	Dietary & supplements	Maternal serum UMFA and folate (not fasted)	Maternal serum UMFA (nmol/L) median = 1.6 Q1: <0.66 Q2: 0.66-1.65 Q3: 1.65-4.69 Q4: >4.69 Maternal serum folate (nmol/L) median=53.2 Q1: <32.6 Q2: 32.6-53.2 Q3: 53.2-74.5 Q4: >74.5 nmol/L
Magdelijns et al., 2011	Netherlands	Oct-2000 – 2007	Cohort study	Health pregnancy women week 10-14 (conventional group). Recruitment	n=2640 pregnant women (Total) n=842 home visit for child	Blood: 35 weeks gestation Supplement use: FFQ: 14 to 34 weeks gestation.	Supplements	RBC ICF (not stated)	Maternal ICF (nmol/L) QU1: ≤ 480 QU2 : 481-643 QU3 : 644-862 QU4 : 863-1139 QU5: ≥1140

				through organic food shops/midwives (alternative group). Children: part of the KOALA Birth Cohort study. Offspring age: Outcomes assessed up to 6–7 years (serial follow-up at 3, 7, 12, 24 months, and older ages until 6-7 years).	age 24 m and n=435 home visit for child age 6-7 y				
Molloy et al., 2020	Australia	2010-2013	Cohort study	Pregnant women 28-32 weeks gestation and their infants at 1 year of age	n=1074 mother-infant pairs	Maternal RBC folate measured at 28-32 weeks gestation FA supplementation recorded for 1st and 2nd trimesters	Dietary/Supplements	RBC folate (not stated)	Maternal RBC folate (nmol/L): >1360 defined as high. <906 defined as low. FA Supplements <500 mcg/d 500-999 mcg/d ≥to 1000 mcg/d
Parr et al., 2017	Norway	1999-2008	Cohort study	Women 18 weeks gestation Offspring assessed 2 7 years	n=95,248 mothers n=114,761 children born 1999 - 2009	Blood: 18 weeks gestation FA intake assessed by FFQ 22 weeks gestation	Dietary/ supplements	Plasma folate (non-fasted)	Total folate intake in pregnancy by quintiles (mcg/day) QU1: <146 QU2 : 147–216 QU 3 : 217–391 QU : 392–577

									QU5 : >578 Plasma folate (nmol/L) Q1 : ≤ 5.91 Q2 : 5.92-8.68 Q3: 8.69-14.69 Q4: ≥14.70
Roy et al., 2018	United States	2006-2011	Cohort study	Mother/infant dyads	n=858 mother-infant dyads	Blood: 2nd trimester	Dietary/ supplements	Plasma folate (not stated)	2nd trimester plasma folate ≥ 20ng/mL
Trivedi et al., 2018	United States	1999-2002	Cohort study	Mother-child pairs from Project Viva.	n= 1279 mother-child dyads	FFQ 11-12 weeks gestation	Dietary/ Supplements	N/A	FA intake (mcg/d): First trimester, Mean=930 Q1:129-660 Q2:661-909 Q3:910-1175 Q4:1176-3497 Second trimester (mcg/d), Mean=1238 Q1:118-937 Q2:938-1357 Q3:1358-1502 Q4:1503-2601
Vereen et al., 2019	United States	Dec 2006-Jun 2011	Cohort study	Mother/infant dyads	n=676 mother-infant dyads	Blood: 2nd Trimester	Dietary/ supplements	Plasma folate (not stated)	Plasma folate (ng/ml) Q1:3.76-12.9 Q2: 12.94-19.2 Q3: 19.23-26.8 Q4: 26.8-85.0
Yang et al., 2015	China	March 2010-March 2011	Case control study	Infants with asthma infant controls	n=362 infants aged under 2.5 years old (total):	FA supplementation during peri-pregnancy	Supplements	N/A	Cumulative dose = Daily dose × total supplementatio

					n=150, Infant asthma cases; n= 212 controls.				n days (mcg/d). Dose categories: Zero Low: <36,000 mcg/d = ~ 400 mcg/d for <3 months Middle: 36,000–72,000 mcg = ~400 mcg/d for 3–6 months High: >72,000 mcg/d =~ 400 mcg/d for >6 months OR 800 mcg/d for 3 months
Congenital Malformations									
Bortolus et al., 2021	Italy	Jul 2009-Feb 2014	Randomised controlled trial	Women of childbearing age (18-44 years) who planned a pregnancy within 12 months.	n= 1060 controls, randomised: n=529 to 4.0 mg FA and n= 531 to 0.4 mg FA. n=505 conceptions randomised: n=267 to 4.0 mg FA and n=238 to 0.4 mg FA.	Preconception & until 12 weeks gestation	Supplements	N/A	High dose FA intake: 4.0 vs 0.4 mg/d FA supplementation

Husen et al., 2022	Netherlands	15 Oct-04 (Ethics approval). Study dates undeclared.	Cohort study [on-going prospective]	Pregnant women 9+6 weeks of gestational age, depending on the time of the first ultrasound	n=166	Preconception	Dietary/Supplements	RBC folate (not stated)	Maternal RBC folate (nmol/L) Median = (min-max) 1343 (466-2919) Q1:466–1078 Q2:1079–1342 Q3 (reference level): 1343-1594 Q4 (elevated):1595-2919
Michels et al., 2012	Netherlands	2003-2007	Case control study	Mothers of children with positional PP and mothers of children without positional plagiocephaly (controls)	n=80 Positional plagiocephaly group and n=55 Control group	Preconception & 1st trimester Survey to evaluate FA supplementation preconception (4 weeks before pregnancy until 8th week gestation)	Supplements	N/A	Defined as: 400 mcg (normal) vs 800 mcg Double FA supplement vs control
Michikawa et al., 2020	Japan	2011-2014	Cohort study	Women in pregnancy were recruited @12th week of gestation.	n=89,235 pregnant women	Preconception & pregnancy FFQ dietary & supplement intake pre-pregnancy (1 year)	Supplement and dietary	N/A	Maternal intake of FA (400 mcg/d) and vitamins B6/B12; Combined intake = folate ≥400 mcg/d + high B6 and/or B12.

									Usual intake: (long-term, habitual dietary folate intake over preceding year)
Large for Gestational Age									
Bortulus et al., 2021	Italy	July 2009 to Feb 2014	Randomised controlled trial	Women of childbearing age (18-44 years) who planned a pregnancy within 12 months.	n= 1060 controls, randomised: n=529 to 4.0 mg FA and n= 531 to 0.4 mg FA. n=505 conceptions randomised: n=267 to 4.0 mg FA and n=238 to 0.4 mg FA.	Preconception to 12 weeks gestation	Supplements	NA	High dose FA intake 4.0 mg/d vs 0.4 mg/d FA supplementation
Xie et al., 2018	China	May 2013-May 2014	Case control study nested in a population-based cohort	Pregnant women 19-24 weeks Cases: Mothers who delivered LGA infants (birth weight >90% for gestational age) Controls: randomly selected at 2:1	n=542 [Cases] n=1084 [Controls]	Blood: 19-24 weeks gestation	Dietary	RBC folate (not stated)	RBC folate concentrations (ng/L) <400 400-570 > 570

				ratio and matched for age and gestational weeks at enrolment among pregnant women from the population.					
Methylation									
Plumptre et al., 2020	Canada	Nov 2010-Jan 2012	Cohort study (PREFORM Study)	Pregnant women 18-45 yrs between 12-16 weeks gestation	n=368 pregnant women (total) [n=289 deliveries at full term].	Maternal blood: a)12-16 weeks gestation b) 37-42 weeks gestation c) cord blood at delivery	Dietary/Supplements	Maternal RBC folate, serum UMFA (non-fasted) Cord RBC folate and UMFA	RBC folate (nmol/L) at 12-16 weeks gestation mean = 2417 Range: 2362-2472 considered very high. 95% participants consumed FA supplements 1,000 mcg/d during pregnancy
Neurodevelopment									
Compan-Gabucio et al., 2021	Spain	2003-2008	Cohort study	Mother-child dyads available at 4–5-year child assessment (665 girls, 664 boys)	n=1329 mother-child pairs	Supplement use self-reported by FFQ administered at 10-13 weeks and 32 weeks, to evaluate FA supplement intake in	Dietary/Supplements	N/A	FA supplementation (mcg/d) <400 400-999 (reference) ≥1,000

						periconceptional period, 2nd trimester (4th-7th month) and entire pregnancy			
Compan-Gabucio et al., 2022	Spain	2003-2008	Cohort study	Pregnant women who gave birth to a live newborn	n=2625 pregnant women	10-13 weeks gestation, 28-32 weeks gestation FFQ dietary intake in preconception to 3 months pregnancy	Dietary/Supplements	N/A	FA intake (mcg/d) < 400 400-999 (recommended dose) ≥1,000 (high dose)
Raghavan et al., 2018	United States	1998-2013	Cohort study	Mother-infant pairs. Mothers of newborns 24-72 hours post-partum.	n=1257 mother infant pairs n=86 ASD child cases and n=1171 neurotypical children]	Blood: 24-72 hours post delivery & questionnaire for supplementation during pregnancy	Supplements	Plasma folate (Not stated)	Supplementation >5 times/week Multivitamin intake = prenatal multivitamin supplements, which included FA Maternal plasma folate (nmol/L): <P10: <14.7 nmol/L P10 to <P90: ≥14.7 to <60.3 ≥P90: ≥60.3 nmol/L (high)
Raghavan et al., 2020	United States	1998-2014	Cohort study	Mothers who delivered a singleton live birth at Boston Medical Centre	n=567 children (total) [n=92 ASD child cases and n= 475	Umbilical cord blood at time of delivery	Dietary	Cord blood UMFA	Cord UMFA (nmol/L) median [IQR] 0.69 [0.36-1.33] Q1(reference):

					neurotypical children]				0.25 Q2: 0.51 Q3: 0.92 Q4: 2.21
Sharman Moser et al., 2019	Israel	2000-2013	Case control study	Children diagnosed with ASD born between 2000 and 2013	n=2009 children with ASD diagnosis prior to age 6 yrs They were matched using a 1:10 ratio to n=19,886 ASD-free children	12 months prior to end of pregnancy Supplement use via dispensing records.	Supplement	N/A	FA supplement dose (mg/d) nil/v.low: < 0.2 low: 0.2- < 0.4 typical: 0.4-< 1 high: 1 -< 3 very high≥ 3
Valera-Gran et al., 2017	Spain	Feb 2004-Jan 2008	Cohort study	Pregnant women (10-13 weeks gestation) mother-child pairs were included in the final analyses.	n=1682 mother child dyads	FFQ: Supplement use & dietary intake assessed from 3 months before conception to 3rd month of pregnancy, and from 4 - 7 months of pregnancy	Dietary/Supplement	N/A	Typical dosages of FA (mcg/d) <400, 400-999 (reference) >1000)
Pre-term Birth									
Chen et al., 2015	Singapore	Jun 2009-Sept 2010	Cohort study	Pregnant women who were attending antenatal care <14 weeks of gestation; dating determined by	n= 986	Blood: 26-28 weeks gestation	Dietary/supplement	Plasma folate (fasted)	Highest quartile median for plasma folate = 57.1 nmol/L (range: 51.2-72.7)

				ultrasound scan					
Olapeju et al., 2019	United States	1998-2014	Cohort study	Mothers who delivered at the Boston Medical Centre recruited 24-72 hours after delivery while still hospitalised.	n= 2313 mothers	Multivitamin supplementation (containing 400–800 mcg FA) 6 months before conception to 3rd Trimester of pregnancy. Maternal interview and structured questionnaire while the mother was still hospitalized after giving birth. Maternal blood 24-72 after delivery Most prenatal multivitamins in the cohort contained **800 mcg FA**, per US standard prenatal formulation	Supplement	Plasma folate	Supplementation frequencies recorded: -none - 1–2 times/week - 3–5 times/week - >5 times/week Plasma folate levels (nmol/L): Q1: 6·6–19·4 Q2: 19·4–30·0 Q3: 30·0–43·8 Q4: 43·8–185·5
Small for Gestational Age									
Bortolus et al., 2021	Italy	Jul 2009-Feb 2014	Randomised controlled trial	Women of childbearing age (18-44 years) who	n= 1060 controls, randomised: n=529 to 4.0	Preconception to 12 weeks gestation	Supplements	NA	High dose FA intake 4.0 mg/d vs 0.4 mg/d FA supplementatio

				planned a pregnancy within 12 months.	mg FA and n= 531 to 0.4 mg FA. n=505 conceptions randomised: n=267 to 4.0 mg FA and n=238 to 0.4 mg FA.				n
Chen et al., 2015	Singapore	Jun 2009-Sept 2010	Cohort study	Pregnant women who were attending antenatal care <14 weeks of gestation; dating determined by ultrasound scan	n= 986 pregnant women	Blood: 26-28 weeks gestation	Dietary/supplement	Plasma folate (fasted)	Highest quartile median for plasma folate = 57.1 nmol/L (range: 51.2-72.7)
Li et al., 2019c	China	Aug 2013-Nov 2013	Cohort study	Women post pregnancy with a live birth	n=7307 women	Supplements before conception & during pregnancy Interviews post-partum month 3	Dietary/supplement	N/A	FA Intake [mcg/d] Median (IQR) Continuous = 299.3 (145.6) T1 =203.3 (76.0) T2 = 299.3 (44.3) T3 = 421.3 (125.4))
Navarrete-Munoz et al., 2019	Spain	May 2004-Aug 2008	Cohort study	mother child dyads	n=2302 mother-child pairs	FA dosages assessed 10-13 weeks and 28-32 weeks gestation FFQ	Dietary/supplement	N/A	FA intake (mcg/d) <1000 (non-user) 1000-499 9≥5000

Nguyen et al., 2017	Vietnam	Oct 2011-May 2012	Randomised controlled trial	Women of reproductive age	n=5011 women (total): n=1,668 multiple micro-nutrient; n=1671 iron and FA; n=1672 FA.	FA supplements in preconception (per day) FA: 2800 mcg FA MM, Multi-mineral + 2,800 mcg FA IFA: 60 mg iron supplement + 2,800mcg FA **FA alone:** 2800 mcg FA weekly preconception; 400 mcg FA daily during pregnancy. **IFA:** Same FA regimen + 60 mg iron. **MM:** Same FA regimen + iron + other micronutrients. FA only, reference group	Supplements	N/A	Not stated
Pastor-Valero et al., 2011	Spain	Feb 2004-Jun 2005	Cohort study	Pregnant Spanish women aged 16+ years	n=840 women (total): n=786 gave birth to live singleton	FA supplements & dietary intake in preconception assessed by FFQ	Dietary/supplement		FA supplement users (mcg/d): moderate, ≤1000; high, >1000 Reference group = non-users of FA supplements Dietary folate intake (mcg/d): QU1 : ≤211·1 QU2 :211·2–

									245·4 QU3 :245·5– 283·7 QU4 :283·8– 332·0 QU5:>332

Legend- FFQ – food frequency questionnaire, NS – not significant, SGA – small for gestational age

The studies investigated the following outcomes in offspring: atopic eczema, food allergy, wheeze and asthma (n=8) [219, 255, 338, 365-369], neurodevelopment and cognitive function (n=6) [318, 319, 327, 370-372], small or large for gestational age (n=6) [316, 362, 373-376], congenital malformations (n=4) [345, 363, 377, 378], impaired methylation related defects (n=1) [379] and preterm birth (n=1) [380]. Studies were conducted in Europe (Italy, Netherlands, Norway, Spain) (n=10) [318, 319, 327, 363, 365, 367, 373, 375, 377, 378], Asia (China, Israel, Japan, Singapore, Vietnam) (n=7) [255, 316, 345, 362, 372, 374, 376], North America (Canada, United States) (n=7) [219, 368-371, 379, 380] and Australia (n=2) [338, 366]. Quality assessment of these offspring outcomes included evaluation of cohort studies (n=20), case control studies (n=4), and randomised-controlled trials (n=2).

2.4.4.3 Findings Related to Outcomes in Offspring

2.4.4.3.1 Allergic Outcomes

The eight studies investigating the association between excess FA and allergic outcomes examined a range of conditions from newborn to childhood. Assessment methods varied, with folate measured in plasma/serum, or RBCF in five studies [338, 365, 366, 368, 369], while four studies assessed folate and FA intake via food and/or supplementation during pregnancy [219, 255, 365, 367]. Blood testing was typically conducted in the third trimester, with measurements including serum [82], intracellular, [365] , and RBCF [366], and UMFA [338].

No significant associations were found between higher maternal folate status, whether assessed as total serum/ plasma folate, RBCF (including levels defined as high, >1360 nmol/L), or unmetabolized folic acid (UMFA),

and offspring allergic outcomes, including atopic disease, eczema, atopic dermatitis, or IgE-mediated food allergy [338, 365, 366]. Comparisons were based on continuous measures and categorical thresholds (e.g., quartiles or high folate cut-offs), with adjusted risk ratios showing no evidence of increased risk across these ranges.

In infancy, respiratory outcomes included asthma or bronchiolitis in four studies [219, 255, 367, 369] and wheeze in three studies [338, 365, 368], while skin-related outcomes such as atopic dermatitis were assessed in one study [368]. Yang et al. (2015) reported that cumulative maternal folic acid supplementation, defined as total dose >72,000 mcg (equivalent to 400 mcg/day for 6 months or 800 mcg/day for 3 months) was associated with a significantly increased risk of infant asthma compared with no supplementation (adjusted OR = 3.16; 95% CI: 1.15–8.71), and the risk was even higher when supplementation began before pregnancy (OR = 3.32; 95% CI: 1.80–6.12) or after conception (OR = 3.65; 95% CI: 1.78–7.48) [255]. Conversely, low-dose supplementation (<36,000 mcg cumulative; roughly 400 mcg/day for <3 months) was associated with a reduced risk (OR = 0.36; 95% CI: 0.17–0.77).

In contrast, a large US cohort found no association between maternal folic acid intake (from food and supplements) and asthma at mid-childhood (7–10 years), with adjusted OR = 0.80 (95% CI: 0.49–1.33) [219]. Additionally, in the Western Australian cohort, UMFA concentrations in the highest quartile (>4.69 nmol/L) were linked to a greater likelihood of medically diagnosed wheeze at 1 year compared with the lowest quartile (<0.66 nmol/L) (adjusted OR = 3.07; 95% CI: 1.15–8.21) [338]. In childhood, one large Norwegian cohort study reported a modestly increased risk of asthma at 7 years among

children whose mothers were in the highest quintile of total FA intake during pregnancy compared with the lowest quintile (aRR = 1.23; 95% CI 1.06, 1.44) [367]. Vereen et al., 2019 found no increased risk of infant bronchiolitis across maternal plasma folate quartiles (Q4 vs Q1: aOR = 0.73 (95% CI: 0.40, 1.31) [369] . Overall, the association was not statistically significant (p = 0.112).

2.4.4.3.2 Congenital Malformations

Four studies examined the link between excess FA intake (via FA supplementation or FA-fortified food) or elevated blood folate concentrations and congenital malformations [345, 363, 377, 378]. These studies investigated various outcomes, including major or minor structural malformations (as defined by the European Surveillance of Congenital Anomalies (EUROCAT) [363], embryonic head volume and circumference [377], positional plagiocephaly [378] and anorectal malformations [345].

Husen et al. (2022), evaluated embryonic head and brain size using 3D ultrasound at 9 and 11 weeks of gestation. In this Dutch cohort, maternal RBC folate quartiles were defined as Q1: 466–1078 nmol/L, Q2: 1079–1342 nmol/L, Q3: 1343–1594 nmol/L (reference), and Q4: 1595–2919 nmol/L. Compared with Q3, when adjusting for gestational age, head volume was significantly larger in Q4 at 9 weeks (β = 0.233; 95% CI: 0.013, 0.452; P = 0.04) and at 11 weeks (β = 0.661; 95% CI: 0.116, 1.205; P = 0.02). Head circumference at 11 weeks was also greater in Q4 (β = 2.745; 95% CI: 0.340, 5.149; P = 0.03). The authors hypothesised that extreme head size, too large or too small, could influence neurodevelopment, but these measurements were not classified as congenital malformations and fall within the range of normal variation [377]. Michels et al. (2012) conducted a retrospective case–

control study in the Netherlands to examine whether excessive folic acid (FA) intake during pregnancy was associated with positional plagiocephaly (PP). Among mothers of infants with PP, 20% reported using double the recommended FA dose (≥800 mcg/day) compared with 6% in the control group ($p < 0.05$). The recommended daily dose in this study was 400 mcg/day from four weeks before conception until eight weeks of gestation. Duration of FA use beyond eight weeks did not differ significantly between groups [378]. In contrast, Michikawa et al. (2020) focused on anorectal malformations (ARM) in a large Japanese birth cohort (n = 89,235). They found no significant association between folate intake alone and ARM, but observed a suggestive inverse association for combined intake of folate (≥400 mcg/day), vitamin B6, and vitamin B12 during early pregnancy (adjusted OR = 0.4; 95% CI: 0.2, 1.0, p=0.05) [345]. Bortolus et al., (2021) reported no difference in major congenital malformations between 4.0 vs. 0.4 mg FA supplementation: RR=0.80; 95% CI 0.31–2.03 [363].

2.4.4.3.3 Small for Gestational Age (SGA) Babies

Six studies explored the association between excess FA during preconception/pregnancy via supplement intake (n=3), supplement and food intake (n=1), plasma folate concentrations (n=3), and SGA babies [316, 362, 363, 373-375]. Two observational studies reported increased SGA risk with FA ≥1,000 mcg/day compared to non-users [373, 375]. In Spain, FA 1,000–4,999 mcg/day and ≥5,000 mcg/day during the periconceptional period were associated with more than double the odds of SGA by weight (OR = 2.21; 95% CI: 1.17, 4.19 and OR = 2.32; 95% CI: 1.06, 5.08, respectively; $p < 0.05$, both) and a tendency toward lower birth weight (β = –73.83 g; 95% CI: –151.71, 4.06; p=0.06) [373]. Pastor-Valero et al. also reported a significantly higher risk of SGA by length with FA >1 mg/day (OR = 5.33; 95% CI: 2.08,

13.7; *p* = 0.001) and a reduction in birth length (β = –0.53 cm; 95% CI: –0.96, –0.09, p=0.012), while the association with SGA by weight was borderline (OR = 2.05; 95% CI: 0.98, 4.28, p=0.07) [375]. Conversely, two studies found decreased SGA risk with higher FA doses. In a randomized trial, 4 mg/day FA vs 0.4 mg/day from preconception to 12 weeks reduced SGA risk (RR = 0.43; 95% CI: 0.19, 0.97; *p* = 0.04) [363], Similarly, a Chinese cohort reported lower odds of SGA in the highest FA intake tertile (median 421.3 mcg/day) vs lowest (median 203.3 mcg/day) (OR = 0.77; p trend = 0.010) [362]. In Vietnam, FA-only supplementation (2,800 mcg/week) was less effective than FA + iron (IFA) or multiple micronutrients (MM) to reduce the risk of offspring being stunted or to improve offspring fine motor skills. IFA reduced stunting risk at 2 years (RR = 0.87; 95% CI: 0.76, 0.99) and improved fine motor development (mean composite score differences IFA and FA = 0.41; 95% CI: 0.05, 0.77; p = 0.03), suggesting iron, not FA excess, was protective [374]. Finally, a Singapore cohort found no association between high maternal plasma folate (>51.2 nmol/L at 26–28 weeks) and SGA risk (OR = 0.97; 95% CI: 0.77, 1.22), although higher folate was linked to longer gestation (β = 0.12 weeks per 1-SD increase in plasma folate 95% CI: 0.02–0.21; P = 0.017) and lower preterm birth risk[316].

2.4.4.3.4 Large for Gestational Age (LGA) Babies

Two studies examined the relationship between excess FA supplementation and RBCF concentrations and large for gestational age (LGA) babies [363, 376]. Xie et al. (2018) conducted a nested case–control study of 542 large-for-gestational-age (LGA) cases and 1,084 appropriate-for-gestational-age (AGA) controls in China. Median RBCF was significantly higher in LGA cases (668.65 ng/mL [IQR: 528.50–835.52]) than controls (654.72 ng/mL [IQR: 496.72–810.29]; *p* = 0.039). After adjustment for maternal age, BMI,

gestational weeks, and infant gender, women with RBC folate 400–570 ng/mL had an OR of 2.38 (95% CI: 1.41–4.03; p = 0.001) for LGA compared to ≤400 ng/mL, and those with >570 ng/mL had an OR of 2.56 (95% CI: 1.55–4.25; p < 0.001). There was a significant trend (p trend = 0.003). Maternal BMI was higher in LGA cases (24.32 ± 2.73 vs 23.09 ± 2.72 kg/m²; p < 0.001), and birthweight was markedly greater (3,875.41 ± 280.85 g vs 3,330.10 ± 238.03 g; p < 0.001) [353]. Bortolus et al. (2021), in an Italian randomized controlled trial of 1,565 women planning pregnancy, compared 4 mg FA/day vs 0.4 mg FA/day during preconception and the first trimester. Offspring of mothers receiving 4 mg FA had significantly higher mean birthweight (3,357 g vs 3,213 ± 462 g; p = 0.01), indicating an increased risk of higher birthweight with high-dose FA [363].

2.4.4.3.5 Neurodevelopment and Cognitive Decline, and Autism Spectrum Disorder (ASD) in Offspring

Six studies assessed the association between excess FA supplement intake or blood concentrations on adverse neurodevelopmental outcomes particularly focusing on attentiveness, cognitive function and Autism Spectrum Disorder (ASD) in the offspring [318, 319, 327, 370-372].

Three cohort studies evaluated cognitive outcomes at preschool (4–5 years) and school age (7–9 years) in relation to maternal folic acid (FA) dose .

At 4–5 years, Compañ-Gabucio et al. (2021) found that, compared with the recommended 400–999 mcg/day in the periconceptional period, both <400 mcg/day and ≥1000 mcg/day were associated with higher omission errors on the Conners' Kiddie-CPT (indicative of inattention): incidence rate ratio (IRR) = 1.14 (95% CI 1.01–1.29; $p = 0.035$) and IRR = 1.16 (95% CI 1.02–1.33; $p = 0.027$), respectively. In boys, low and high FA doses

were also associated with slower hit reaction time (HRT)—the mean response time in milliseconds (β denotes the regression coefficient): β = 34.36 ms (95% CI 10.01–58.71; $p = 0.006$) for <400 mcg/day, and β = 33.18 ms (95% CI 6.10–60.25; $p = 0.016$) for ≥1000 mcg/day; HRT variability (HRT-SE, also in milliseconds) was higher with ≥1000 mcg/day (β = 3.31 ms; 95% CI 0.53–6.09; $p = 0.019$). These IRRs are each estimated versus the recommended dose; they are similar in magnitude to one another, and the study did not test a formal dose–response contrast between the two non-recommended categories. In girls, low and high FA doses were not associated with HRT [318]. At 7–9 years, Compañ-Gabucio et al. (2022) reported that <400 mcg/day (second trimester, months 4–7) was associated with poorer alertness on the Attentional Network Test (ANT): β = 18.70 ms (95% CI 7.51–29.89; $p \approx 0.001$) in all children, and β = 30.01 ms (95% CI 12.96–47.01; $p < 0.001$) in girls. For working memory (N-back), the d′ score (detectability; unitless) improved in girls whose mothers used ≥1000 mcg/day across pregnancy: 2-back d′ β = 0.28 (95% CI 0.01–0.56; $p < 0.05$) and 3-back d′ β = 0.32 (95% CI 0.08–0.56; $p < 0.05$); conversely, boys exposed to <400 mcg/day in the second trimester had lower 2-back d′: β = –0.25 (95% CI –0.49 to –0.01; $p < 0.05$). *(Note: the two β values here are separate effects for the 2-back and 3-back indicators, respectively.)* [319]. Complementing these findings at 4–5 years, Valera-Gran et al. (2017) observed that >1000 mcg/day during the periconceptional period was associated with lower cognitive scores, including global verbal (β = 22.49; 95% CI 24.71, 20.27; $p = 0.028$) and verbal memory (β = 23.59; 95% CI 26.95, 20.23; $p = 0.036$) compared with 400–999 mcg/day [327].

Three studies examined the association between high folic acid exposure

and ASD risk [370-372]. Two of these studies reported significant associations between elevated maternal folate biomarkers and increased ASD risk [370, 371]. In the Boston Birth Cohort, Raghavan et al. (2018) found that mothers with plasma folate concentrations ≥60.3 nmol/L (90th percentile) measured 24–72 hours postpartum had a 2.4-fold higher risk of having a child with ASD compared to those in the middle 80th percentile (adjusted HR = 2.4; 95% CI: 1.3–4.4; p = 0.007) [370]. Similarly, Raghavan et al. (2020) reported that cord blood unmetabolised folic acid (UMFA) in the highest quartile (Q4; median = 2.21 nmol/L) versus the lowest quartile (Q1; median = 0.25 nmol/L) was associated with a markedly increased ASD risk among children of black ethnicity (adjusted OR = 9.85; 95% CI: 2.53–38.31; p < 0.001) [371]. In contrast, Sharman Moser et al. (2019), using pharmacy dispensing data in a large Israeli cohort, found no significant association between cumulative FA supplement use from 12 months prior to conception through pregnancy, even at doses >3 mg/day and ASD risk which typically evaluate suspected ASD cases under the age of 6 years (adjusted OR = 1.01; 95% CI: 0.60–1.70; p > 0.05) [372].

Table 7- Dose of folate during preconception and pregnancy considered excessive by authors of individual studies

Author (publication year) [ref number]	**Duration and FA dose considered excessive**	**Folate blood concentration considered excessive by authors**
Increased Risk of allergic disease		
Best et al (2021) [90]	Not available	UMFA >4.69 nmol/L 36-40 weeks' gestation
Parr et al (2018) [118]	>578 mcg per day from both food and supplements (FA equivalents) during pregnancy. Nearly all took at least 400 mcg/day of supplemental FA	Plasma 18 weeks' gestation correlated with total folate intake but there was no association
Yang et al (2015) [122]	400 mcg per day for 6 months or 800 mcg/day for 3 months in peri pregnancy and throughout early pregnancy	Not measured
Increased Risk of LGA*		
Bortolus et al, 2021 [130]	4 mg/day in preconception & first trimester	Not measured

Xie et al, 2018 [129]	Not available	RBCF > 906 nmol/L (19-24 weeks' gestation)
Risk of ASD & Adverse cognitive development		
Raghavan et al, 2018 [123]	Multivitamin supplement intake > 5 x per week during third trimester. Level of FA not specified	Plasma folate ≥ 60.3 nmol/L; 24-72 hours post delivery
Raghavan et al, 2020 [124]	Not available	Plasma folate ≥ 60.3 nmol/L UMFA cord concentrations at delivery 2.21 nmol/L
Valera-Gran et al, 2017 [67]	FA supplement ≥ 1,000 mcg/day in periconception (adverse cognitive development)	
Increased Risk of GDM		
Chen et al, 2021 [99]	≥ 400 mcg/day i.e.: a supplement every day from prepregnancy throughout pregnancy	RBC ≥904 nmol/L 9-13 weeks' gestation
Cheng et al, 2022 [53]	Not available	Serum folate > 43.2 nmol/L in pregnancy
Lai et al, 2018 [100]	Not available	Plasma folate ≥ 44.5 nmol/L; 26-28 weeks' gestation
Li et al, 2022 [101]	Not available	Serum folate ≥ 32.99 nmol/L; 24-28 weeks' gestation
Li et al, 2019 a [115]	Not available	Serum folate ≥27.58 nmol/L; 24-28 weeks' gestation
Li et al, 2019 b [63]	≥800 mcg/day from at least 4 weeks prepregnancy – 16 weeks' gestation	Not measured
Liu et al 2020 [102]	Not available	RBC ≥862.5 nmol/L 12 weeks' gestation
Liu et al, 2023 [103]	Not available	Serum folate >45.3 nmol/L; prior to 24 weeks' gestation
Saravanan et al, 2021[104]	Not available	Plasma folate >45 nmol/L; 26-28 weeks' gestation
Xie et al, 2019 [105]	Not available	RBCF >906 nmol/L; 19-24 weeks' gestation

2.4.4.3.6 Preterm Birth

Two studies investigated the association between high plasma folate concentrations and preterm birth [316, 380]. Olapeju et al., 2019 conducted an observational study in the USA among 7,576 mothers enrolled in the Boston Birth Cohort, a predominantly urban, low-income, minority population at high risk for PTB. Plasma folate was measured within 24–72 hours postpartum in a sub-sample (n = 2,313) [380]. Higher plasma folate

concentrations were associated with a reduced likelihood of PTB. Specifically, women in the highest quartile (>43.8 nmol/L) compared with the lowest quartile (6.6–19.4 nmol/L) had a significantly lower risk of PTB (adjusted OR = 0.74; 95% CI: 0.56, 0.97; $p < 0.05$). Excess plasma folate (>45.3 nmol/L) was also associated with reduced odds of spontaneous PTB (aOR = 0.72; 95% CI: 0.55, 0.95, p=0.021) and medically indicated PTB (aOR = 0.58; 95% CI: 0.40, 0.82, p=0.002). In this study, folic acid supplement use was assessed via postpartum interview, and frequent multivitamin intake (≥3 times/week) throughout pregnancy was similarly associated with lower PTB risk. In contrast, Chen et al., 2015 investigated maternal folate status during pregnancy in Singapore in a multiethnic Asian cohort (n = 986) [316]. Plasma folate was measured at 26–28 weeks gestation (median: 34.4 nmol/L). Higher maternal folate concentrations were associated with longer gestational age (β = 0.12 weeks per SD increase; 95% CI: 0.02, 0.21; p = 0.017) and a borderline reduction in overall PTB risk (aOR = 0.79; 95% CI: 0.63, 1.00; p = 0.05). When restricted to spontaneous PTB, the association remained similar (aOR = 0.76; 95% CI: 0.56, 1.04). Unlike Olapeju et al., supplement use was not assessed; the study focused on biomarker data. Neither study found an increased risk of PTB with higher folate levels; rather, both suggested that higher maternal folate status, whether measured during late pregnancy or postpartum, was linked to a reduced likelihood of PTB. Table 7 summarises the level of FA and/or blood concentrations considered to be excess by the authors.

2.4.4.3.7 Fetal Epigenetic Outcomes and Maternal Folate Status

In a Canadian cohort study, maternal and cord blood folate concentrations were examined in relation to fetal epigenetic markers In a Canadian cohort study, maternal and cord blood folate concentrations were examined in relation to fetal epigenetic markers [379]. Maternal blood was collected at 12–16 weeks gestation and at delivery (37–42 weeks), and cord blood was collected at delivery, with serum folate, RBC folate and UMFA measured at each timepoint. Serum and RBC folate and plasma UMFA concentrations in early and late pregnancy and cord blood were not significantly associated with global DNA methylation (5mC) in cord blood mononuclear cells. However, an inverse association was observed with DNA hydroxymethylation (5hmC): maternal RBC folate in early pregnancy correlated negatively with 5hmC ($r = –0.16$, $p = 0.04$), and cord plasma UMFA showed a stronger inverse correlation ($r = –0.23$, $p = 0.004$). Subgroup analysis revealed that infants born to mothers with high RBC folate (>2860 nmol/L) and low serum vitamin B12 (<167 pmol/L) in early pregnancy had significantly lower global DNA methylation (mean 5mC: 5.16% vs. 5.50%, $p = 0.01$) and higher birth weight (mean: 3697 g vs. 3393 g, $p = 0.01$) compared to those with lower folate and higher B12 status [379]. These findings suggest that maternal one-carbon nutrient imbalance may influence fetal epigenetic programming and growth. Maternal blood was collected at 12–16 weeks gestation and at delivery (37–42 weeks), and cord blood was collected at delivery, with serum folate, RBCF and UMFA measured at each timepoint. Serum and RBCF and plasma UMFA concentrations in early and late pregnancy and cord blood were not significantly associated with global DNA methylation (5mC) in cord blood mononuclear cells. However, an inverse

association was observed with DNA hydroxymethylation (5hmC): maternal RBCF in early pregnancy correlated negatively with 5hmC (r = –0.16, *p* = 0.04), and cord plasma UMFA showed a stronger inverse correlation (r = –0.23, *p* = 0.004). Subgroup analysis revealed that infants born to mothers with high RBCF (>2860 nmol/L) and low serum vitamin B12 (<167 pmol/L) in early pregnancy had significantly lower global DNA methylation (mean 5mC: 5.16% vs. 5.50%, *p* = 0.01) and higher birth weight (mean: 3697 g vs. 3393 g, *p* = 0.01) compared to those with lower folate and higher B12 status. These findings suggest that maternal one-carbon nutrient imbalance may influence fetal epigenetic programming and growth.

2.4.5 Discussion

This systematic review found that both elevated blood folate concentrations and excess synthetic FA supplementation taken by women in preconception and during pregnancy were associated with several adverse outcomes in both offspring and mothers. Furthermore, the papers in this review indicate that synthetic FA intake may also have harmful effects at levels below the established UL. In offspring, some studies reported an increased risk of asthma, autism, inattention large for gestational age babies, small for gestational age and pre-term birth. However, these findings were not consistent across all studies; several investigations found no significant associations between maternal folate status and these outcomes.

For pregnant women, excess or high FA supplementation was consistently linked to an increased risk of GDM. Across all ten studies reviewed, higher folic acid (FA) exposure, whether through supplementation or elevated blood folate concentrations, was associated with an increased risk of gestational diabetes mellitus (GDM). Reported associations included FA doses ≥800

mcg/day or prolonged use from preconception through mid-pregnancy [325, 348] and elevated serum (>32.9 nmol/L) or RBC folate (>862.5 nmol/L) concentrations [347, 350, 353]. Adjusted odds ratios ranged from 1.58 to 2.76, and some studies observed a dose-response trend [351, 353]. However, definitions of “high” exposure varied, and no universal threshold or consistent gradient was established. Evidence from two studies suggests that longer duration of FA supplementation (from preconception through mid-pregnancy) may increase GDM risk compared with shorter use [364, 381]. Overall, most studies examined FA dose or blood folate concentrations rather than timing, with higher FA intake (>800 mcg/day) and elevated serum (>32.9 nmol/L) or RBCF (>862.5 nmol/L) consistently associated with increased GDM risk [347, 350, 353].

Two recent meta-analysis provide additional context beyond the studies included in this review [349, 382]. Xu et al. 2023, reported that increased serum folate concentrations, were more strongly associated with GDM risk than RBCF, suggesting that the biomarker used may influence observed associations [382]. Li et al. (2022b) found that both RBCF and serum/plasma folate were linked to higher GDM risk, with the highest versus lowest categories showing OR for RBCF (OR 1.96) and plasma/serum folate (OR 1.23) [349]. They also reported an 8% increase in GDM risk for every 200 ng/ml increase in RBCF. These findings highlight that elevated folate status, regardless of whether measured as serum or RBCF, may confer increased GDM risk, although the strength of association differs by biomarker. Given these results, clinicians should consider monitoring FA supplementation, particularly in populations exposed to mandatory fortification and voluntary high-dose supplement use, such as in Australia, where cumulative intake

may exceed recommended levels.

2.4.5.1 Metabolic Disease and Gestational Diabetes Mellitus

Our review found a consistent association between higher FA intake via supplementation or elevated blood folate concentrations and increased GDM risk, although definitions of 'excess' exposure and timing varied across studies. Most studies examined FA dose or biomarker levels rather than duration directly. To provide context beyond our review, we note an animal study [383] showing that peri gestational exposure to high FA programmed offspring for metabolic dysfunction, including insulin resistance and altered adiponectin signalling, mechanisms relevant to GDM pathophysiology.. Within human studies, a Chinese cohort (Lili et al., 2019) that reported a U shaped relationship between FA supplementation and duration and GDM risk [381]. Women who did not take FA or those that took 400 mcg/day FA supplementation >90 days had a higher incidence of GDM compared to those who took FA for less than 60 days (adjusted OR 3.45; 95% CI 1.01–11.8). This study did not measure blood folate, so findings relate to duration rather than folate status. They also observed that prolonged FA use correlated with adverse lipid profiles, lower HDL-c concentrations and higher triglycerides and LDL-c concentrations particularly in the second trimester. While causality was not tested, these lipid patterns are recognised independent predictors of GDM in other cohorts [384]. Together, these findings suggest that cumulative FA exposure may influence metabolic pathways relevant to GDM, but evidence remains limited and heterogeneous.

2.4.5.2 Elevated folate status and GDM risk.

Several studies in our review reported that higher blood folate concentrations were associated with increased GDM risks, although thresholds varied. For example, RBC folate levels greater than 904 nmol/L in the first trimester and serum folate greater than 27.6 nmol/L in third trimester increased risk of GDM [347, 350, 353]. These cut offs are study specific and should not be interpreted as universal thresholds.

To provide context beyond our review, an Australian study found that women who supplemented FA 400-800 mcg/day had higher serum folate compared to those who did not supplement and those taking ≥800 mcg had the highest levels [385]. The authors also found that RBC folate in the post fortification era was significantly higher (3-fold) than before fortification irrespective of FA supplementation (prefortification with FA supplementation median 623 nmol/L vs post fortification 1512 nmol/L, $p<0.001$) suggesting the effects of fortified foods may be significantly raising RBC folate levels. The authors propose that the effects of excess FA on the placenta and insulin secretion are a result of long-term and not short-term exposure to high levels of FA [385]. The mechanisms linking excess FA to GDM may involve an imbalance between folate and vitamin B12, with high blood concentrations of folate and low concentrations of Vitamin B12 exacerbating the effects of vitamin B12 deficiency [322, 325, 356, 381]. Deficiency of vitamin B12 causes the 'methyl block' phenomenon where an impairment of methyl uptake into the methionine cycle leads to elevated homocysteine concentrations, contributing to insulin resistance, reduced β cell function and oxidative stress

[328, 347, 348, 352, 386, 387]. Another proposed mechanism is that high FA intake reduces *MTHFR* activity, also leading to elevated homocysteine [280, 314, 387]. These effects are also thought to be mediated by reductions in adiponectin, a hormone involved in glucose regulation and lipid metabolism [313, 321, 323, 388]. UMFA may also play a role, potentially interfering with cellular folate uptake and contributing to disruptions in one-carbon metabolism, further increasing the risk of GDM [196]. This may explain why GDM, which affects 14% of pregnancies worldwide [387] has been rising in countries like Australia where rates tripled between 2008 and 2018 to 16% following mandatory folic acid-fortification [347]. The health effects of GDM for both the mother and offspring are significant, with a GDM diagnosis during pregnancy potentially predisposing the mother to increased risk for certain diseases like cardiovascular disease [389], elevated blood pressure, disturbed lipid profiles [381, 384], metabolic syndrome [390] and type 2 diabetes [389]. A meta-analysis (35 studies) of the association between metabolic syndrome and GDM in pregnant women found that women with a history of GDM and the offspring exposed to GDM in utero have higher risks of developing metabolic syndrome later in life compared to those with no history of GDM [390]. Another study consisting of 231,271 births in Israel also found that offspring had a higher rate of long-term hospitalisations, diabetes mellitus and obesity compared to those not exposed [391]. The mechanism as to why the offspring are affected by GDM in pregnancy is thought to be related to DNA methylation in oocytes which have been shown to affect the expression of genes critical for development and metabolic processes in the offspring. For example, altered methylation patterns can influence genes involved in metabolic pathways, cellular signaling, and organ development,

which may predispose the offspring to various health issues later in life [297].

2.4.5.3 Neurological Development of the Offspring

It was found that excess FA supplement intake >1000 mcg per day is associated with deficient attentional function, poor working memory and an increased likelihood of ASD. A wide body of published literature supports that ASD is linked to epigenetic programming in the embryo and specifically, paternal semen DNA methylation [392-394]. Folate is critical for nucleotide synthesis and methylation [395] to prevent uracil misincorporation and DNA instability [396, 397]. High circulating folate concentrations in elderly patients (>45.3 nmol/L) have been shown to alter methylation [398]. A study in non-pregnant adults aged 18-40 years in the United States evaluated FA intake in food (not supplements) via a food diary and found that an FA food intake of >200 mcg Dietary Folate Equivalents (DFE) may contribute to genomic instability [399]. Excess FA intake alters the function of the genome and affects gene expression in the offspring [400]. Murine models consistently show that offspring development and neurodevelopment are affected by high dose FA intake with doses equivalent to human doses of 800 mcg or greater of FA intake per day. A dose of 800 mcg/day was associated with greater anxiety-like behaviour and hyperactivity, and neurotransmitter genes were down regulated by more than two-fold [401]. Doses of 4 mg of FA/day were associated with short-term memory impairment in mice [314], and ASD risk [402]. A study by Girotto et al. (2013), in experiments with rats, showed that high dose FA was associated with changes in brain synaptic transmission and higher seizure susceptibility in their offspring [403]. A review of 22 papers in 2016 by Gao et al, into the association between FA supplementation and

blood folate concentrations during pregnancy and neurodevelopment in the offspring, found only one study showed a harmful effect of neurodevelopmental issues at high dose (>1000 mcg/day) of FA supplementation yet found that 'the magnitude of the protective effect of folic acid supplementation observed was quite small" [404]. A Norwegian study of 85,176 children found that women who took less than 400 mcg FA from 4 weeks before pregnancy to 8 weeks' gestation was associated with a lower risk of autistic disorder in the offspring and of particular note was that none of their foods were fortified with FA [405]. Sweeney et al. (2006) investigated the presence of UMFA in participants after eating more than two slices of fortified bread over an eight-hour period, and found that UMFA concentration increased with increasing number of slices consumed and they suggested that FA may accumulate in the body leading to negative health effects [406]. Frye et al. (2020) reported that some children with ASD have cerebral folate deficiency, a condition where folate transport into the brain is impaired despite normal blood folate levels. This occurs because autoantibodies against the folate receptor alpha (FRα) block its function, reducing folate entry into the central nervous system. These autoantibodies are immune-mediated, not caused by excess FA intake, but high FA may exacerbate imbalances by increasing unmetabolized folic acid (UMFA) [407]. Supporting this, Akiyama et al. (2022) observed that high-dose FA supplementation in children with cerebral folate deficiency led to persistently low CSF 5-MTHF despite elevated serum folate, which improved when FA was reduced or replaced with folinic acid, suggesting FA can competitively inhibit 5-MTHF transport across the blood–CSF barrier [408]. Tang et al. (2022) further hypothesized that UMFA may generate immunomodulatory metabolites (e.g.,

6-formylpterin) that interfere with folate transport and immune regulation [409].

2.4.5.4 FA and Blood Folate Concentration Measurement and Exposure

The studies in this review used different values to define high FA intake and high blood concentrations. There is significant variability in how folate intake is assessed, with some studies relying on food frequency questionnaires or self-reported supplement use, which introduce recall bias and unreliable data, particularly when data is collected retrospectively, such as postpartum interviews for preconception dietary intake analysis [316, 362, 380].

Measuring plasma/serum folate concentrations in the third trimester does not accurately capture early pregnancy folate status, which is crucial for assessing risks like preterm birth and fetal development [380, 410, 411]. Moreover, plasma/serum folate levels measured in late pregnancy do not accurately capture FA exposure during the preconception period or early gestation, when adequate folate is crucial for preventing neural tube defects and supporting fetal development [316, 380]. This raises important questions about the current guidelines for FA supplementation during pregnancy, as both human and animal studies point to risks of folate overload, particularly disruption in neurodevelopment and metabolic health [106, 331, 412].

While in some studies, elevated serum folate levels (Chen et al., 2015)) seemed to be linked to a reduced risk of small for gestational age babies, evidence from other studies in this review highlighted potential risks of excessive serum folate levels [316, 380, 413].

The WHO recommendation to prevent NTDs is that a minimum RBC folate

of 906 nmol/L [100] be achieved, however, no real 'excess' levels have been defined. be achieved, however, no definitive 'excess' levels have been defined. This review highlights that throughout different stages of preconception and pregnancy, biological health impacts may be seen at levels lower than currently considered the minimum range to prevent NTDs. For example, RBCF greater than 906 nmol/L was associated with large for gestational age babies at 19-24 weeks gestation [376]. Liu et al. (2023) reported that RBCF of >862.5 nmol/L during the first trimester increased the risk of GDM [350]. Chen et al. (2021) found that RBCF greater than 963 nmol/L [347] was associated with an increased risk of GDM, as did Xie et al (2019) with RBCF >906 nmol/L at 19-24 weeks gestation [353]. These studies imply that the 906 nmol/L may be the high end of which RBCF should not be exceeded. A Canadian study found that the average DFE intake for Canadians, excluding supplements was 442 mcg of both natural food folate and fortified foods and if supplements were added to this the overall intake was significantly higher for supplement users ($p<0.001$) compared to non-supplement users [47]. An Australian study found that folic acid-fortification resulted in 63% higher maternal folate concentrations with RBCF consistently >1512 nmol/L irrespective of supplement use, and this increase in RBCF was contributing to altered hormones that regulate glucose metabolism [41]. This shift towards higher RBCF concentrations following folic acid-fortification has been found in other countries like Canada [414]. A study conducted in New Zealand found that in women of reproductive age, who received either 140 mcg or 400 mcg supplement per day had similar increases in plasma and RBC folate concentrations over a 12 week period, however women who took 400 mcg of FA achieved levels >906 nmol/L in just six weeks compared to

834.5 nmol/L in those on 140 mcg of FA/day [415]. This is concerning given the most popular prenatal supplement in Australia contains 800 mcg of FA [308].

For these reasons, researchers have consistently called for a review of high FA intake and blood concentrations [39, 44, 87] because we do not yet understand the implications of exceeding RBCF concentrations highlighted in this review as 'excess'. Emerging evidence from human studies supports this concern. Husen et al. (2022) demonstrated U-shaped associations between maternal RBC folate and embryonic head size, noting that folate acts as a key methyl donor in one-carbon metabolism. Excessive FA intake may alter DNA methylation and gene expression, potentially influencing neurodevelopmental outcomes [377]. These safety assessments do not consider the potential epigenetic effect of elevated folate exposure [310, 416, 417].

Optimal serum/plasma folate concentrations are considered to be 13.5-45.3 nmol/L by the WHO [97]. While some associations in this review were non-significant, some studies suggest that concentrations outside this range may infer risk in key areas such as GDM and neurodevelopment. For example, plasma levels >27.5 nmol/L (12.2ng/mL) were associated with an increased risk of GDM [364] and above 32.9 nmol/L (14.6 ng/ml) [349] and plasma levels > 19.02 ng/mL considered to be overdose by the authors [317]. In the United States, since fortification, serum folate concentrations are believed to have increased 2.5 times across the entire population 4-80 years of age [305]. Clearly upper limits need to be defined in all life stages and specifically in preconception and pregnancy. The problem to date is the inconsistency in reporting methods and this was highlighted in an Australian study in 2020

which questioned results from a study reporting RBCF using an immunoassay rather than microbiological methods and found that levels had been greatly over-reported in the initial study [340]. This issue is discussed further in the limitations section below. The WHO recommendations above do not take into account baseline blood concentrations of the participants and do not take into account the high risk versus the low risk individual [100].

2.4.5.5 Unmetabolised Folic Acid (UMFA)

Few studies which met eligibility criteria for the inclusion in this review measured UMFA. Measurement methods for UMFA vary in accuracy and are not widely available [418-420].

In this review, two studies measured UMFA. Best et al. (2021) found no consistent associations with allergic disease except for increased wheeze risk in the highest maternal UMFA quartile [338], and Raghavan et al. (2020) reported a markedly increased risk of ASD in offspring when cord blood UMFA was in the highest quartile (>2.21 nmol/L) compared with the lowest [371]. Other studies report associations with elevated UMFA, for example, in umbilical cord blood, an association between UMFA > 0.51 nmol/L and an increased risk of ASD was found [421] and in a non-pregnancy related study elevated UMFA was inversely associated with natural killer cell cytotoxicity in post-menopausal women [57]. UMFA has also been shown to inhibit the enzyme *DHFR* which may reduce intracellular folate and affect methylation capacity [422, 423]. Given the importance of 5MTHF for DNA synthesis and repair during rapid fetal growth, these findings raise concerns about the long-term effects of high UMFA concentrations on offspring health. The clinical significance of UMFA presence remains unclear, with some studies reporting low or undetectable levels of UMFA even with low FA supplementation [226,

337]. The potential effects of UMFA on fetal development, including risks of insulin resistance, asthma, and obesity in offspring, as well as perturbations in DNA methylation, require further investigation.

2.5 Limitations

Consideration must be given to the impact of analytical methods used to measure serum/plasma folate, RBCF, and UMFA concentrations in the cited studies. There are several published and commercial methods available, including automated assays that utilise folate binding protein (FBP) [424], microbiologic assays involving organisms such as *Lactobacillus casei* [425, 426], high pressure liquid chromatography with fluorescence and ultraviolet detection [427, 428], and liquid chromatography tandem mass spectrometry [335, 418].

Each of these methods have their own strengths and limitations such as analytical sensitivity and specificity, or the ability to quantify different derivatives of folates such as tetrahydrofolate, 5-MTHF and UMFA [334]. In addition to differences in method, assays may have differences in aspects such as calibrators, pre-analytical extraction techniques, and choice of equipment. The above factors could lead to analytical variation that, in the worst-case scenario, can result in poor agreement across methods. Unfortunately, the impact of this is not always apparent until comparisons are made across studies.

Fazili et al conducted an international ‘round-robin’ investigation to assess agreement between different serum folate LC-MS/MS methods [429]. Calibrators, standard reference material, and pooled serum were distributed

to 13 laboratories; all measured 5-MTHF and FA, some measured other folate forms. Median imprecision for pooled serum ranged from 1.70-11.0% and 3.9-23.0%, with recoveries ranging from 59-138% and 67-198% for 5-MTHF and FA, respectively. Mean relative bias ranged from -24 to +30% and -47% to +578% for 5-MTHF and FA, respectively. Overall, this highlights the poor precision and agreement between 5-MTHF and FA measurements using LC-MS/MS methods.

RBCF measurements also suffer from a high degree of between-laboratory variability. Several variables such as composition and volume of lysing reagent, duration of lysis step, and measurement uncertainty of haematocrit may all contribute to this [430]. In 1996, Gunter et al. performed an international 'round-robin' investigation and distributed pooled whole-blood to 20 research laboratories. Overall coefficient of variation (CV) was 35.7% for RBCF across the pools with the largest variation occurring at critical low concentrations [431]. Pfeiffer et al. performed a similar comparison focusing on microbiological growth assays for both RBC and serum folate [432]. Measured geometric means ranged from 39.5-59.2 nmol/L and 1120-1380 nmol/L for serum and RBCF respectively. Additionally, the study identified that changing the calibrator from 5-MTHF to FA could increase the results by up to 32%.

The poor agreement between assays highlights the limitations of establishing cut-offs for guidelines or recommendations due to a lack of assay standardisation. Despite these differences, WHO guidelines for optimal serum and RBC folate concentrations in women of reproductive age do not specify the analytical method used to determine recommendations [433], however the cited studies state a microbiological assay was used with no

mention of possible analytical variation and disagreement [104]. This also highlights the difficulty of comparing studies that specify associations with specific blood folate concentrations as results may not be comparable across methods. While the review was developed with input from a health librarian and involved searches of seven journal databases in addition to clinical trial registries and handsearching, it must be acknowledged that the omission of some databases (such as Web of Science) may have resulted in some manuscripts being missed.

2.6 Conclusion

While FA supplementation is essential for preventing neural tube defects, excessive /high intake during preconception and pregnancy may carry risks, particularly for gestational diabetes and neurodevelopmental disorders in offspring. Given the widespread use of FA supplementation and mandatory food fortification, there is a pressing need to clearly define safe upper limits for FA supplementation and adequate RBC and serum blood folate concentrations. Additionally, standardising folate testing procedures is essential for accurately assessing and managing folate exposure. Testing other forms of folate like 5-MTHF may assist in further understanding the doses and adverse health impact, if any, of excessive use. These conclusions have far-reaching implications for governments worldwide, particularly in countries with mandatory fortification, where FA supplementation is commonly recommended at a minimum of 500 mcg/day and RBCF levels above 906 pmol/L are recommended to prevent NTDs. The potential risks of excessive FA intake need to be addressed. It is important to consider that excess FA may have unwanted effects and there is a need to define what ‘excess’ means in both supplementation and all blood

concentrations.

2.7 Chapter Conclusion

Although alternative folate forms, including 5-MTHF, have been proposed as potential substitutes for FA, FA remains the only folate form with robust randomised controlled trial evidence demonstrating a reduction in NTD risk. To date, no trials have directly evaluated whether 5-MTHF reduces NTD incidence, and therefore its efficacy for NTD prevention remains unproven. Consequently, current public health recommendations continue to prioritise FA for primary NTD prevention.

Concerns regarding potential adverse outcomes associated with high FA exposure and elevated blood folate concentrations are derived predominantly from observational studies, which are inherently susceptible to residual confounding, exposure misclassification, and co-supplementation effects. Reported associations, including those with gestational diabetes and neurodevelopmental outcomes, are inconsistent across populations and vary by country, socioeconomic context, and assessment methodology. In many settings, FA is consumed as part of multi nutrient supplements, making it difficult to isolate its independent effects. As such, elevated serum folate or unmetabolised folic acid may reflect supplement use rather than a direct causal relationship.

This chapter presented two systematic literature reviews that collectively establish the foundational evidence for the thesis. The first review demonstrated that women of reproductive age, particularly in countries with mandatory FA fortification, are frequently exposed to total FA intakes that may exceed the upper tolerable intake level. The second review examined the biological implications of excessive FA exposure, identifying associations

between high FA intake or elevated folate biomarkers and adverse maternal and offspring outcomes. While causality cannot be inferred, these findings highlight biologically plausible concerns that warrant further investigation.

Together, these reviews identify critical gaps in current public health policy and clinical practice, particularly the lack of personalised approaches to folate supplementation that account for individual metabolic variability, including common genetic polymorphisms affecting folate metabolism. They provide a rationale for exploring alternative folate forms such as 5-MTHF, highlighting a significant gap in the evidence base, as its role in neural tube defect prevention has not been directly investigated, despite well-established biochemical plausibility and its position as the biologically active form of folate.

Importantly, the findings presented in this chapter underpin the mixed-methods research design of the thesis. The pilot randomised controlled trial (Chapter 4) was designed to assess the feasibility and biochemical relevance of personalised folate supplementation rather than to establish clinical efficacy. Qualitative interviews with trial participants (Chapter 5) explored the emotional and experiential dimensions of recurrent pregnancy loss care, while a national survey of health professionals (Chapter 6) examined current prescribing practices and implementation barriers. By synthesising existing evidence and identifying key biological and systemic gaps, Chapter 2 provides the rationale for the empirical phases of the thesis and supports its overarching aim: to evaluate the feasibility of using 5-MTHF compared with FA in couples experiencing recurrent pregnancy loss. The following chapter outlines the methodology used to operationalise these research objectives within a mixed-methods framework.

Chapter 3 Methodology

3.1 Chapter Introduction

This chapter outlines the mixed method research design used to investigate the feasibility and clinical relevance of personalised folate supplementation in couples experiencing RPL. Building on the evidence gaps identified in Chapter 2, the methodology was developed to address the thesis's overarching aim: to evaluate whether 5-methyltetrahydrofolate (5-MTHF) may offer a safer and more effective alternative to folic acid (FA), particularly in individuals with *MTHFR* polymorphisms.

A mixed methods approach was adopted to reflect the complexity of RPL and the need to understand both biological and psychosocial factors. The design integrates quantitative and qualitative data to explore not only clinical outcomes but also the lived experiences of affected couples. The research was organised into distinct but interrelated phases, each aligned with specific research objectives and feasibility domains.

Phase 1 involved a pilot randomised controlled trial (RCT) comparing FA and 5-MTHF supplementation in couples with RPL. This phase was designed to assess feasibility domains such as recruitment, adherence, acceptability, and biochemical outcomes, including changes in folate biomarkers and unmetabolised folic acid (UMFA). Embedded within this trial was a qualitative

sub-study (Phase 1A), which explored participants' experiences of RPL and their perceptions of care.

Accordingly, Phase 2 (the health-care professional knowledge, attitudes and practice survey) was embedded alongside the pilot RCT to identify implementation barriers and facilitators, characterise variation in current prescribing practices, and assess readiness for clinical adoption, thereby informing PRECIS-2 domain choices, CONSORT-recommended progression criteria, and site-readiness for a future definitive randomised controlled trial.

Phase 2 comprised a national survey of health professionals who support couples with RPL. This phase aimed to investigate current prescribing practices, awareness of folate metabolism, and readiness for clinical change. Together, these phases provide a comprehensive framework for evaluating both the clinical and implementation feasibility of personalised folate strategies.

By detailing the rationale, design, and procedures of each study component, this chapter establishes the foundation for the results presented in Chapters 4 through 6. It also demonstrates how the research design was intentionally structured to build a cohesive and policy-relevant evidence base for improving RPL care.

3.2 Design and Methodology

This research adopted a mixed methods experimental design, integrating quantitative and qualitative methods across two interrelated components:

- A randomised controlled trial (RCT) pilot study with an embedded participant qualitative sub-study
- A practitioner survey

This design allowed for comprehensive evaluation of the clinical feasibility, acceptability, and early signals of effectiveness of the proposed intervention (via the RCT pilot), as well as collection of contextual insights from both participants and practitioners to inform a future full-scale trial. The integration of these components reflects a pragmatic-explanatory approach, balancing real-world applicability with methodological rigour.

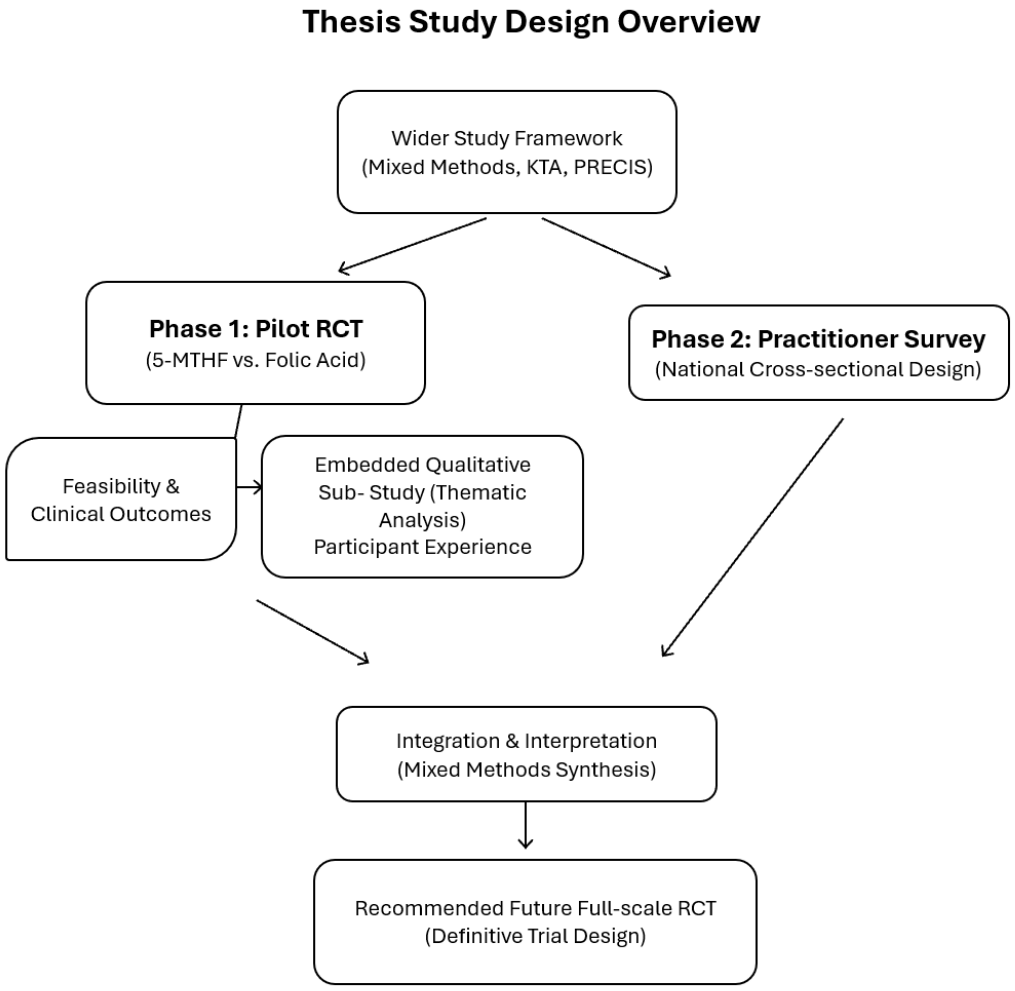


Figure 7- Presents a flow diagram of the multiple phases of this mixed methods exploratory project

Design decisions across all three study components were informed by the **Pragmatic–Explanatory Continuum Indicator Summary (PRECIS)** framework [434], which considers ten key trial domains such as participant eligibility, intervention flexibility, and analysis strategy. The PRECIS wheel (see Figure 8) visually represents where each domain falls on the continuum from explanatory (centre of the wheel) to pragmatic (outer edge).

This framework supports the alignment of study elements with their intended purposes:

- The RCT pilot study, including the embedded qualitative component, is positioned between pragmatic and explanatory poles to reflect real-world conditions while retaining control over variables.
- The practitioner survey is designed pragmatically to capture frontline clinical perspectives relevant to implementation.

Using this continuum as a scaffold ensures that the study produces findings that are both scientifically robust and practically meaningful.

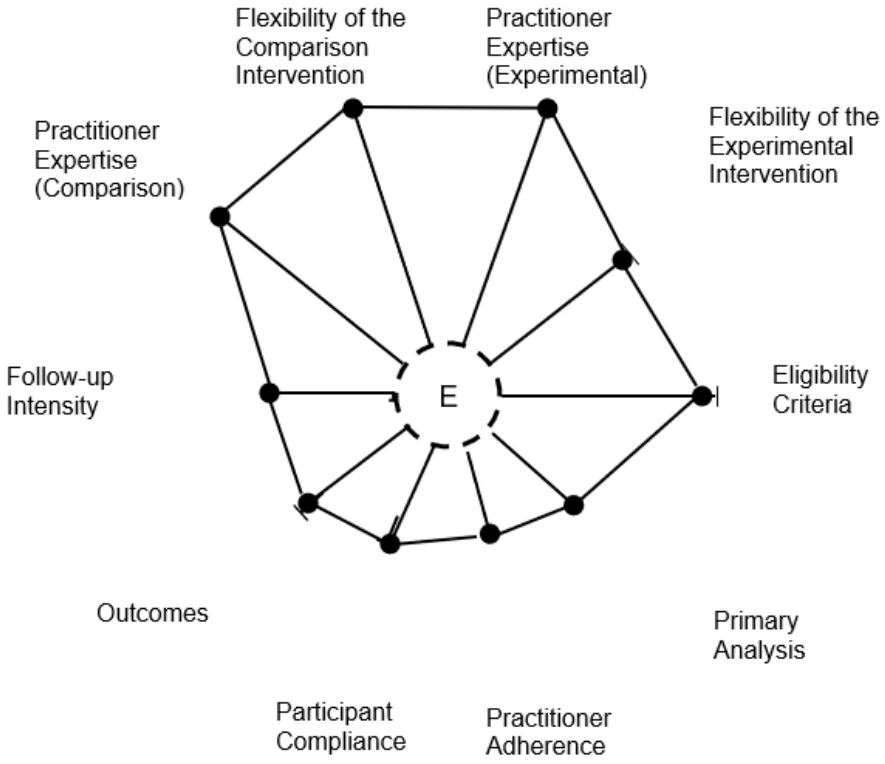


Figure 8- The pragmatic-explanatory continuum indicator (PRECIS) wheel. The Wheel represents the pragmatic-explanatory design. The closer to the spoke the more explanatory and the further away the more pragmatic the approach.

3.3 Mixed Methods Rationale

This study employed a mixed methods approach, drawing from both pragmatic and explanatory paradigms to comprehensively address the research question. The integration of quantitative and qualitative data across the pilot RCT (including its embedded sub-study) and the practitioner survey, ensured that the design captured both measurable outcomes and the rich, contextual understanding of trial delivery and user experience.

Mixed methods are especially suitable for health services research, where the complexity of care pathways and participant experiences often demand multiple forms of evidence [435].

In this study, qualitative methods were used developmentally to enhance and inform the quantitative trial component [436, 437].

Specifically, qualitative data, drawn from naturally occurring recruitment interviews:

- Helped refine trial protocols and implementation strategies
- Provided insights into recruitment, retention, and participant expectations
- Supported evaluation of intervention acceptability and procedural feasibility
- Identified contextual barriers and enablers that could affect delivery and scalability
- Gave a voice to participants whose experiences of RPL are often underrepresented in clinical research

The participant sub-study adopted an inductive thematic analysis approach,

guided by a constructivist paradigm, to explore the lived experience of RPL and participants' perspectives on the trial process. This ensured the participant voice was meaningfully embedded within the core trial analysis.

The integration of qualitative insights into clinical trial design and interpretation is well-supported in the literature [435, 438]. Mixed methods enhance trial quality by contextualising outcome data and guiding necessary adaptations. As Hunter and Armour (2019) [439] emphasise, such integration generates explanatory power that can clarify why particular outcomes occur, not just whether they do. In this study, quantitative methods were used to measure key trial outcomes and assess intervention feasibility, while qualitative methods explored how participants and practitioners experienced the intervention and trial procedures.

This methodological orientation also aligns with the Knowledge-to-Action (KTA) Framework [440], which views knowledge translation as iterative, multidirectional, and context-driven. The KTA model includes:

- Identifying the problem
- Synthesising existing knowledge
- Analysing contextual fit
- Designing interventions
- Implementing and evaluating outcomes
- Supporting research use and scale-up

By combining the quantitative structure of a pilot trial with the qualitative depth of participant narratives and practitioner perspectives, this study was positioned to produce actionable insights that inform future trial design,

service delivery, and reproductive health policy.

The National Institutes of Health (NIH) and other best practice guidelines advocate for the intentional integration of mixed methods in clinical trials, particularly during pilot and feasibility stages [435, 441]. This integration enhances trial quality by ensuring that the intervention is not only evidence-based but also contextually appropriate and implementable.

By combining qualitative depth with quantitative breadth, this study was well-positioned to not only test a novel intervention, but also to generate actionable knowledge that can be used to inform clinical practice, shape future trials, and support healthcare policy.

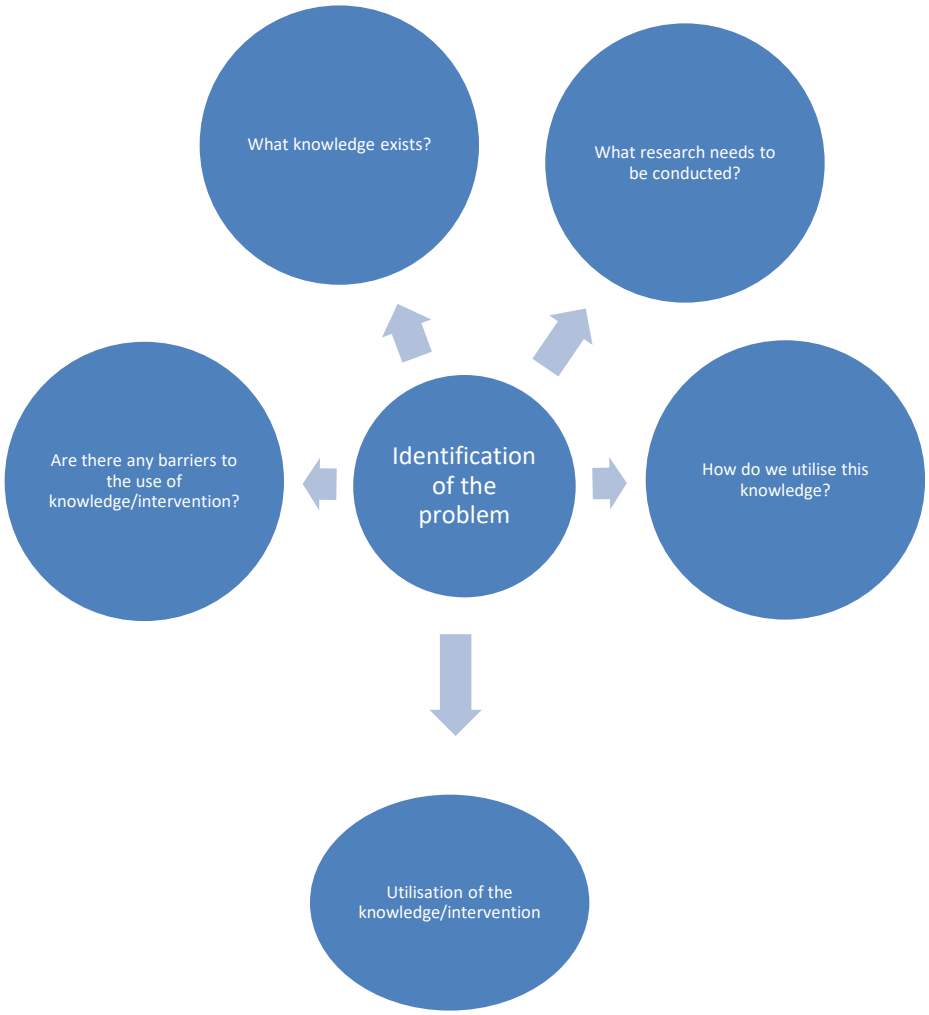


Figure 9- Conceptional framework of the knowledge transfer process adapted from Ward et al. (2009)

3.4 Thesis Phases

This thesis was structured into two distinct empirical phases, each building upon the findings of the previous to explore the role of FA in RPL. The phases were designed to address the overarching research aim through a combination of quantitative, qualitative, and mixed methods approaches.

Informational Stage: Consisted of two comprehensive systematic literature reviews.

Systematic Review one: Investigated FA intake among women in preconception and pregnancy across countries with mandatory folic acid-fortification. This review aimed to quantify exposure and assess this against current recommended guidelines.

Systematic Review two: Examined the potential adverse effects of elevated FA intake, including both supplemental use and high blood folate concentrations. This review explored associations between excess folate and reproductive and offspring outcomes and its association with reproductive outcomes.

Findings from these reviews informed the design and rationale for the subsequent phases.

Phase One: An interventional pilot trial comparing FA and 5-MTHF supplementation in women and men during preconception and pregnancy. This phase included a qualitative sub-analysis of participant interviews to explore women's clinical experiences and perceptions of care in the context of RPL, adding depth and context to the trial findings.

Phase Two: A cross-sectional survey of healthcare practitioners involved in fertility and pregnancy care. The survey assessed health professionals' knowledge, attitudes, and prescribing practices regarding FA use in couples experiencing RPL. This phase aimed to identify clinical practice patterns and inform future educational and policy initiatives.

3.5 Study Components

3.5.1 Randomised Controlled Trial Pilot Study

The RCT pilot study was the core component of this research. It assessed the feasibility, acceptability, and preliminary outcomes of the intervention compared to a control condition in a real-world setting. Participants were randomly assigned and monitored across the trial duration.

Male partners were included in the trial because recurrent pregnancy loss is a couple-level condition, and paternal factors contribute materially to embryo viability and pregnancy maintenance [157]. Men contribute 50% of the genetic material to the embryo, and folate-dependent one-carbon metabolism plays a critical role in spermatogenesis, DNA synthesis, and epigenetic regulation [442]. Disrupted folate metabolism in men has been associated with impaired sperm quality, increased DNA fragmentation, and altered DNA methylation patterns, which have been linked to adverse reproductive outcomes, including miscarriage [443].

In addition, common polymorphisms in folate-metabolising genes, particularly *MTHFR*, are associated with male infertility phenotypes, providing a plausible biological pathway through which paternal folate status may influence reproductive outcomes [80]. Despite this, men are rarely included in preconception or recurrent pregnancy loss interventions. Their inclusion in this pilot trial was therefore intentional, to assess the feasibility, acceptability, and biochemical relevance of a couple-based personalised folate approach and to inform the design of future definitive trials.

Data collected included:

- Standardised outcome measures (pre/post intervention)
- Adherence and retention rates
- Adverse events and safety outcomes

In addition, an embedded qualitative sub-study was conducted using recruitment interviews. Although originally intended for study briefing and consent, these conversations revealed rich personal narratives regarding participants' experiences of recurrent pregnancy loss. This data underwent thematic analysis to identify:

- How participants experience and understand RPL
- How they perceive the relevance and acceptability of the intervention
- Feedback on trial logistics, consent, and burden

This embedded sub-study supports interpretation of trial outcomes and ensures participant experiences directly inform future intervention refinement.

3.5.2 Practitioner Survey

A cross-sectional online survey was distributed to healthcare professionals involved in the care of individuals with RPL. The survey examined:

- Clinical relevance and acceptability of the intervention
- Barriers and facilitators to implementation in clinical practice
- Suggestions for improving alignment with existing service delivery models

The findings have helped contextualise trial outcomes and contribute to the

design of a scalable intervention for future application.

3.6 Feasibility Domains of the Study

To assess feasibility, a range of domains were monitored throughout the study, including recruitment, retention, adherence, protocol fidelity, and participant acceptability. These outcomes were used to guide trial refinement and ensure readiness for a future full-scale RCT. (See Table 8 for specific feasibility indicators across study phases.)

3.7 Randomised Clinical Trial Pilot Study – Phase 1

3.7.1 **Design of the Pilot Trial**

The pilot trial has been designed using the Pragmatic–Explanatory Continuum Indicator Summary (PRECIS) framework [434], which assesses ten key domains of trial design. These are represented visually in the PRECIS wheel (see Figure 8), where the distance from the spoke indicates whether design elements are more pragmatic (outer rim) or explanatory (centre).

Table 8 - Feasibility indices across study phases

Feasibility Domains	**Study Outcome**	**Phase of the study in which the assessment is conducted. (Chapter)**
Acceptability	Acceptability of the proposed study protocol to eligible individuals who enquire about study involvement	Phase 1 pilot trial (Chapter 4)
	Acceptability of the study protocol to individuals who withdraw from the study	
	Acceptability of the study protocol to participants who complete the study	
Demand	Demand for a 5-MTHF prenatal multivitamin among couples with recurrent pregnancy loss	Phase 1 pilot trial (Chapter 4) Participant Survey (Chapter 5)
Implementation	Participant adherence to data collection requirements	Phase 1 pilot trial (Chapter 4)
	Side effects associated with the dose of 5-MTHF or folic acid	
	Adherence to the dietary requirement of avoiding folic acid fortified foods	
	Adherence to the abstinence of trying to conceive for 2 reproductive cycles following the introduction of the multivitamin	
	Effective support provided through the online meetings	
	Adherence with blood collection requirements	
	Practicalities and adherence by participant and Dr for the confirmation processes	
Practicalities	Hours required by researchers	Phase 1 pilot trial (Chapter 4)
	Resources required by researchers	
	Practicality and effectiveness of recruitment methods including screening and eligibility processes	
	Effectiveness and practicality of the participant and researcher blinding process	
	Practicalities, ease of use of the online booking system and meeting scheduling system	
	Practicalities associated with clinician's adherence to the study protocol	
	Participant retention overall and between the groups	
	Practicalities of data collection processes	
	Variability of data collected throughout the study	Phase 1 pilot trial (Chapter 4)
	Treatment effects/outcome expectations	
	Research team challenges in conducting the study	
Integration	Implementation of 5-MTHF prenatal multivitamin use into existing policy and practice frameworks regarding couples with recurrent pregnancy loss	Phase 2 Practitioner Survey (Chapter 6)
Efficacy testing	Efficacy rates of each group in achieving a viable pregnancy to inform sample size calculations for future study	Phase 1 pilot trial (Chapter 4)
	Change in UMFA	Phase 1 pilot trial (Chapter 4)
	Changes in vitamin B12, folate and homocysteine status	Phase 1 pilot trial (Chapter 4)

	MTHFR polymorphism variance	Phase 1 pilot trial (Chapter 4)
Interpretation	To identify appropriate pathways for conducting a larger trial	Phase 1, 2

This framework ensures a balanced approach that considers both internal validity and external applicability, aligning with the feasibility objectives of a pilot study while preparing for a definitive trial.

This study was a pilot randomised controlled trial (RCT) designed to estimate the effect of 5-methyltetrahydrofolate (5-MTHF) compared to folic acid in reducing the risk of pregnancy loss among individuals with a history of recurrent pregnancy loss (RPL). The trial assessment consisted of a range of feasibility metrics related to study conduct and participant experience. These include:

- Adherence to the intervention dosage
- Compliance with dietary instructions, particularly the avoidance of folic acid–fortified foods
- Feasibility of abstaining from conception attempts for two reproductive cycles after beginning the intervention
- Acceptability and logistics of blood sample collection
- Withdrawal and retention rates
- Monitoring and reporting of safety outcomes.

To ensure practical feasibility, participants had to reside in Australia and have access to reliable internet and postal services. This virtual design enhanced accessibility and efficiency while preserving study oversight, a model increasingly relevant in the post-pandemic clinical research environment. The pilot trial played a pivotal role in evaluating both the scientific and operational viability of a larger-scale RCT. As noted by Abbott (2014), pilot

studies are essential in determining whether a full trial is feasible, while Thabane et al. (2010) highlight their utility in identifying necessary modifications to trial procedures and estimating preliminary treatment effects.

Table 9 - PRECIS domains illustrating the explanatory or pragmatic approach to each domain

Key Domains	**Pragmatic or Explanatory**	**Justification**	**Data Collection Method**
The eligibility criteria for trial participants	Pragmatic	Identify study participants with the condition of interest from as many sources as possible	Strict inclusion and exclusion criteria set
The flexibility in which the experimental intervention is applied	Explanatory	Strict instructions are given on how to administer the intervention	Specific instructions were given regarding dose, time of dose, any medications that would affect the intervention, specific directions for managing complications or side effects of the intervention
The degree of practitioner expertise in applying and monitoring the experimental intervention	Pragmatic	The intervention would be applied by all practitioners and in the full range of clinical settings, regardless of their expertise	Practitioner survey
The flexibility in which the comparison intervention is applied	Pragmatic	Usual practice management which is the use of folic acid-based multivitamins would be compared to the methyl folate version	Practitioner survey/Participant exit survey
The degree of practitioner expertise in applying and monitoring the comparison intervention	Pragmatic	What are the benefits and harms of the intervention in comparison with usual practice settings of interest	Practitioner survey/Pilot trial
The intensity of follow-up trial participants	Explanatory	Study individuals are followed with many more frequent visits and more extensive data collection	Ongoing monthly meetings with an 'end of study' survey
The nature of the trial's primary outcome	Explanatory	Is the trial feasible, incorporating patient attitudes and defined measurements?	Pilot study, participant exit survey, practitioner survey
The intensity of measuring participants' compliance with the prescribed	Explanatory	Compliance is measured closely	Ongoing meetings, regular assessment points, compliance evaluation

intervention and whether compliance improving strategies are used			
The intensity of measuring practitioner's adherence to the study protocol and whether adherence improving strategies are used	Explanatory	There is close monitoring of how well the participating clinicians are adhering to even the minute details in the trial protocol and manual of procedures	Practitioner survey
The specification and scope of the analysis of the primary outcome	Explanatory	Will this work under ideal conditions? Exclude noncompliant participants, exclude patients to be found ineligible	Pilot study, participant exit survey, practitioner study

3.7.2 Trial setting

The trial was conducted online only, led by researchers from the School of Public Health at University of Technology Sydney. All interactions with participants were virtual via phone or video conferencing, and the documents and investigational products were posted to participants. Participants had to reside in Australia.

3.7.3 Study Outcomes and Outcome Measures

The pilot trial addressed the study's overarching objectives through a series of specified outcomes, which were assessed using predefined outcome measures (see Table 10). These included both clinical and feasibility outcomes relevant to evaluating the intervention and informing the design of a future definitive trial.

Table 10- Data collection methods for study outcomes

Pilot Study Outcome Measure	**Data collection instrument/s (platform)**
A minimum participant conversion rate of 50% for eligible individuals who enquire about the study	Participant screening form (Qualtrics)
Acceptance of randomisation by 60% of enrolled participants	Initial Baseline collection of information form (REDCap)
A participant withdrawal rate no higher than 30% of enrolled participants	Six-weekly questionnaire for participants (REDCap)
A completion rate of 70% in those participants that consent to inclusion in the trail	Six-weekly questionnaire for participants (REDCap)
Prevalence of use, type of folate, dose and source of prescription/ recommendation of folate supplement among individuals eligible for study participation who are using a folate supplement prior to study involvement	Initial Baseline collection of information form (REDCap)
Rate of consenting participants that complete all baseline assessments within two weeks	Six-weekly questionnaire for participants (REDCap)
Duration to completion of all baseline assessments for participants that did not complete within the allocated two-week timeline	Six-weekly questionnaire for participants (REDCap)
Incidence of adverse events identified through the study and their possible relationship to the intervention	SUDS – Subjective Units of Discomfort scale DASS-21 anxiety and depression scale Adverse Event report Adverse Event Log
24-hour and 7-day recall of diet protocol adherence at each pilot study meeting (Wks 8, 14 and every 6 weeks thereafter)	Participant Diary
Rate of participants that adhere to the abstinence requirement	Six-weekly questionnaire for participants (REDCap)
Study participants' perceptions of the support provided through online meetings	Participant Exit Survey (Qualtrics)
Rate of consenting participants that completed blood collections within two weeks of the requirements	Six-weekly questionnaire for participants (REDCap)
Duration to completion of all blood collections for participants that did not complete within the allocated two-week timeline	Six-weekly questionnaire for participants (REDCap)
Rate of consenting participants and their practitioners that completed the confirmation sheets within two weeks of the requirements	Six-weekly questionnaire for participants (REDCap)

Number of researcher hours required to manage the pilot study	Time tracking software
Financial, capital, and perishable resources required to run the pilot study	Logistics Log (Microsoft Excel)
The dollar cost per participant for recruitment including the most cost-effective method used for recruitment. Recruitment rates per week/month, recruitment and screening/eligibility process evaluation	Participant Screening information (Qualtrics) Logistics Log (Microsoft Excel)
Participants' self-reported perception of whether they had been allocated the pilot study or control product	Participant exit survey (Qualtrics)
Participant perceptions of the online booking system including ease of use, errors, failures in booking procedures.	Participant exit survey (Qualtrics)
Rate of errors associated with booking procedures using the online booking system	Six-weekly questionnaire for participants (REDCap)
The rate of clinicians' adherence to providing signed study protocol documents including: confirmation of recurrent pregnancy loss,18-22 week gestational scan and confirmation of viable pregnancy	History of recurrent pregnancy loss form Drs confirmation of pregnancy form – 22 weeks
Rate of participant retention amongst the allocation groups, and reasons for study withdrawal	Participant exit survey (Qualtrics)
Rate of participant missing data	Six-weekly questionnaire for participants (REDCap)
Participant time taken for document completion	Six-weekly questionnaire for participants (REDCap)
Researcher time taken to check and finalise participant documents, and to meet with participants	Time tracking software
Protocol changes required to achieve objectives	Logistics log (Microsoft Excel)
Time to pregnancy versus expected time to pregnancy in the intervention group (2-4 reproductive cycles post the abstinence period)	Six-weekly questionnaire for participants (REDCap)
The rate of success in achieving the 22-week gestation (≥30% success rate for the intervention group)	Six-weekly questionnaire for participants (REDCap)
Other challenges experienced by researchers not captured through other outcome measures	Researcher Protocol Changes and Challenges record (Microsoft Excel)
Enrolled participants' behaviours and attitudes towards: (1) taking 5-MTHF vs folic acid; (2) abstaining from conceiving for two full cycles at study onset; (3) withdrawal from the pilot study seven months from study onset if not pregnant; (4) avoiding folic acid fortified foods.	Participant exit survey (Qualtrics)
Rate of participants in the 5-MTHF arm versus folic acid arm that achieve 22 weeks' gestation.	Six-weekly questionnaire for participants (REDCap)
Difference in concentrations of UMFA at baseline and again at 6 weeks	Six-weekly questionnaire for participants (REDCap)
Difference in concentrations of serum vitamin B12, methylmalonic acid, serum folate, red cell folate and homocysteine at baseline and at time points throughout the pilot study	Six-weekly questionnaire for participants (REDCap)
The variance of *MTHFR* polymorphisms in the pilot study participants overall and between groups	Six-weekly questionnaire for participants (REDCap)

1. Insurmountable problems with the necessary process: STOP, do not proceed with the main trial 2. Problems identified are surmountable: modification will be necessary in full study protocol and further pilot testing may be necessary 3. Few or no major problems: a full study can proceed without modifications or further testing.	All of the above

3.7.4 Eligibility Criteria for Trial Participants

3.7.4.1 Inclusion Criteria

Non-pregnant women of childbearing age between 20-49 years and their reproductive partners were eligible if they reported a history of two or more pregnancy losses and were interested in conceiving in the future. Pregnancy loss was defined according to the European Society of Human Reproduction and Embryology (ESHRE), a clinical loss, after the missed menstrual period or a positive pregnancy test, excluding biochemical pregnancy losses [157]. History of pregnancy loss was confirmed by a letter from a medical doctor *(see Appendix 10, History of recurrent pregnancy loss)*. The Australian Government defines women of childbearing age as those between 15 – 49 years. However, the average age of mothers in 2019 was 29.4 years [444, 445] and women aged 15-19 years accounted for only 2.2% of all births [444]. Given the relatively low proportion of teenage pregnancies and the increased health risks associated with pregnancy and birth at younger ages [446], this study included women aged 20 to 49 years.

Furthermore, the following criteria applied:

- Medical confirmation of two or more pregnancy losses before 22 weeks
- Aged 20-49 years of age
- Ability to speak and understand English sufficiently to comprehend the purpose and risks of the study and to provide consent
- Ability to attend a pathology centre for specimen collection

3.7.4.2 Exclusion Criteria

- Pregnant or lactating women
- Couples who were undergoing assisted reproductive technologies
- Men and women taking folate antagonist medication (phenytoin, valproic acid, valproate sodium, carbamazepine, methotrexate, trimethoprim hydrochloride, trimethoprim sulphate, trimethoprim, aminopterin sodium, phenobarbital, phenobarbital sodium, primidone, divalproex)
- Individuals with a known sensitivity to any ingredients in the products
- History or presence of psychiatric disorders, autoimmune disease, and cancer
- History of gynaecological and/or genetic problems– antiphospholipid syndrome, blocked fallopian tubes, Pelvic inflammatory disease, Primary ovarian insufficiency, endometriosis, Uterine fibroids, Factor V Leiden.

3.7.5 Interventions

3.7.5.1 Investigational Product

The investigational/intervention product was a prenatal multivitamin. This was not commercially available; however, it was modelled on existing commercial formulas with a history in the marketplace. This bespoke manufacture of both products was necessary to control the dosage of the folic acid and 5-MTHF and the appearance of the product to ensure blinding. Each multivitamin contained 2,500 mcg of folate, delivered across three capsules as either 5-MTHF or folic acid. This dosage was derived from findings in a previous case-series study examining folate prescribing practices in individuals with *MTHFR* polymorphisms, which demonstrated

that doses at or above this level were commonly prescribed in real-world clinical settings and were associated with measurable biochemical responses [447]. The dose was therefore selected to reflect observed clinical practice and to ensure sufficient metabolic exposure to allow assessment of biochemical feasibility rather than to represent a recommended prenatal intake.

The ingredients of the multivitamin (in three capsules) were:

Beta carotene	1500 mcg
Thiamine	44 mg
Biotin	300 mcg
Riboflavin 5 phosphate	25 mg
Nicotinamide	30 mg
Pantothenic acid	100 mg
Pyridoxal 5 Phosphate	15 mg
Methylcobalamin	500 mcg
5-MTHF	2,500 mcg
Iodine	200 mcg
Ascorbic acid	250 mg
Vitamin D	1000IU
Vitamin E	200IU
Calcium	100 mg
Magnesium	50 mg

Zinc	25 mg
Selenium	140 mcg
Manganese	5 mg
CoQ10	50 mg
Choline	200 mg
Inositol	25 mg
Chromium	40 mcg
Alpha Lipoic acid	30 mg

The excipients included leucine, ascorbyl palmitate, colloidal anhydrous silica, vegetable hard capsule (Hypromellose and purified water).

Iron was intentionally excluded from the investigational multivitamin because the formulation was designed for use by both female participants and their male partners. Routine iron supplementation is not indicated for men and may be inappropriate in iron-replete individuals. Exclusion of iron therefore ensured suitability of the formulation for a mixed-sex study population and avoided unnecessary supplementation unrelated to the aims of the trial.

The dosage was three capsules per day in the morning after breakfast. Participants were instructed to titrate the dose to mitigate side effects. Week one , one capsule per day, Week two, 2 capsules per day and Week three and ongoing 3 capsules per day for the first trimester. Two capsules were given in trimester 2 and one in trimester 3.

3.7.5.2 Control

The control product replicated the above exactly with a substitution of 2,500 mcg of 5-MTHF for 2,500 mcg of FA. Dosage replicated the above i.e. 3

capsules per day.

3.7.5.3 Product Preparation, Appearance, and Supply

The investigational and control products were prepared to match in appearance and odour. Both products appeared as capsules in plastic containers labelled for the trial and produced specifically for this study in accordance with standard GMP practices.

The labels identified the products as either 'Prenatal Multivitamin A' or 'Prenatal Multivitamin B'. The research team were blinded to the ingredients of each product. Each container contained 90 capsules, equivalent to a one-month supply. The label was identified by the trial name and contained further information, including the investigator's name and contact details, ethics and trial registration numbers. The label is shown below.

Folate Trial for recurrent miscarriage
Supplement: **Prenatal Multivitamin A (or B)**

Participant No:

Directions for use: 3 capsules in the morning after eating

University of Technology Sydney HREC No: ETH22-7218

Chief Investigator: Carolyn Ledowsky Ph: 0434 370 394

Email: carolyn.j.ledowsky@student.uts.edu.au

ANZCTR:U1111-1283-5932
STORE BELOW: 25°C in a cool dry place out of sunlight.

EXPIRY DATE: BATCH NUMBER:

Figure 10 - Trial multivitamin label

3.7.5.4 Other Medications

No other vitamin or mineral supplements, herbal products or pharmaceutical products were permitted during the trial period unless recommended by a qualified health professional. Participants were required to advise the

research team if this occurred and provide evidence of a health professional prescription.

3.7.5.5 Storage

All products were stored in a dry, and daylight protected locked room. The room was temperature controlled to ensure all products were kept below 25°C.

3.7.5.6 Dispensing of Study Drug

The investigational product and the control product were dispensed by a member of the research team via Australia post. The researcher documented the dispensing of each product in the Product Dispensing Log Record which was kept in the participant's chart file. Each time a product was dispensed, the date, the product ID, the quantity, batch number, and expiry of the product was recorded. The researcher provided each participant with the products for a three-month period.

3.7.5.7 Return or Destruction of Study Drug

At the final trial meeting, participants were asked to provide a count of remaining capsules. This was recorded in the participant's file in REDCap. Participants were then asked to dispose of any remaining product through normal household waste or invited to continue using it until finished.

3.7.5.8 Criteria for Discontinuing or Modifying Allocated Interventions

A participant could discontinue their intervention at any time. Participants were free to withdraw from the trial at any point without question or penalty. This was explained to the participants when they either contacted the researcher via email or phone, or at one of the follow-up visits. The withdrawal of the participant from the trial was documented by the researcher in the REDCap system, and they were immediately sent the trial exit survey

to complete.

Treatment dose could be modified in response to Safety Advisory Board recommendations following mild or common expected adverse events such as nausea and vomiting in early pregnancy. In this case, participants were asked to cease product use for three days to see if symptoms subsided. If symptoms persisted, a titrated dose protocol could be implemented as per the revised and final ethics approval document.

A participant was encouraged to consult their GP or other specialist medical doctor to advise their inclusion in the trial. Once pregnant, the participant was again advised to consult their GP and/or fertility specialist for concomitant care throughout the trial.

3.7.5.9 Participant Requirements

Participants were prohibited from taking the following during the trial:

• Any multivitamin or product containing folic acid

• Any food containing folic acid

• Any vitamin or mineral supplement that had not been approved by the researchers

• Any medication listed in the exclusion criteria for this study

3.7.5.9.1 Participant Dietary Requirements

For this trial, all participants were required to consume a diet that avoided folic acid fortified foods and supplements. A list of foods to avoid and foods was supplied to each participant *(see Appendix 11, A list of folic acid foods)*.

3.7.5.9.2 Participant's Timeline

A schematic of the participant timeline can be found in *Figure 11*.

3.7.5.9.3 Participant Screening

The trial was advertised via social media. The social media advertisement

included a link to an initial screening questionnaire (conducted via Qualtrics) requiring consent for data collection for screening purposes and asking participants for health-related information to check eligibility against inclusion and exclusion criteria *(see Appendix 12, Qualtrics Participant screening form)*. If the participants were deemed eligible, they were asked to indicate consent for being contacted by the researcher and selected that they would like to receive further information and to provide their preferred contact details.

Through the screening instrument, a meeting with the researcher was scheduled at a time suitable for the participant using the Acuity Timetable and Scheduling platform. Acuity scheduling® is a 24/7 online scheduling assistant to allow participants to set, change, or cancel appointments at their leisure. Prior to the confirmation interview the participant was emailed the participant information sheet *(see Appendix 13 and Appendix 14, Participant Information Sheet for females and males),* consent form, contact details and any further information they required about trial participation. If participants were deemed ineligible, the Qualtrics survey displayed a message indicating that they were not eligible to participate in the trial. The display also contained advice to discuss their fertility with their General Practitioner (GP).

3.7.5.9.4 Confirmation Interview

All participants deemed eligible met with the researcher via phone or videoconferencing to discuss the pilot trial procedures and were given an opportunity to ask any additional questions they had. Researchers reiterated the voluntary nature of the trial and reinforced the participants' ability to withdraw from the trial at any time. Participant eligibility was also confirmed by the researcher during the Confirmation Interview. When participants indicated that they required no additional information or had no further

questions, and that they had sufficient time to consider their participation and the implications of such participation, they were sent a link to an e-consent form via REDCap and enrolled into the trial. REDCap is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages, and 4) procedures for importing data from external sources [448]. For the e-consent, the REDCap e-consent framework provided standardised tools to collect the electronic informed consent. All questions from the original consent form were integrated into the survey, so to participate, participants needed to confirm that they had read and understood all the information they had received. Once all boxes were ticked, and the participant had added the date, they could then add their signature.

Upon providing consent, a PDF was generated with the participant responses, displaying all the responses and the signature of the potential trial participant. Participants were then invited to review the PDF and certify that all the information in the document was correct. The documents were stored in the file repository on the REDCap server and could be downloaded and shared with the participant. The REDCap e-consent has been implemented in various research institutions worldwide and is complying with regulatory requirements for e-consent.

Following this meeting, the researcher emailed participants a participant study pack containing the details about the trial including appointment information, dietary modification instructions, requirement to abstain from attempting to conceive for first eight weeks of study involvement, and their

pathology testing referral forms for blood and genetic tests to be completed prior to the baseline appointment. Participants were asked to have their medical doctor complete the Confirmation of Eligibility form confirming the participant has a history of recurrent pregnancy loss *(see Appendix 10, History of recurrent pregnancy loss)*.

3.7.5.9.5 Baseline Visit

Within one week following the Confirmation Meeting, the participant met with the researcher to initiate trial participation. The researcher asked the participant to provide information about their demographic information, dietary intake, fertility history, current menstrual cycle information *(see Appendix 15, REDCap initial baseline collection of information)*. Participants were invited to ask any questions that had arisen since the confirmation interview. The researcher then discussed and clarified the required dietary modifications, the titration protocol for introduction of the prenatal multivitamin, confirmed blood collection had been completed and checked receipt of Confirmation of Eligibility Form from participant's medical doctor. Participants were reminded that they should not attempt conception for two reproductive cycles from commencement of the prenatal multivitamin. All remaining meetings for this study were scheduled for the participant at this Baseline Visit. All data was entered into REDCap during this Baseline Visit. At the completion of the Baseline Visit, the researcher randomised the participant to a study arm via the REDCap block randomisation sequence and sent the appropriate study product to the participant via mail.

3.7.5.9.6 Product Tolerability and Adherence Visit

At Week 2, the researcher met with the participant via phone or videoconferencing where they were asked about product tolerability and whether any adverse events occurred within the first two weeks of study

involvement *(see Appendix 16, Product tolerability and adherence visit checklist)*. The researcher answered any questions the participant had and provided guidance regarding study protocols including required dietary changes. Participants were reminded of the date of their next meeting and advised that they could contact the researcher at any stage with questions or concerns.

Figure 11- Study Flow Chart of participants visits

3.7.5.9.7 Follow-Up Meetings

At week eight, the researcher met with the participant to check adherence to protocol (product use, collection of blood work and dietary modifications), tolerability, and occurrence of adverse events *(see Appendix 17, Six-weekly REDCap questionnaire for participants)*. One week prior to the meeting, participants were emailed a pre-meeting questionnaire to identify areas of potential concern or discussion, to help prioritise the meeting discussion points. During the meeting, participants were asked to provide details of their menstrual cycle and any confirmation of pregnancy together with any issues they may have had with the dietary requirements. During this time, the participant was monitored for any signs of distress. Participants were sent sufficient product for use until the next meeting.

If pregnancy was suspected, the woman was asked to confirm pregnancy via a pregnancy test kit (they were required to take a photo and email it to the researcher). If the woman was under the care of either a GP or fertility support specialist once pregnancy was confirmed, they were asked to advise the specialist of the trial and give them the Trial Information Pack for Doctors *(see Appendix 18, Trial Information Pack for Doctors)*. This pack explained the trial and supplied contact information for the researcher should the GP or specialist wish to contact the researchers. Should the patient see a GP or specialist at this time, they were requested to ask their medical doctor to sign the Confirmation of Pregnancy form following confirmation via an 8-week *scan (Appendix 19, Dr's confirmation of pregnancy form 8 weeks)*. The GP or specialist was also asked to sign the Confirmation of Viable Pregnancy form at 22 weeks *(Appendix 20, Dr's confirmation of pregnancy 22 weeks)*. This meeting was repeated every four to six weeks (Weeks 14, 20, 26, 32, 38, 44) until study completion or participant reported a live birth.

3.7.5.9.8 Follow-up Emails

Participants received regular emails to confirm participant progress and remind them to contact the research team with any questions or queries.

3.7.5.10 Sample Size

The sample size for this pilot trial was eleven couples in each arm (22 people).

3.7.5.11 Recruitment

Consumer and community involvement was sought through pregnancy loss support groups including the Pink Elephants Support Network and the Gidget foundation. These groups were approached to assist with the recruitment strategy. There was a rolling recruitment period from March to October 2023 to endeavour to achieve the goal of 30 couples in each arm.

This trial aimed to recruit from the general public around Australia. The main form of advertising was on social media with advertisements for the trial placed on Facebook, Instagram, Twitter, and LinkedIn *(see Appendix 21, Advertising flyer)*. The trial was also listed on the UTS website, and a Faculty of Health UTS media release announced the trial. A dedicated webpage was developed to describe the goals of the trial, including contact details so that interested parties could contact the researchers about possible recruitment.

A 30% drop out rate of participants was factored into the numbers.

3.7.5.12 Assignment of Interventions

3.7.5.12.1 Sequence Generation

Randomisation was performed with block randomisation, using random block sizes (1:1), with an equal probability of a participant being in either size block. The blockrand library in R (version 4.2.1) was used to create the allocation sequence and uploaded into a REDCap database, where the randomisation

was conducted.

3.7.5.12.2 Allocation Concealment

All researchers and participants were blinded to the group allocation. The researcher uploaded a spreadsheet to the REDCap randomisation module containing the sequence of groups (A and B). The code A or B corresponded with the group label on the investigational product container, it did not provide any additional information about the content of the container.

3.7.5.12.3 Implementation

The researcher conducting the regular participant interviews was the only researcher that had access to the randomisation sequence and administrative rights to the REDCap database. The researcher screened the participants and enrolled them into the trial. Once a participant had met all the inclusion criteria, signed the consent form, and was formally enrolled, then the researcher entered the participant's information into the REDCap online database, for randomisation purposes. Once the data was entered, the researcher allocated the participant via the inbuilt randomiser, and the system provided the group allocation such as: "Participant receives product A", or "Participant receives product B". These group labels A and B align with the labels on the interventional products. The group allocation was noted in the participant's forms and files.

3.7.5.12.4 Blinding (Masking)

All researchers were blinded to the participant allocation. The manufacturer of both products labelled each of the products (A or B), and as such was the only one aware of the contents of the products, they were not involved in any of the study procedures and did not know which group each participant was assigned to.

3.7.5.12.5 Unblinding Procedures

Participants could only be unblinded in a number of specific circumstances,

most of which were safety related. Unblinding was required in the following situations:

• An unexpected serious adverse event occurs. This is called an emergency unblinding, where it is essential for clinical diagnostic or treatment decisions that the investigational product is known, so that its impact on the adverse event could be evaluated.

• The Advisory Safety and Monitoring Board (ASMB) requests unblinding. This would only affect blinding of the lead researcher, whereas the other researchers and participants remain blinded.

• When the study was concluded, it was necessary for the researchers to know the allocation, to analyse the data according to the trial analysis plan.

The safety of the participants was considered paramount. If a serious adverse event did occur, the participant was told to cease the investigational product immediately, and the research team followed the adverse event procedures. There were no instances throughout the trial where unblinding was required. There was only one serious adverse event where the ASBM was called in to monitor the event, which was eventually deemed unrelated to the trial product. The researcher received notice of the serious adverse event, then immediately informed the head of the safety monitoring board, who then informed the ASBM within 2-3 hours. It was determined within 24 hours if the adverse event could be attributed to the investigational product. The researcher involved in participant management contacted the participant daily and their treating physician(s) via email correspondence and the incidence was deemed unrelated.

3.7.6 Methods: Data Collection, Management, and Analysis

3.7.6.1 Data Collection Methods

The primary outcome of this pilot trial was feasibility, including recruitment, retention, adherence, and acceptability, to inform the design of a future definitive trial comparing 5-MTHF and folic acid in couples with recurrent pregnancy loss.

Data was collected online (REDCap) for the primary and secondary outcomes. All data was exported to Stata statistical analysis software for analysis. At baseline, basic sociodemographic and clinical data were collected, which was used to describe the sample, as well as serve as confounding variables in the statistical analyses.

Table 11- Overview of time points and data collection methods for females in the trial

	Pre-screening	**Confirmation interview**	**Baseline (Day 0)**	**Follow up Week 2,3**	**Follow up Weeks 8,12,16,20, 24,28,32**	**Follow up Week 44**
Screening	**x**	**x**				
Written informed consent		**x**				
Randomisation			**x**			
Sociodemographic information, medical history (CRF)			**x**			
***MTHFR* Polymorphisms**			**x**			
Homocysteine blood test			**x**			
Serum B12 blood test			**x**			
Serum folate blood test			**x**	**x**	**x**	**x**
Red cell folate blood test			**x**	**x**	**x**	**x**
Safety (AE)				**x**	**x**	**x**

Compliance, medications, adverse events (Diary)			**x**	**x**	**x**	**x**
Accounting for investigational products				**x**	**x**	**x**
End-of-trial evaluation- exit survey						**x**

The primary outcome was assessed via qualitative and quantitative methods. The pilot trial included an exit survey to evaluate key components of the pilot study protocols and to gauge attitudes towards the study as a whole.

1. Pilot study

2. Participant exit survey

Safety: Adverse events (AE) were collected throughout the pilot study. They were defined as any symptom, sign, illness, or experience that develops or worsens in severity during the course of the study, including intercurrent illnesses or injuries and abnormal diagnostic results. Participants were asked at each follow-up to provide information on any adverse event since the last visit. A detailed description of the visits can be found in Table 11.

3.7.6.1.1 Blood Test Summary and Completion

Due to budget constraints, baseline blood tests were only conducted for females. Males were only required to complete the *MTHFR* gene polymorphism test via a buccal swab sent to their home. All blood tests were conducted via the same pathology testing company, Nutripath Pty Ltd, who distributed the referrals to each of the female participants, to have collected in their local pathology collection centre, under the same strict guidelines for each collection. The testing of folate was performed as follows:

MTHFR gene test- Method PCR

Vitamin B12 –Method Automated chemistry Roche

Red cell folate- Method Automated chemistry Roche

Serum folate- Method Automated chemistry Roche

UMFA/MMA- Method LCMS/MS/MS

Homocysteine – Samples kept on ice, centrifuged within 30- 60 minutes and plasma promptly separated according to Roche method.

The following baseline tests were completed for the females:

- Homocysteine – this gives an indication of methylation capacity. If homocysteine is low, it can affect the uptake and amount of folate available for methylation and S-adenosylmethionine (SAMe) and if elevated, indicates low folate, vitamin B12 and/or B6 i.e. methylation factors.
- Unmetabolised folic acid – this is essential to analyse whether the intervention group presents with significantly reduced concentrations of UMFA following the removal of FA from both dietary and supplemental sources.
- *MTHFR* polymorphisms – it is important to evaluate whether those that repeatedly miscarry show any higher variance of *MTHFR* polymorphisms given that this gene regulates metabolism of 5-MTHF.
- Red cell folate – this has been shown to fluctuate according to levels of folic acid vs 5-MTHF (and other derivatives of folate such as tetrahydrofolate)
- Serum folate Serum B12

Due to a lack of funding the following tests were not conducted:

- Methylmalonic acid – more of a predictive marker of vitamin B12 status and research shows that excess folic acid consumption exacerbates vitamin B-12 insufficiency, but a biochemical/physiological mechanism has not yet been identified. It is hypothesised that excessive intake of folic acid depletes serum holotranscobalamin, thereby decreasing active vitamin B-12 in the circulation and limiting its availability for tissues.

Follow-up tests – After two reproductive cycles, women underwent a follow-up blood test that consisted of folate derivatives including unmetabolised folic acid.

3.7.6.1.2 Compliance, Monitoring, and Retention

Treatment adherence was monitored by the researcher via follow-up meetings and emails. Adherence to the dietary guidelines was also reviewed during follow-up meetings. Sufficient adherence was achieved when the participant was taking at least 80% of the expected investigational products. At each meeting, the researcher reviewed the diet of each of the participants to ensure that no foods fortified with folic acid were consumed. A list of foods to eat and avoid was supplied initially and reviewed at each meeting.

If a participant did not attend a scheduled meeting, the researcher contacted the participant, reminded the participant of the study requirements, and tried to understand why the participant was not adhering to the requirements. The researcher attempted to address any questions or concerns at this time and reminded the participant that they were, at any time, able to withdraw from the study if they wished to do so. If the participant could not be contacted, then the research assistant undertook five attempts to contact them via

phone, email, or mail. If the participant did not respond to any of the five attempts of contact, then they were informed that they would be treated as lost to follow-up cases. Unless the participant withdrew consent from providing their data, even the data of non-compliant participants were included in the analysis. Only participants who returned the end-of-trial assessment documents were eligible to receive the gift card as a token of appreciation for their participation.

3.7.6.2 Data Management

Data collected on hard copy forms by participants (i.e. those forms completed by their doctors) were scanned and uploaded into the UTS database by the researcher. Additional information was uploaded to the REDCap database and although it did contain identifying information, this was not exported into the dataset for data analysis. REDCap has been specifically developed for research and provides a secure environment for data management [448].

All hard copy files were scanned and uploaded to the UTS database and were then the originals were destroyed once uploaded. Files for statistical analysis were de-identified using the participant code and kept on password protected drives on UTS computers, accessible to those involved in the clinical trial only. Before analysis, all data were checked for plausibility (via descriptive statistics such as mean, median and range), and recoded to create summary scores of the questionnaires. The syntax and a log of any data processing were stored in the same folder as the original data. Upon study completion, all electronic files were saved in STASH.

3.7.6.3 Statistical Methods

All data was exported into Stata 17. Analyses were conducted on an intention-to-treat (ITT) basis, and additional analyses were conducted on the

per-protocol (PP) population as well. Descriptive statistics summarised the data as either means and standard deviations or medians with interquartile range depending on the data distribution, and as absolute or relative frequencies. Parametric tests (t-tests for continuous variables and chi-square tests for continuous variables) were applied for exploratory purposes only. 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.

3.7.6.4 Subject Populations for Analysis

The intention to treat analysis included all participants enrolled in the trial, if they provided at least the baseline information (CRF, *MTHFR* test, blood tests). Missing data was completed using the Markov chain Monte Carlo multiple imputation method in Stata. A set of 50 imputations were generated, and the mean score was used for the ITT analyses. In addition, a per-protocol sensitivity analysis was conducted, where only adherent participants (at least 80%) were included, and only available time points were analysed. This provided information about the magnitude of the effect in relation to compliance.

3.7.7 Monitoring

3.7.7.1 Data Monitoring

A Data Monitoring Committee (DMC) was not used for this study.

3.7.8 Adverse Events

Adverse events (AEs) were defined as any unintended medical occurrences in a participant receiving an intervention, regardless of whether they were causally related to the treatment. In the context of this trial, AEs encompass

any unfavorable or unintended signs, symptoms, or diseases temporally associated with the use of the investigational product, regardless of whether they are considered related to the intervention. Establishing a clear definition of AEs is essential for ensuring consistent identification, documentation, and reporting throughout the study, and for safeguarding participant safety.

3.7.8.1 Adverse Event Definition

An adverse event (AE) was any symptom, sign, illness, or experience that developed or worsened in severity during the course of the study. Intercurrent illnesses or injuries were regarded as adverse events. Adverse events were documented (*Appendix 22 – Adverse Events log and Appendix 23 – Adverse Events Report Form*) Abnormal results of diagnostic procedures were considered to be adverse events if the abnormality:

- Resulted in study withdrawal
- Was associated with a serious adverse event
- Was associated with clinical signs or symptoms
- Led to additional treatment or to further diagnostic tests
- Was considered by the investigator to be of clinical significance

Adverse events were classified as serious or non-serious. A serious adverse event was any AE that was:

- Fatal
- Life-threatening
- Requiring or prolonging a hospital stay
- Resulting in persistent or significant disability or incapacity
- A congenital anomaly or birth defect

- An important medical event

Important medical events were those that were not immediately life-threatening but were clearly of major clinical significance.

All adverse events that did not meet any of the criteria for serious adverse events were regarded as non-serious adverse events. All non-serious adverse events (Table 12) followed a Non-Serious Adverse Event protocol *(Appendix 24, Non-serious adverse events protocol)*.

Table 12 - Non-serious adverse events

Neurological symptoms	Headaches
	Anxiety
	Dizziness
Hormonal Changes	Change in cycle length
	Reduced menstrual flow
	Increased menstrual flow
	Menorrhagia
Skin Symptoms	Eczema
	Rash
	Pruritis
	Erythema
Stomach/Bowel	Constipation
	Diarrhoea
	Cramping
	Nausea
General	Muscle pain

	Cold or flu
	COVID-19
	Food Poisoning
Pregnancy	Nausea
	Vomiting
	Hyperemesis Gravidarum

The prenatal multivitamin was discontinued until symptoms subsided (up to one week), and the non-serious adverse events protocol followed. The participant was then encouraged to titrate the dose of the multivitamin back up to the desired three capsules per day. If they were unable to tolerate the product again, they were allowed to introduce niacin (vitamin B3) multiple times throughout the day to mitigate the side effects and recommence the multivitamin slowly. Should they be unable to do so, the participant was withdrawn from the trial. A summary of reported non-serious adverse events was shared with the ASMB every two weeks. Nausea and vomiting in pregnancy meant that the participant could discontinue the trial product for three days and then titrate back to either one, two or three capsules as tolerated. A reduced dose was permitted throughout the trial. When the ASMB was notified of serious adverse events, the information provided included:

• Study identifier

• Study Centre

• Participants ID

• A description of the event

• Date of onset

• Current status

• Whether study treatment was discontinued

• The reason the event was classified as serious

• Investigator's assessment of the association between the event and study treatment

An overview of the standard adverse event management and reporting lines is shown in *Figure 13*. This does not include the reporting of significant safety issues which were immediately reported to the HREC and the TGA.

3.7.8.2 Adverse Events Monitoring

It was the responsibility of the researcher to oversee the safety of the study and safety monitoring including careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a distress protocol that was implemented where required (see Section 3.7.10 Auditing, Monitoring, and Inspecting). Medical monitoring included a regular assessment of the number and type of serious adverse events and referral back to the participant's GP or specialist if necessary.

3.7.8.3 Advisory Safety and Monitoring Board

An Advisory Safety and Monitoring Board (ASMB), consisting of a group of professionals experienced in clinical care and/or clinical research, was established to provide additional safety and oversight to this trial.

The ASMB for this trial consisted of:

- A fertility specialist: Dr Natasha Andreadis, Fertility Specialist, City Fertility
- A nutritionist and clinical trials specialist: Dr Janet Schloss, PhD
- A general practitioner: Dr Wendy Burton, Chair of the Antenatal/Postnatal Specific Interest Group for the Royal Australian College of General Practitioners
- A registered psychologist: Carrie Thomson-Casey

The ASMB was only contacted if a serious adverse event occurred. All members of the board, including a senior member of the research team, were contacted via email, and a meeting was organised within 24 hours after the researchers learned about the event to discuss the event. All meetings of the ASMB and the outcomes were documented in the adverse events section of the trial.

3.7.8.4 Harms

At each contact with the participant, the researcher sought information on adverse events by specific questioning. The researchers screened the feedback form for any adverse events not reported directly by the participant. Information on all adverse events was recorded immediately in the Adverse Event Log document using the CTCAE *(Appendix 22, Adverse Events log)*. All clearly related signs, symptoms, and abnormal diagnostic procedures results were recorded in the source document, Adverse Events Report Form *(Appendix 23, Adverse events report)*.

All adverse events that occurred during the study period were recorded. The clinical course of each event was followed until resolution, stabilisation, or until it had been determined that the study treatment or participation was not

the cause. Fourteen adverse events were reported during the trial. Three were related to Arm A (methyl) with two deemed unrelated to the trial product, and the third adverse event was tiredness. Eleven adverse events were related to Arm B (folic acid), and all were related to the folic acid product.

The regular meetings also identified if participants were smoking cigarettes and/or consuming alcohol. Both alcohol and smoking have been shown to affect the ability to conceive, and can contribute to pregnancy complications, and the WHO recommends that all pregnant women should be asked about their tobacco use, exposure to secondhand smoke, and alcohol intake at every care visit and that no smoking or alcohol is considered safe throughout pregnancy [449]. Participants were asked if they were aware that both smoking and alcohol use during preconception and pregnancy may be problematic and asked if they would like to consult with a health care professional to discuss this.

The safety protocol for the trial can be found in Appendix 25 and the Office of Research audit in Appendix 26.

3.7.9 Data Analysis and Management

See Appendix 27 – STASH data management plan

3.7.10 Ethics and Dissemination

3.7.10.1 Research Ethics Approval

The pilot trial was conducted according to common standard guidelines for clinical trials as stated by the Declaration of Helsinki ICH-GCP guidelines for clinical research [450], and the NHMRC National Statement on Ethical Conduct in Human Research 2025 (Updated 2025) [451], as well as applicable government regulations and institutional research policies and procedures. The study was approved by the University of Technology Sydney

Human Research Ethics Committee (ETH22-7218 16/5/22).

All participants in this study were provided a participant information sheet and a consent form describing the pilot trial and providing sufficient information for subjects to make an informed decision about their participation in this study (see appendices). The forms in the appendices were submitted with the protocol for review and approval by the Human Research Ethics Committee at the University of Technology Sydney. Formal written consent of a participant was obtained before that participant commenced any aspect of the study, via the approved consent form. The consent form was signed by the participant and the representative of the investigator who is obtaining the consent.

3.7.10.2 Protocol Amendments

This protocol and amendments were submitted to the University of Technology Sydney, Human Research Ethics Committee(s), for formal approval of the study conduct.

3.7.10.3 Consent or Assent

The consent for this pilot study was obtained by the researcher who is qualified and has received study-specific training. A first consent was obtained via the online survey, to allow the researcher to contact the participant to receive further information, and for full screening. After the full screening, the researcher confirmed the eligibility and commenced the consent process.

Participants were asked to sign and return the consent form once they 1) had received the information pack, 2) had a chance to read the participant information sheet, 3) had a chance to seek additional information or discuss the study with the researchers, and 4) had sufficient time to consider their

participation and the implications of such participation. The consent form could be signed at any stage after that, without any researcher present to prevent any coercion. Researchers repeatedly highlighted that participation is absolutely voluntary at any stage.

The researchers then countersigned the consent form and returned a copy to the participants for their files. An electronic consent form was automatically generated into a pdf format and stored electronically in REDCap.

3.7.10.4 Confidentiality

Information about the pilot study participants was kept confidential and managed according to the requirements of the Australian standards of Good Clinical Practice. The regulations required a signed participant authorisation informing the participant of the following:

- What protected health information (PHI) will be collected from subjects in this study.
- Who will have access to that information and why?
- Who will use or disclose that information?
- The rights of a research subject to revoke their authorisation for use of their private health information (PHI).

In the event that a subject revoked authorisation to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorisation.

All information that was collected from participants that could be identified was kept on a locked computer under password protection. No information was collected in hard copy form.

All dissemination material contained aggregated data only, presenting the pilot trial findings via summary measures. As such, confidentiality would not be breached.

3.7.10.5 Declaration of Interests

The researchers on this project declare that there are no competing interests in relation to this study. Carolyn Ledowsky is the founder of *MTHFR* Support Australia which is a company that assists patients with naturopathic, and nutritional advice in relation to *MTHFR* and other genetic polymorphisms that may affect biochemical pathways leading to health issues.

We are unaware of any direct benefits beyond what would be experienced by any health practitioner undertaking clinical research relevant to their clinical practice. There is also no financial benefit that we would see in relation to the prenatal multivitamin as this is a unique formula for this trial and does not replicate the existing market product.

3.7.10.6 Access to Data

The participant-level dataset will not be shared, and only the pilot trial researchers and the head of the work unit at the UTS Australian Research Centre in Complementary and Integrative Medicine will have access to the final pilot trial dataset.

3.7.10.7 Records Retention

In accordance with ethics approval and participant information and consent forms, hard copy forms were scanned and uploaded, and all data stored in a re-identifiable (coded) format in the UTS database for 15 years as required by the UTS HREC. All data will then be destroyed.

3.7.10.8 Ancillary and Post-pilot Trial Study Care

All participants were contacted one-month post-pilot study completion to

ensure the health and safety of the participant. No harm or disturbance occurred. Upon completion or following withdrawal of the study, participants completed an exit survey designed to give further insight into the pilot study specifics.

3.7.10.9 Dissemination Policy

The findings of the study will be published in Carolyn Ledowsky's thesis, have been submitted to a journal for publication and have been presented at national and international conferences. The investigators will also provide a summary of the study findings to the participants via a letter, as well as to the public and health care practitioners via public presentations.

3.7.10.9.1 Plans for Granting Public Access to the Full Protocol and Data Set

The full protocol and data set will be made available upon reasonable request.

3.7.11 Facilities and Resources

The resources utilised for this study were:

• REDCap – through UTS

• Stata – through UTS

3.7.12 Study Feasibility and Risk Assessment

3.7.12.1 Potential Risks to the Study

3.7.12.1.1 Funding

There were considerable costs associated with the production of the prenatal multivitamin. Therefore, funding was sought from outside suppliers to help support this cost. The following funding was applied to this project:

1. Balchem Corp donated the choline for the trial product together with a $20,000 USD manufacturing grant to assist Factors group in the manufacturing of the product

2. Manufacturing was carried out by Factors Group – they are registered by the TGA to produce products for clinical trials, and they offered to produce the multivitamin products at cost price.

3. Methyl folate ingredient was donated by Apropol AG

4. Blood testing was conducted by Nutripath Pty Ltd at a cost price

3.8 Participant Interviews – Embedded Qualitative Sub-study-Phase 1A

3.8.1 Study Setting

This embedded qualitative sub-study was conducted as part of the pilot randomised controlled trial to support evaluation of trial feasibility, acceptability, and implementation context. The sub-study analysed naturally occurring recruitment and follow-up interviews conducted during participant screening, consent, and trial participation. Rather than functioning as a standalone exploration of RPL, the qualitative component was designed to contextualise participants' engagement with their health professional regarding their previous pregnancy losses and inform interpretation of trial feasibility outcomes within real-world care pathways.

3.8.2 Design

In this study, thematic analysis was applied to address implementation-focused questions related to intervention acceptability and feasibility rather than to generate an independent theory of RPL.

Thematic analysis is a flexible and widely used method in qualitative research that facilitates the identification, analysis, and interpretation of patterns or themes within qualitative data [452-454]. Through a structured yet adaptable approach, it enables researchers to uncover how participants make sense of their experiences and the significance they attribute to them.

An inductive approach was taken, underpinned by a constructivist paradigm, to enable themes to emerge from the data without imposing pre-existing theoretical frameworks [453, 455]. A constructivist epistemology acknowledges that meaning is co-constructed between researcher and participant, and that multiple, contextually bound realities exist [454, 456]. This philosophical stance is particularly appropriate for exploring the nuanced and subjective nature of pregnancy loss, as it positions participants as experts in their own experience and allows for a rich, interpretive understanding of the data.[457].

3.8.3 Study Aim

To explore how women with RPL experience and navigate care pathways in the context of participation in a personalised folate intervention trial, and to identify factors influencing engagement, acceptability, and perceptions of folate-related care.

3.8.4 Participants

Participants included women who experienced two or more pregnancy losses and engaged in recruitment interviews after responding to advertisements for the pilot trial. Some later entered the trial and completed exit interviews.

3.8.5 Inclusion Criteria

- Women with a history of two or more pregnancy losses.
- Those who participated in recruitment interviews for the pilot trial.
- Those who completed exit surveys post-trial.

3.8.6 Exclusion Criteria

Individuals who responded to trial advertisements but were not eligible and therefore did not participate in recruitment interviews.

3.8.7 Outcomes

The primary outcome of this sub-study was a set of inductively derived themes relevant to trial feasibility and implementation, including:

- Factors influencing willingness to engage in a personalised folate intervention
- Perceptions of prior folate-related advice and supplementation experiences
- Barriers and enablers to participation, adherence, and trust in care
- How interactions with healthcare providers shaped expectations of the intervention

These findings helped explain patterns of recruitment and engagement in the pilot trial, supported interpretation of feasibility outcomes, and highlighted practical considerations for delivering personalised folate interventions within real-world RPL care.

3.8.8 Data Collection

Data were drawn from naturally occurring recruitment interviews, email correspondence, and trial meetings conducted as part of the pilot trial screening and consent process and ongoing interviews. While the primary purpose of these interviews was to inform participants about the study and assess eligibility, many participants voluntarily shared personal reflections on their experiences of pregnancy loss. With appropriate consent, interviews were transcribed verbatim, providing a rich source of qualitative data for thematic analysis.

3.8.9 Data Analysis

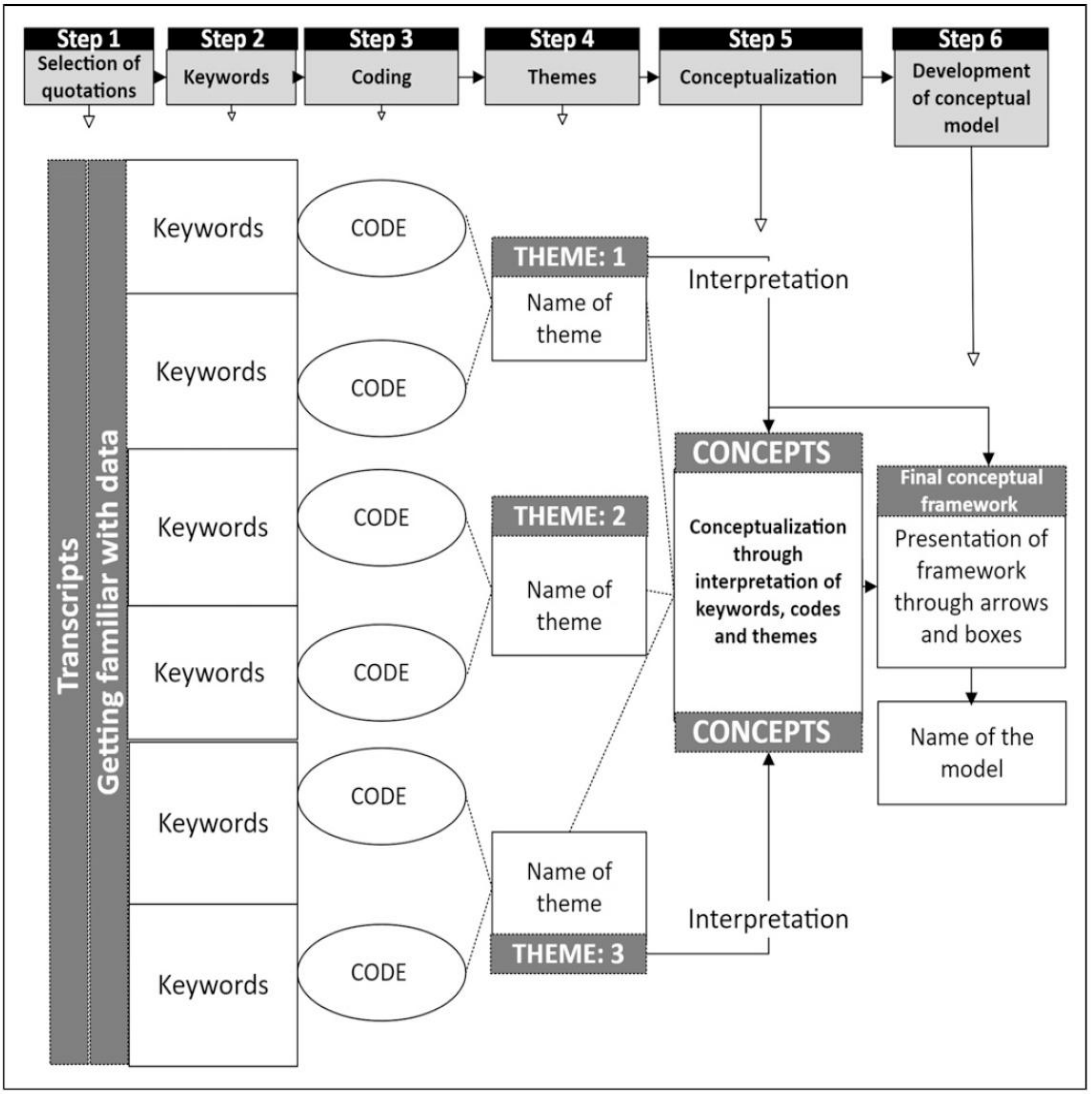
Data was analysed using the systematic thematic analysis process developed by Naeem et al [453] (figure 14), starting with the identification of key terms and concepts in the transcripts. This structured approach allows for both descriptive and conceptual insights:

- Familiarisation with the data
- Generating initial codes
- Searching for themes
- Reviewing themes
- Defining and naming themes
- Producing the final report

Analysis was supported by NVivo software, which assisted with data management, coding, and tracking analytical decisions. Initial coding emphasised recurring words, phrases, and narrative elements. Codes were then refined and grouped into broader themes, which were synthesised into a visual conceptual framework representing the experience of RPL.

This framework represents participants' experiences of RPL and their interactions with care systems, grounded in data and developed through iterative analysis and interpretation.

Figure 12- Process used for thematic analysis (from Naeem et al, 2023 [453])



3.8.10 **Reflexivity**

Reflexivity was embedded throughout the research process to acknowledge the potential influence of the researchers' assumptions, positionalities, and experiences with the subject matter [455]. Memos and reflective notes were kept during the coding and analysis phases to document analytical decisions and maintain transparency in the development of themes.

3.8.11 Trustworthiness and Rigour

To enhance the credibility and trustworthiness of the findings, several strategies were employed: while member checking may not be feasible given the context in which the data were collected, peer debriefing with supervisors supported critical engagement with the analysis. A clear audit trail was maintained throughout the process, and descriptive and illustrative quotes were used to support the transferability of the findings.

3.8.12 Ethical Considerations

Ethical approval for this sub-study was obtained as a component of the original trial ethics approval (ETH23-8644) and all procedures followed ethical standards for research involving human participants. Although the interviews were initially conducted as part of the recruitment process for a pilot randomised clinical trial, verbal and/or written consent was obtained from all individuals prior to the interviews taking place. Participants were informed about the purpose of the conversation and their right to ask questions, decline participation, or withdraw at any time.

During these recruitment interviews, many participants voluntarily shared personal reflections and experiences related to RPL. Given the richness of this material and its relevance to understanding lived experiences of RPL, the data were deemed suitable for secondary analysis using a qualitative framework. This decision aligns with ethical guidelines around the appropriate and respectful use of data collected with participant consent, especially when the content shared extends beyond administrative or informational interactions and into meaningful narrative accounts [458, 459].

All data were anonymised during transcription, and identifying information was removed to protect participant confidentiality. Participants' narratives are

presented in a way that maintains their privacy and dignity, using pseudonyms and omitting specific personal details. The use of these interviews for qualitative analysis is grounded in a commitment to represent participants' voices authentically and with care.

3.9 Cross-sectional Survey of Clinicians - Phase 2

3.9.1 Study Setting

This study was conducted via an online clinician's survey via the Royal Australia College of General Practitioners (RACGP), the Australian College of Midwives (ACM), the Fertility Society of Australia (FSA) and the Perinatal Society of Australia and New Zealand (PSANZ).

3.9.2 Design

This survey utilised a framework to evaluate the potential for or actual public health and population impact. The project engaged the participants collaboratively to consider their attitudes towards the policy of using folic acid versus 5-MTHF [460, 461].

Using an ontological positivist approach through an advocacy/participatory lens, the research contained an action agenda to assist bringing about change in policy, action of frontline GPs in collaboration with others [462].

This survey drew on grounded theory research [462] to understand the views of clinicians towards the use of folic acid, the processes they follow for patients with recurrent pregnancy loss and their interactions with their patients.

With the above approach this survey utilised the RE-AIM framework that includes five dimensions (Table 13) – reach, effectiveness, adoption, implementation, and maintenance [463] that assist researchers in programme planning, implementation, evaluation and reporting for health

research [464].

Table 13 - The RE-AIM framework dimensions

Reach	Why people accept or decline participation. Description of participants vs nonparticipants
Effectiveness	Are the quantitative effectiveness findings meaningful to clinicians i.e. does it help them make decisions and/or achieve their goals? What outcomes are meaningful to the clinician and how did the intervention fare on these outcomes?
Adoption	Would they participate and implement the change? Would they participate in the change?
Implementation	Would the intervention be implemented? What are the key variables in this being achieved: staff, setting, time, communication, tools, resources required?
Maintenance	Program sustainability and reasons why people would continue or discontinue the intervention

3.9.3 Study Aim

The aim of this study was to explore the experiences and attitudes of clinicians who treat patients for recurrent pregnancy loss regarding the following:

- To understand prescribing in regard to prenatal multivitamins and folate specifically
- Recommendations and testing procedures they follow for patients with RPL
- To identify resources that health professionals require to effectively support their patients who suffer RPL
- To understand what research would be required to change their prescribing practices should that be warranted
- To identify what protocols, if any, are prescribed to the male reproductive partner

3.9.4 Participants

All clinicians (including GPs and specialists) who treat patients for RPL.

3.9.4.1 Inclusion and Exclusion Criteria

All clinicians were eligible if they consulted with patients who experience recurrent pregnancy loss and speak fluent English. They were excluded if they do not consult with female patients for fertility.

3.9.5 Outcomes

This study provided updated data as to the prescribing, protocols, and awareness of Australian clinicians to couples with RPL. The findings have provided a deeper understanding as to the knowledge and awareness that these health professionals have regarding preconception care for women and men, multivitamins prescribed, and the research that would be required to change practice behaviour. The study has assisted with information to ensure that health professionals are equipped to offer advice and respond to the needs of patients. The outcome of this research will help to provide education and resources to health professionals to provide improved patient outcomes and support for those with recurrent pregnancy loss.

3.9.6 Data Collection

Study-specific survey questions were developed based on the literature regarding prescribing protocols and attitudes towards the practitioners' ability to support couples with recurrent miscarriage. The survey included five sections: 1. Sociodemographic, time in practice, 2. Attitudes towards how equipped they are to support couples with recurrent miscarriage, 3. Knowledge regarding existing preconception health advice and supplements, 4. RPL protocols, prescribing and testing 5. Folate prescribing, dosing and use.

The online survey responses were collected via an online data collection survey platform called Qualtrics using a database of practitioners through the RACGP, ACM, FSA, and PSANZ who expressed interest in participating in the research.

3.9.7 Data Analysis

Data was analysed using Stata and descriptive statistics were primarily used to analyse and report the survey responses. Descriptive statistics were used to analyse the data and describe awareness of existing protocols and differences regarding preconception care. Predictive analysis helped to develop initiatives and enhance existing protocols for couples with RPL history.

3.9.8 Data Management

No data was kept on any platform outside the UTS secure servers. This was an anonymous survey with data collected via Qualtrics.

3.9.9 Ethical Considerations

A full ethics application was submitted to UTS HREC and approved (ETH23-8411)

3.9.9.1.1 Research Ethics Approval

The trial was conducted according to common standard guidelines for clinical trials as stated by the Declaration of Helsinki ICH-GCP guidelines for clinical research [450], and the NHMRC National Statement on Ethical Conduct in Human Research 2025 (Updated 2025) [451], as well as applicable government regulations and institutional research policies and procedures. This protocol and any amendments were submitted to the University of Technology Sydney Human Research Ethics Committee(s), for formal approval of the study conduct.

3.9.9.1.2 Consent or Assent

Consent was sought from participants before commencement of the survey. Participants were invited to enter a $200 book prize voucher competition and entered a second survey to enter their details. No details were collected in the first survey.

3.9.9.1.3 Confidentiality

No personal identifiable information was collected during the survey.

3.9.9.1.4 Declaration of Interests

The researchers on this project declare that there are no competing interests in relation to this study. Carolyn Ledowsky is the founder of *MTHFR* Support which is a company that assists patients with naturopathic and nutritional advice in relation to *MTHFR* and other genetic polymorphisms that may affect biochemical pathways leading to health issues.

3.9.9.1.5 Access to Data

The participant-level dataset will not be shared, and only the research staff and the head of the work unit at the UTS Australian Research Centre in Complementary and Integrative Medicine will have access to the final dataset.

3.9.9.1.6 Records Retention

In accordance with ethics approval and participant information and consent forms, data will be stored on the UTS stash website for the duration of the study as well as the following 15 years as required by the UTS HREC. All data will then be destroyed.

3.9.9.1.7 Dissemination Policy

The findings of the survey will be published in Carolyn Ledowsky' thesis, and have been published in the journal of Scientific reports and presented at national or international conferences. The investigators will also provide a summary of the study findings to the participants via a letter, as well as to the

public and health care practitioners via public presentations.

3.9.9.1.8 Plans for Granting Public Access to the Full Protocol and Data Set.

The full protocol and data set will be made available upon reasonable request.

3.10 Chapter Conclusion

This chapter has outlined the rationale and structure of the thesis's mixed method research design, developed to investigate the feasibility and clinical relevance of personalised folate supplementation in couples experiencing RPL. The integration of quantitative and qualitative methods was essential to address the complexity of RPL, which encompasses biochemical, genetic, emotional, and systemic dimensions.

The pilot RCT (Phase 1) was primarily designed to assess feasibility, focusing on recruitment, adherence, acceptability, and biochemical outcomes. The embedded qualitative sub-study (Phase 1A) enriched this assessment by exploring participants' lived experiences, providing context for feasibility findings, and identifying gaps in current care. The practitioner survey (Phase 2) extended the feasibility evaluation to the clinical setting, examining prescribing practices, folate prescribing practices, and readiness for change.

Together, these phases form a cohesive and purposefully sequenced investigation. The systematic reviews (Chapter 2) identified key risks and evidence gaps, which informed the design of the pilot trial. This chapter has established the methodological framework that supports the thesis's overarching aim and ensures that each phase contributes meaningfully to the development of a personalised, evidence-informed approach to RPL care. The following chapter (Chapter 4) presents the results of the pilot randomised

controlled trial, focusing on feasibility outcomes such as recruitment, adherence, acceptability, and biochemical measures.

Chapter 4 Results - Feasibility of a Randomised Clinical Trial Comparing 5-Methyltetrahydrofolate and Folic Acid Prenatal Multivitamins in Couples with Recurrent Pregnancy Loss

4.1 Chapter Introduction

As outlined in Chapter 3, this phase of the research involved a pilot RCT comparing 5-MTHF and FA supplementation in couples experiencing RPL. The trial was designed to evaluate the feasibility of conducting a larger-scale study, with a particular focus on recruitment, adherence, acceptability, and biochemical outcomes.

This chapter presents the results of the pilot trial, focusing on feasibility outcomes and preliminary biochemical data. By reporting the findings of Phase 1, this chapter contributes directly to the thesis's overarching aim: to assess whether personalised folate supplementation is both clinically relevant and practically implementable in the context of RPL care.

4.2 Publication

This manuscript was published in the Journal of Nutrition Research in February 2026. (Appendix 28)

Carolyn Ledowsky, Vanessa Scarf, Kris Rogers, Amie Steel,

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

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(https://www.sciencedirect.com/science/article/pii/S0271531725001587)

4.3 Introduction

An estimated 23 million miscarriages occur annually, accounting for approximately 15.3% of all pregnancies[8, 9]. Recurrent pregnancy loss (RPL), defined as two or more consecutive losses prior to 20-22 weeks gestation—affects a subset of these women, yet in more than 50% of cases, no identifiable cause can be found [465]. This idiopathic nature leads to significant emotional and financial stress[8], with many couples pursuing assisted reproductive technologies (ART) despite low success rates and substantial costs [20].

Folate supplementation is one of the most widely implemented strategies to reduce pregnancy loss [466]. Folic acid (FA), the synthetic and oxidised form of folate, is recommended globally to prevent neural tube defects (NTDs) [30, 31] and is mandated for food fortification in several countries, including Australia [467]. However, in countries with mandatory fortification, combined dietary intake and prenatal multivitamin use frequently result in FA intakes exceeding the established upper tolerable intake level, particularly among women of reproductive age [48]. Recent research has raised concerns about the accumulation of unmetabolised folic acid (UMFA), particularly in populations consuming both fortified foods and supplements[44-46]. Although the long-term clinical implications of UMFA are still under investigation, observational associations have been reported with cancer[87], infertility[196, 204], altered immune function [87, 196], and metabolic disturbances have been reported [44].

FA is first reduced by the enzyme *DHFR* to tetrahydrofolate and then converted by methylenetetrahydrofolate reductase [468] to the active form,

5-methyltetrahydrofolate (5-MTHF) [86, 87]. Genetic polymorphisms in *MTHFR* - particularly C677T and A1298C impair this latter step, reducing enzymatic activity and altering folate metabolism [77, 78, 113]. These variants are common, with C677T homozygosity present in up to 14% of Caucasians and heterozygosity in up to 25% of the general population [115]. Impaired folate metabolism due to *MTHFR* polymorphisms has been linked to infertility, miscarriage, and poor reproductive outcomes in both sexes [79-81, 83-85].

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 [89]. 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 unmetabolised folic acid accumulation [86, 89, 90, 93, 94, 469] . Clinical studies suggest that 5-MTHF may result in greater increases in plasma and red cell folate concentrations compared with FA, particularly in individuals with *MTHFR* C677T and A1298C variants [88]. However, findings are not consistent across all studies and the extent to which circulating folate indices reflect tissue folate availability or translate into clinical benefit remains uncertain. 5-MTHF has been shown to improve folate biomarkers and reduce homocysteine concentration (which may contribute to vascular dysfunction and pregnancy loss) [470, 471]. Despite this, few studies have evaluated its use in RPL populations.

In the context of recurrent pregnancy loss (RPL), these considerations are particularly relevant. RPL is a heterogeneous condition in which metabolic, vascular, epigenetic, and placental mechanisms have been implicated [157,

162], and disturbances in folate metabolism have been proposed as a potential contributing factor [442]. Women and couples affected by RPL are frequently advised to take higher-dose folate supplements in clinical practice [157], often for prolonged periods and without clear guidance regarding optimal folate form, genetic variability, or cumulative exposure [148, 235]. Despite this, there remains limited evidence assessing whether differences in folate formulation, dose, or metabolic handling translate into meaningful biochemical or clinical differences in RPL populations. This uncertainty highlights an important gap in the evidence base and underpins the rationale for examining folate form and exposure specifically within couples experiencing recurrent pregnancy loss.

Although numerous studies, including large clinical trials, have compared 5-methyltetrahydrofolate (5-MTHF) and folic acid across a range of populations [121, 472-474], the findings from these studies cannot be assumed to translate directly to RPL populations. Existing folate studies have largely examined biomarker responses in general or mixed populations and have not evaluated the feasibility, acceptability, or implementation challenges of delivering a controlled folate formulation in couples experiencing RPL, nor have they routinely incorporated partner-inclusive designs [163, 451]. Furthermore, the identification of biomarkers predictive of pregnancy loss requires large, longitudinal studies and is not achievable within small exploratory trials. Accordingly, a pilot feasibility study is a necessary and appropriate first step to determine whether a definitive randomised controlled trial comparing folate formulations in couples with RPL is practicable, acceptable, and methodologically sound [475].

In addition to metabolic differences, folate form and formulation

characteristics may influence biological exposure, particularly in the context of multi-nutrient supplements. Factors such as stability, excipient interactions, and storage conditions have been identified as potential considerations when evaluating folate interventions [476, 477], and may have implications for the interpretation of biochemical outcomes .

Preconception care has traditionally focused on women, despite evidence that male factors may contribute up to 70% of all infertility cases [110, 478]. Most RPL studies exclude male participants [176, 198, 479, 480] despite growing evidence that folate status and *MTHFR* polymorphisms also affect male fertility [182, 481]. 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 [482, 483]. Without this preliminary step, large trials risk failure due to unforeseen logistical or design challenges.

In reproductive health research, and especially in recurrent pregnancy loss (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 [484]. 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 [482, 483]. Reporting preliminary efficacy indicators, such as biomarker

trends, is acceptable only to inform whether a full trial is warranted, not to claim effectiveness [482], 2014].

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

4.4 Methods

4.4.1 Study Design and Setting

This study is registered with the Australian New Zealand Clinical Trials Registry (Trial id: ACTRN12622001577707, registered 20/12/2022). Utilising an exploratory framework, this was a double-blind, randomised 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 was developed to ensure the welfare of all participants.

The study Recruitment commenced March 2023 and concluded 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.

4.4.2 Ethics and Consent

The study was approved by the University of Technology Sydney Human Research Ethics Committee (ETH22-7218 16/5/22).

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.

4.4.3 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 organisations 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 multi-pronged 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.

4.4.4 Participants

Inclusion criteria:

- 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 and were interested in conceiving again.
- Females were aged between 20 and 49 years.
- Reproductive partners of eligible females (there were no age restrictions for male partners).
- Willingness of both partners to participate in a couple-based intervention and biomarker assessment

Exclusion criteria:

- Current pregnancy at enrolment
- Known chromosomal, anatomical or endocrine disorders
- Females currently lactating
- Females currently undergoing ART
- Any use of medications known to interfere with folate metabolism,
- or had a diagnosed medical or genetic condition likely to confound study outcomes.

4.4.5 Randomisation and Blinding

Following confirmation of eligibility and baseline data collection, couples were randomly assigned using computer generated block randomisation in a 1:1 ratio to either the intervention group (5-MTHF) or control group. The randomisation 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 pre-packaged 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 colour. 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 minimised performance and ascertainment bias.

4.4.5.1 Intervention

The investigational product was a bespoke prenatal multivitamin, manufactured under controlled conditions to allow strict standardisation of folate form, dose, capsule appearance, and excipients, thereby ensuring double blinding. The formulation was not commercially available.

Both intervention arms were identical in composition except for folate form. Iron was deliberately excluded, as the formulation was designed for use by both female and male participants, and routine iron supplementation is not

indicated for men.

4.4.5.1.1 Supplement Composition (total daily dose – three capsules)

Beta carotene	1500 mcg
Thiamine	44 mg
Biotin	300 mcg
Riboflavin 5 phosphate	25 mg
Nicotinamide	30 mg
Pantothenic acid	100 mg
Pyridoxal 5 Phosphate	15 mg
Methylcobalamin	500 mcg
5-MTHF	2,500 mcg
Iodine	200 mcg
Ascorbic acid	250 mg
Vitamin D	1000 IU
Vitamin E	200 IU
Calcium	100 mg
Magnesium	50 mg
Zinc	25 mg
Selenium	140 mcg
Manganese	5 mg
CoQ10	50 mg

Choline	200 mg
Inositol	25 mg
Chromium	40 mcg
Alpha Lipoic acid	30 mg

The excipients included leucine, ascorbyl palmitate, colloidal anhydrous silica, vegetable hard capsule (Hypromellose and purified water).

4.4.5.1.2 Dose Rationale

The total daily folate dose was 2,500 mcg, delivered across three capsules. This dose did not represent a recommended prenatal intake but was selected for methodological reasons in a pilot feasibility context.

Specifically, the dose was derived from:

1. Observed clinical prescribing practice, as documented in a prior case-series study examining folate dosing in individuals with *MTHFR* polymorphisms [447].
2. The need to ensure sufficient metabolic exposure to allow meaningful comparison of biochemical responses between folate forms within a small pilot sample.

While the established tolerable upper intake level (UL) for folic acid is 1,000 mcg/day, individuals with RPL are frequently prescribed higher doses in practice. The present study did not seek to endorse such dosing, but rather to evaluate feasibility and biochemical response under real-world conditions.

Each multivitamin contained 2,500 mcg of folate (in the three capsule dose) as either 5-MTHF or as folic acid. This dosage was derived from previous study data [447]. 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 folic acid-fortified foods (e.g., commercial breads and cereals) for the duration of the study. A food list was provided that included FA fortified and non-fortified 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 eight 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, that 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 minimising 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.

The product was manufactured in a GMP compliant facility by Factors Group in Canada.

4.4.5.2 Blood Biomarkers

All blood collection for the study was conducted via Nutripath Pty Ltd who supplied the collection forms and instructions. Each female participant was instructed to conduct a fasting blood collection at baseline and again following two reproductive cycles. The collection was conducted at Douglass

Hanly Moir pathology centres Australia wide, following the same instructions. Blood samples for total homocysteine (tHcy) analysis were collected under standardised conditions using EDTA tubes. Samples were processed promptly, with plasma separated within one hour of collection to minimise ex vivo homocysteine generation. Plasma tHcy was quantified using Abbott method, with results interpreted as a functional biomarker of folate-dependent one-carbon metabolism rather than as a predictive clinical outcome. This method involved immediate centrifugation at 4 degrees Celsius and plasma aliquoted promptly. This assay is routinely used in Australian clinical pathology laboratories and demonstrates strong agreement with chromatographic methods for clinical assessment. All folate biomarkers i.e.: serum folate and red cell folate were analysed using LCMS at Nutripath Pty Ltd with calibration and quality control procedures performed according to accredited laboratory standards. UMFA concentrations were quantified using LC-MS/MS.

4.5 Outcome Measures

4.5.1 **Primary Outcome**

The primary outcome of this study was feasibility, and as such outcomes were measured according to the recognised domains of feasibility studies [475, 485]. 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 non-participant 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 folic acid fortified foods and adherence to abstinence of trying to conceive for 2 reproductive cycles following the introduction of the multivitamin. Practicalities was 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 two months on the multivitamins, change in blood biomarkers and *MTHFR* polymorphism variance amongst the cohort.

4.6 Data Analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at 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 categorical variables) were applied for exploratory purposes only and should not be interpreted as evidence of efficacy. These analyses were intended to illustrate potential trends and variability rather than to draw statistical inferences. Given the pilot nature of the study and the sample size inferential findings are interpreted cautiously. Non-parametric tests such as Mann-Whitney U and Fisher's exact tests are considered more appropriate for 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 Arm 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 specialising in the stability assessment of raw materials and finished products.

4.7 Results

Participant progression through screening, eligibility assessment, randomisation, and follow-up is summarised in Figure 15. A total of 766 individuals responded to the initial screening questionnaire and were assessed for eligibility. Of these, 665 individuals were excluded prior to invitation to interview, most commonly due to not meeting recurrent pregnancy loss criteria, current pregnancy, use of assisted reproductive technologies, or inability to be contacted.

One hundred and one participants were eligible for interview, with 56 completing recruitment interviews. Twenty-three couples subsequently provided informed consent to participate in the trial, of whom 22 couples were randomised to receive either folic acid (n = 11 couples) or 5-methyltetrahydrofolate (n = 11 couples). One couple withdrew prior to allocation. One couple was lost to follow-up after commencing the intervention.

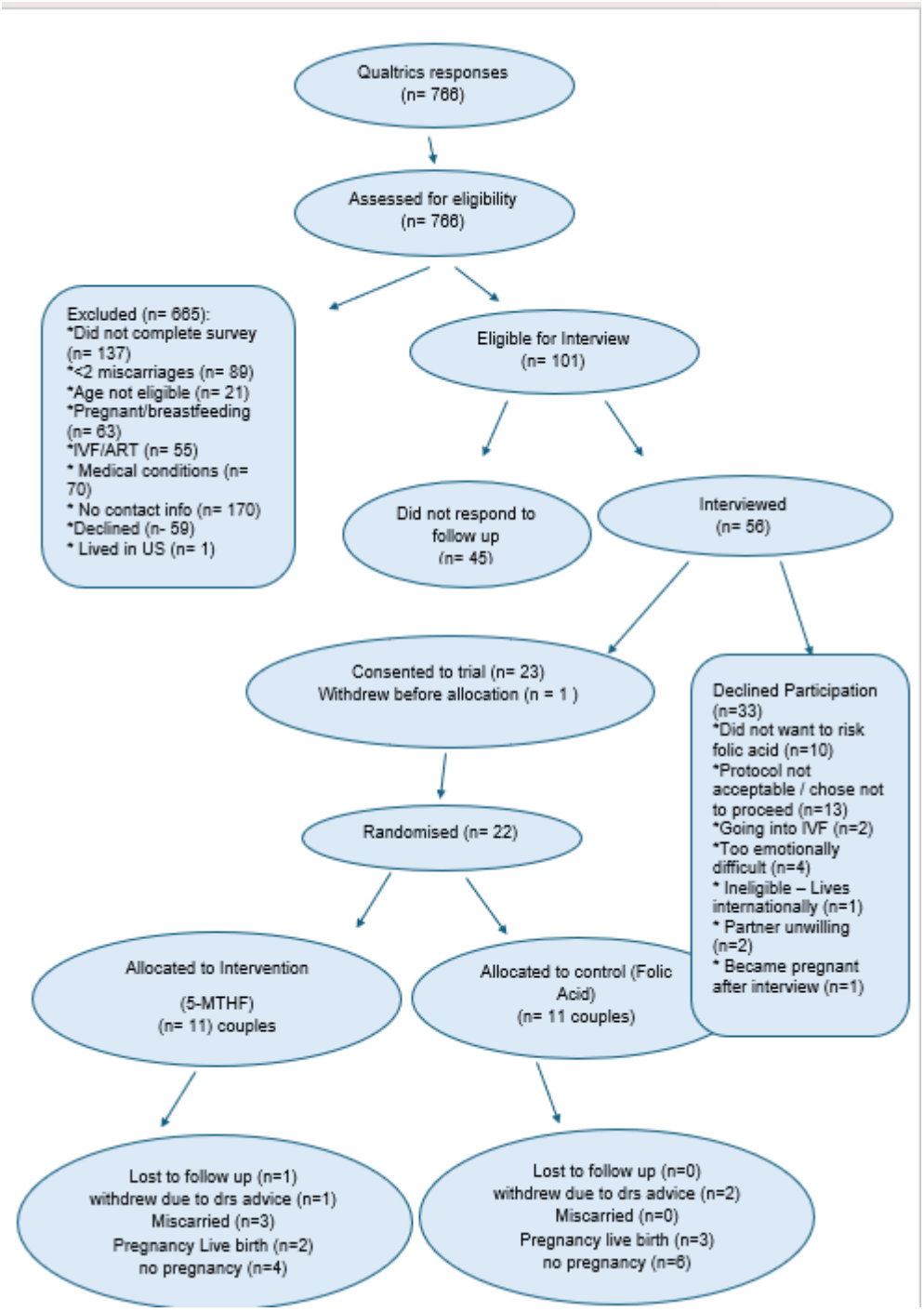

Figure 15- CONSORT ***flow diagram for a feasibility trial in couples with recurrent pregnancy loss.*** *The figure illustrates participant flow through the study: 766 individuals screened, 101 eligible, and 23 couples enrolled. Couples were randomized 1:1 to prenatal multivitamins containing either 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.*

IVF – In vitro fertilisation, ART – Assisted reproductive technology, 5-MTHF – 5-methyltetrahdyrofolate,

4.7.1 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.

4.7.1.1 Demographics

The mean age of female participants in MTHF-A was 37 years (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 14.

4.7.1.2 MTHFR Genotype Distribution

Table 14 summarises the distribution of *MTHFR* polymorphisms among male and female participants by study arm. These data are presented descriptively to characterise the sample and to inform feasibility considerations for future trials; the study was not powered to assess genotype-specific differences by sex or intervention.

Across both treatment arms, the observed distribution of *MTHFR* genotypes differed between male and female participants. A greater proportion of males were observed to carry homozygous *MTHFR* variant genotypes compared with females; however, no statistical comparisons were undertaken, and these findings should be interpreted as descriptive only. In the MTHF-A arm,

11.1% of male participants were homozygous for either the A1298C or C677T variant, while in the FA-B arm this proportion was 36.3% (9.1% for A1298C and 27.2% for C677T). In contrast, approximately 11% of female participants in each arm were homozygous for any *MTHFR* variant.

These genotype distributions highlight the heterogeneity of the study population and support the inclusion of both sexes in future, adequately powered studies designed to investigate potential genotype- and sex-specific effects.

Table 14- 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)	**FA-B** (n = 11)	**MTHF-A** (n = 11)	**FA-B** (n = 11)
	mean (SD)	***mean (SD)***	***n (%)***	***N (%)***
Age (years)	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 (15)
Missing	2	1	2	0
	***n* (%)**	***n (%)***	***n (%)***	***n (%)***
Education Level	n = 11(%)	n=10 (%)	n=7 (%)	n=8 (%)
Year 10	0 (0)	0 (0)	0 (0)	0 (0)
Year 12	0 (0)	0 (0)	1 (14)	2 (25)
Trade qualification	0 (0)	0 (0)	0 (0)	0 (0)
Diploma	5 (45)	2 (20)	0 (0)	5 (63)
Degree	3 (27)	8 (80)	6 (86)	0 (0)
PhD	3 (27)	0 (0)	0 (0)	1 (13)
Employment status	(n=11)(%)	(n=11)(%)	(n=10)(%)	(n=11)(%)

Characteristic	**Female participants**		**Male participants**	
	MTHF-A (n = 11)	**FA-B** (n = 11)	**MTHF-A** (n = 11)	**FA-B** (n = 11)
	mean (SD)	***mean (SD)***	***n (%)***	***N (%)***
Full time work (35 hrs per week)	3 (27)	6 (55)	9 (90)	11 (100)
Part time work (< 35 hrs 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 hours per week	8 (73)	6 (55)	5 (45)	5 (45)
5-10 hours per week	3 (27)	5 (45)	3 (30)	4 (36)
More than 10 hours per week	0 (0)	0 (0)	2 (20)	2 (18)
Missing			1	0
***MTHFR* genotype**	(n=9)	(n=11)	n=10	(n=11)

Characteristic	Female participants		Male participants	
	MTHF-A (n = 11)	**FA-B** (n = 11)	**MTHF-A** (n = 11)	**FA-B** (n = 11)
	mean (SD)	***mean (SD)***	***n (%)***	***N (%)***
MTHFR A1298C heterozygous	3 (33)	2 (18)	4 (40)	1 (9.1)
MTHFR A1298C Homozygous	1 (11)	0 (0)	1 (10)	1 (9.1)
MTHFR C677T Heterozygous	2 (22)	7 (64)	2 (20)	2 (18.2)
MTHFR C677T Homozygous	0 (0)	1 (9.1)	1 (10)	3 (27.2)
MTHFR Compound Heterozygous	3 (33)	1 (9.1)	0 (0)	2 (18.2)
Nil Polymorphisms	0 (0)	0 (0)	2 (20)	2 (18.2)

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.

4.7.1.3 Reproductive History of Female Participants

Table 15 summarises 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 characterise 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.

Table 15- Reproductive history of female participants in a feasibility trial of prenatal multivitamins (5-mthyltetrahydrofolate 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 (>=5mg) 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.

4.7.2 Acceptability

4.7.2.1 Recruitment

The CONSORT flow diagram (figure 15) outlines participant recruitment, screening, eligibility, randomisation, and follow-up throughout the pilot trial. 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 folic acid 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 recurrent pregnancy loss was too emotionally difficult to engage with. Other reasons included plans to begin 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 randomisation. Reasons for drop out include (Dr told her it was better to do IVF, due to hospitalisation 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).

4.7.3 Implementation and Adherence

4.7.3.1 Adherence Rates and Protocol Deviations

Adherence to supplementation and dietary restrictions was high across both arms, with descriptive adherence rates reported in Table 16. These findings indicate strong protocol compliance.

4.7.3.2 Acceptability of the Trial to Participants

Overall, the trial demonstrated a high level of acceptability among participants across both study arms (Table 16). 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 16. In this pilot study, acceptability was defined at the study-level as participants' willingness to enrol, consent, randomise, and initiate supplementation, rather than being determined solely by completion rates. Non-completion was therefore

interpreted as a feasibility and retention issue rather than an indicator of intervention-specific unacceptability.

4.7.4 Efficacy

4.7.4.1 Pregnancy Viability

There were two successful pregnancies in MTHF-A and 3 in FA-B. Three miscarriages were recorded in MTHF-A and none in FA-B. The remaining participants either did not conceive during the study follow-up period or withdrew from the trial prior to achieving pregnancy, as detailed in the CONSORT flow diagram (Figure 15). These pregnancy outcomes are reported descriptively only and should not be interpreted as evidence of efficacy.

4.7.4.2 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 versus MTHF-A (mean of 1.27 vs 0.64 per participant) mean difference -0.64 (95% CI: -1.5,0.27), p= 0.16.

Table 16 - Acceptability of Trial components completed by 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%, 2%	8 (73)	39%, 93%
Acceptability* (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± (missing 5/3)	91 (11)	79, 103	92 (11)	83, 102
Acceptability in withdrawers only± *(missing 8/9)*	76 (25)		100 (0)	
*Level of support in online meetings** (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 *** (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

Characteristic	**MTHF-A (**n=11)		**FA-B** (n=11)	
	n (%)	**95% CI**	***n (%)***	***95% CI***
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%

Characteristic	**MTHF-A (**n=11)		**FA-B** (n=11)	
	n (%)	**95% CI**	***n (%)***	***95% CI***
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 two weeks	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.

*Scale: 0 = not acceptable, 100 = very acceptable
** Scale: 1 = very supported, 5 = not at all
*** Scale: 1= easy, 5 = difficult
± response numbers may vary due to different stages of data collection or to missing data

Table 17- Blood biomarkers and other secondary outcomes at two months post randomisation for female participants

Biochemical indicators of folate and Vitamin B12 status	**MTHF-A**			**FA-B**				
	Baseline (n=9)	**Follow up* (n=7)±**	**Change from baseline**	**Baseline (n=11)**	**Follow up* (n=8)**	**Change from baseline**	**Between arm difference at follow up**	**95% CI**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Homocysteine umol/L	8.22 (2.47)	-	-	7.96 (2.51)	-	-	-	-
Tetrahydrofolate nmol/L	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
5-MTHF nmol/L F/U n=6 (A), n=8 (B)	30.8 (16.4)	79.3 (30.6)	+50.0 (33.0)	31.2 (14.6)	60.6 (17.4)	+28.9 (19.9)	19	-0.14,51
Holotranscobalamin pmol/L	95.8 (31.7)	81.0 (45.8)	-18.3 (62.0)	74.3 (27.3)	54.9 (46.0)	-31.0 (58.9)	26	-25,78
Unmetabolised folic acid (UMFA) nmol/L	0.61 (1.17)	0.39 (0.22)	-0.32 (1.55)	0.41 (0.20)	2.78 (5.27)	+2.37 (5.38)	-2.4	-6.8,2.0

Data are presented as mean ± SD for continuous variables. Change from baseline values represent follow-up minus baseline values, with positive values indicating an increase and negative values indicating a reduction.

Analyses were descriptive only and not powered for statistical significance. P values refer to between-group comparisons at follow-up only and were calculated using non-parametric tests.

Serum folate and red blood cell folate are reported separately elsewhere and are not included here to avoid redundancy.

Abbreviations: 5-MTHF, 5-methyltetrahydrofolate; FA, folic acid; UMFA, unmetabolised folic acid; SD, standard deviation.

Table 18- Baseline bloods by MTHFR polymorphism

Characteristic	**Overall** N = 20[1]	***MTHFR* C677T Homozygous** N = 1[1]	***MTHFR* A1298C Homozygous** N = 1[1]	***MTHFR* C677T Heterozygous** N = 9[1]	***MTHFR* A1298C Heterozygous** N = 5[1]	***MTHFR* Compound heterozygous** N = 4[1]
Red cell folate (nmol/L): baseline	1,135 (114)	1,199 (NA)	NA (NA)	1,116 (162)	NA (NA)	1,140 (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 (umol/L): baseline	8.08 (2.43)	10.00 (NA)	11.70 (NA)	7.34 (2.61)	8.26 (2.00)	8.13 (2.56)
[1] Mean (SD)						

Data are presented as mean (SD). Genotype-specific results are reported descriptively only. In this cohort, no female participants were homozygous wild type for the MTHFR C677T or A1298C polymorphisms; therefore, results for a homozygous normal reference group could not be reported. Genotype categories presented reflect the observed distribution of variants within the study population. Given the small subgroup sizes, no inferential statistical comparisons by genotype or over time were undertaken.

4.7.4.3 Biomarker trends

Blood biomarker changes from baseline to follow-up were evaluated for participants receiving either methyl folate (MTHF-A) or folic acid (FA-B) and biomarker changes from baseline are highlighted in Table 17. At baseline, 5-MTHF and folate concentrations were lower in those participants with the homozygosity in the *MTHFR* gene (Table 18) and homocysteine concentrations were higher. These comparisons are exploratory and not powered for statistical significance. 5-MTHF concentrations increased in both groups, with the observed mean change in MTHF-A was (mean increase 50.0 nmol/L, SD 33.0) compared to FA-B (mean increase 28.9 nmol/L, SD19.9). Holotranscobalamin (active vitamin B12) concentrations showed a downward trend in both groups, less pronounced in MTHF-A. The most significant patterns emerged in unmetabolised folic acid (UMFA) concentrations. UMFA concentrations decreased in the methyl folate group but rose markedly in the folic acid group, with some participants exceeding the 1-2 nmol/L threshold considered beyond physiological capacity for metabolism. These trends are descriptive and intended to inform hypotheses for future trials.

4.8 Discussion

This pilot study demonstrated that the trial design was feasible in terms of recruitment, randomisation, intervention delivery, and biomarker collection, and acceptable to a proportion of participants willing to engage in a couple-based, double-blind folate intervention [182, 185]. These findings align with prior research highlighting the importance of participant-centred approaches in reproductive health intervention design [182, 185]. Participant satisfaction was high across both study arms, with slightly greater acceptability reported in the folic acid group, and no significant adverse

effects were observed, supporting the established safety profiles of both folic acid and methyl folate in reproductive-aged populations [88, 90, 94, 121, 124, 336, 472, 486]. 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 [11, 12]. Blinding was successfully maintained, minimising performance and ascertainment bias [471] 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 [487, 488] . Retention was high, with only one participant fully lost to follow-up. Incomplete biomarker data at follow-up were observed among retained participants due to laboratory limitations, specifically the refusal by some Australian pathology services to perform red blood cell folate testing, rather than participant withdrawal or protocol non-adherence. Available biomarker data showed low variability, supporting internal consistency of the collected measurements, while highlighting laboratory access as an important feasibility consideration for future trials.

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 [11, 489, 490]. Studies suggest that a more personalised recruitment through clinical settings may better support ethical engagement and informed decision-making in this group [11, 12]. Overall, the study procedures were robust, and with minor refinements, particularly in recruitment and screening, future trials may be further optimised 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. Genotype distributions are reported descriptively only and no conclusions regarding internal validity or population prevalence can be drawn given the small sample size, A higher proportion of homozygous *MTHFR* variants was observed among male participants, while exploratory, this observation may raise considerations for the role of paternal folate metabolism in reproductive outcomes and supports the inclusion of male partners in future adequately powered studies. *MTHFR* variants, particularly C677T and A1298C, are known to impair folate metabolism, disrupt DNA methylation and elevate homocysteine concentrations [81, 300], factors linked to oxidative stress, sperm DNA fragmentation and impaired spermatogenesis [192]. The C677T homozygous genotype may reduce enzyme activity by up to 70% [81] and has been associated with reduced sperm quality and increased infertility risk, especially in Asian populations [491, 492] . Similarly, A1298C homozygosity has been linked to implantation failure and embryonic aneuploidy [81]. However, findings remain inconsistent across studies, with some reporting no significant associations, likely due to ethnic variability, dietary folate intake and methodological differences [81, 493]. 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 [494]. These observations suggest a rationale for exploring *MTHFR* genotyping and targeted folate strategies in future research, but confirmation through adequately powered randomised controlled trials is essential before any clinical recommendations can be made.

4.8.1 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 folic acid, suggesting a metabolically favourable profile for 5-MTHF. Unlike folic acid, 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 1-2 nmol/L threshold considered beyond physiological capacity for metabolism [70]. This aligns with previous findings that even low doses of folic acid (200–400 mcg/day) can result in UMFA accumulation due to limited *DHFR* activity [70, 71].

Additionally, participants with the *MTHFR* C677T homozygous genotype exhibited 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 potential 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.

4.8.2 Stability of 5-MTHF Within Complex Nutritional Products

Stability testing of the investigational products was not prespecified in the original trial protocol and was therefore not assessed during the conduct of the study. Following completion of the trial and unblinding of group allocation, post-hoc stability testing was initiated in response to unexpected biochemical variability, as part of a feasibility evaluation to inform future trial design. Independent analysis of retained product samples indicated degradation of both folate forms over time, with greater loss observed in the 5-methyltetrahydrofolate formulation. These findings are presented here as an implementation and formulation consideration, rather than as a primary trial outcome.

Independent analysis revealed that the actual 5-MTHF content per capsule had degraded from the intended 833 mcg (with three capsules being the intended dose of 2,500 mcg) to as low as 199 µg in retained samples. The observed degradation may plausibly relate to know stability challenges associated with 5-MTHF in multi nutrient formulations. Previous studies have demonstrated that methylated folates are sensitive heat, light, pH changes, and humidity [495], and that excipient selection, salt form and capsule microenvironment can influence degradation rates [476, 477]. Hygroscopic ingredients such as choline salts may further exacerbate degradation by attracting moisture within the capsule matrix [496]. 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. While these post-hoc findings limit interpretation of dose exposure in this pilot study, they provide important guidance for future trials. Specifically, future studies should

incorporate prespecified stability testing, moisture-controlled formulation strategies, and packaging that minimises environmental exposure throughout the intervention period. These considerations are essential to ensure accurate evaluation of nutrient interventions and to avoid misinterpretation of efficacy in future definitive trials.

4.8.3 Vitamin B12 Metabolism and Clinical Implications

This pilot study observed changes in vitamin B12 biomarkers over the intervention period, with decreases in holotranscobalamin (active B12) concentrations noted in both intervention arms. Given the small sample size and absence of statistically significant differences, these findings are reported descriptively only and should not be interpreted as indicative of differential effects between folate forms. While the decline in holotranscobalamin appeared numerically less pronounced in participants receiving 5-MTHF, the study was not powered to assess differences in vitamin B12 metabolism, however these observed trends may reflect differences in cobalamin handling between folic acid and 5-MTHF, as proposed in metabolic studies [44, 497]. The hypothesis that 5-MTHF may exert a protective effect on vitamin B12 metabolism warrants further investigation. While the folic acid 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 highlight the importance of monitoring vitamin B12 status in folate-based interventions and support further investigation in adequately powered trials, where prespecified hypotheses regarding folate-cobalamin interactions can be rigorously tested.

4.8.4 Strengths and Limitations

As a feasibility study, this pilot trial was designed to assess the practicality of the study design, recruitment processes, and trial procedures rather than to determine clinical effectiveness. While the study demonstrated several methodological strengths including a randomised, double-blind design, high participant retention, and the successful use of digital platforms for recruitment and data collection, the small sample size and short intervention period limit the generalisability of the findings. The study was not powered to detect statistically significant differences in clinical, biochemical, or genetic outcomes, and all analyses were therefore descriptive only. Results should be interpreted with caution, and no comparative or inferential conclusions can be drawn.

In addition, although male participants were intentionally included to acknowledge the emerging importance of male factors in reproductive health, the scope of male-specific analyses was limited. While no male participants were taking folic-acid–based supplements prior to trial commencement, comprehensive assessment of male folate status and broader pre-conception exposures was not undertaken. This constrains the ability to explore sex-specific or genotype-related differences and highlights an important gap in the current literature.

Finally, genetic data were reported descriptively and were not powered for statistical comparison between genotype groups or by sex. As such, genotype prevalence should not be interpreted as indicating relative risk or biological effect. These limitations primarily inform the refinement of study protocols and underscore the need for future, adequately powered trials that routinely include males and incorporate comprehensive assessment of folate

status, folic acid exposure, and relevant genetic variables when evaluating the clinical efficacy of folic acid versus 5-MTHF in reproductive health contexts.

4.9 Conclusion

This feasibility study demonstrated the practicality of conducting a randomised trial comparing 5-MTHF and folic acid in a reproductive-age population. Although limited by sample size, incomplete biomarker data and formulation instability, patterns in UMFA measurements were observed among participants receiving folic acid, 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 periods, and comprehensive biochemical profiling. While the findings support continued investigation into personalised folate supplementation strategies, particularly in populations with genetic variation affecting folate metabolism, no conclusions regarding genotype prevalence or sex-based differences or comparative clinical efficacy can be drawn from this study.

As a feasibility study, this work was not designed to determine clinical efficacy. Observed biomarker patterns are presented descriptively and warrant further investigation in larger, adequately powered, and well-controlled trials to evaluate the comparative effects of 5-MTHF and folic acid on reproductive and metabolic health, particularly in the context of emerging concerns regarding excess folic acid exposure.

4.10 Chapter Conclusion

This chapter presented the findings from a pilot randomised controlled trial (RCT) comparing 5-MTHF and folic acid supplementation in couples with RPL. The trial was designed to assess the feasibility of conducting such a study, with particular attention to recruitment, adherence, acceptability, and the collection of biochemical outcome measures.

The results demonstrated that the study procedures were feasible and that participants were willing to engage with the trial protocol, including the use of individualised supplementation within the constraints of the study design. By establishing feasibility and identifying key areas for further investigation, this chapter contributes directly to the overarching aim of the thesis.

Furthermore, it included male partners, demonstrating that men can successfully participate in preconception research, a critical but often overlooked aspect of reproductive health. The trial also showed that dietary FA restriction is achievable, supporting the feasibility of implementing food-based protocols in future large-scale studies.

The following chapter builds on these findings by examining the emotional and experiential dimensions of RPL care from the participants' perspectives, providing important contextual insight into the acceptability and perceived impact of current approaches to RPL care.

Chapter 5 Results – How Can Patient Narratives Help Reimagine Care for Those Who Feel Unheard and Unsupported in Recurrent Pregnancy Loss? A Secondary Qualitative Study.

5.1 Chapter Introduction

Phase 1A of the research involved a qualitative sub-study embedded within the pilot RCT presented in Chapter 4. While the RCT focused on clinical feasibility and biochemical outcomes, this phase was designed to explore the lived experiences of participants navigating RPL. This qualitative component extends the feasibility assessment by examining emotional, relational, and systemic factors that cannot be captured through quantitative measures alone.

While Chapter 4 focused on the feasibility, procedural outcomes, and biochemical measures of the pilot randomised controlled trial, it did not examine participants' subjective experiences or emotional responses to recurrent pregnancy loss. The qualitative component presented in this chapter (Phase 1A) was therefore designed to explore these perspectives directly, drawing on participant narratives collected during the trial process.

This chapter does not extend or reinterpret the quantitative findings of the RCT, rather, it provides an interpretive account of participants' lived experiences of RPL, trial participation, and care pathways. By situating these narratives within the broader literature on RPL care, this qualitative sub-study

aims to contextualise feasibility findings without presupposing or advancing results prior to their analysis.

5.2 Publication

This chapter has been submitted to the Women's Reproductive Health journal and is currently under review.

5.3 Introduction

Recurrent pregnancy loss (RPL), defined as the loss of two or more pregnancies, affects approximately 1–2% of women of reproductive age [498]. Despite its prevalence and profound emotional toll, RPL remains a complex and often misunderstood condition, both medically and socially. While clinical guidelines have evolved to reflect a more nuanced understanding of pregnancy loss, the lived experiences of those affected reveal persistent gaps in care, recognition, and support [498-500].

Historically, miscarriage and stillbirth have been under recognised within both clinical practice and the scientific literature, frequently framed as discrete biomedical events, rather than complex experiences with profound and enduring psychological, emotional, and social consequences [11, 499]. This cultural minimisation has contributed to a lack of public discourse, limited health literacy, and inconsistent clinical responses, particularly in early pregnancy loss [501]. Although recent years have seen a shift toward more empathetic and inclusive care, the implementation of best practices remains uneven across healthcare systems [502]. International research underscores the need for individualised, trauma-informed care that acknowledges the unique grief of each parent, regardless of gestational age [500]. Women and their partners consistently report feeling unprepared, unsupported, and excluded from decision-making processes [11, 503, 504]. Moreover,

partners, especially fathers, often experience disenfranchised grief, receiving little to no formal support despite their emotional involvement [198].

Clinical practice guidelines, such as those reviewed by Hennessy et al. (2021), offer a framework for managing RPL, yet their quality, consistency, and applicability vary widely [498]. Many lack stakeholder involvement, fail to address the psychosocial dimensions of loss, and are not routinely implemented in practice [162, 499, 505]. This gap between policy and lived experience highlights the need for research that centres the voices of those most affected. The predominant management strategies for RPL currently focus on identifying and treating underlying causes such as antiphospholipid syndrome, uterine anomalies, endocrine disorders, and autoimmune diseases [160, 161, 498]. However, many treatments remain controversial, and consensus is lacking across guidelines [498]. Additionally, while methylenetetrahydrofolate reductase (*MTHFR*) polymorphism testing is not currently recommended in standard guidelines due to limited research, the studies cited by Hennessy are now over a decade old [498]. The role of *MTHFR* continues to be explored in clinical and patient-led contexts [287, 447, 506].

This study contributes to a growing body of qualitative research by exploring the lived experiences of individuals affected by RPL. While not generalisable, the findings offer valuable insights that can inform future research and support the development of more compassionate and inclusive care practices.

5.4 Objectives

This study aimed to explore the experiences, needs, and perceptions of women and their partners who participated in a clinical RCT trial pilot study

comparing FA and 5-MTHF in the context of RPL. Specifically, the objectives were to examine participants' reproductive histories and care pathways, and to identify themes arising from their experiences of RPL care. This included exploration of participants' knowledge, beliefs and decision-making surrounding folate supplementation, including awareness, attitudes and perceptions of FA and 5-MTHF where applicable. The study further sought to examine how these lived experiences aligned with or diverged from existing clinical guidelines and global research. By situating the findings within the broader context of international research and policy, this study contributes to a growing body of evidence advocating for more compassionate, informed, and equitable care for families navigating the complexities of pregnancy loss.

5.5 Methods

5.5.1 Study Design

The qualitative component (Phase 1A) was designed as an unstructured interview sub-study embedded within the pilot RCT described in Chapter 4. Rather than employing a predesigned questionnaire, qualitative data were elicited through two open prompts administered at predefined points in the trial process. During the initial screening interview, participants were asked to describe their reproductive history to establish eligibility. At the conclusion of the trial, participants were invited to consent to further discussion or participation in future research. In response to these prompts, some participants voluntarily shared reflections on their experiences of recurrent pregnancy loss, trial participation, and care pathways.

This approach was intentionally exploratory and aligned with the feasibility objectives of the broader study, allowing participants to prioritise issues of

personal significance rather than responding to structured questions. The qualitative data therefore represent a supplementary, interpretive sub-analysis rather than a comprehensive qualitative evaluation, offering additional insights beyond the trial's primary feasibility outcomes [507]. It also incorporated elements of supra-analysis, a form of secondary analysis where the aims and focus of the secondary study extend beyond the scope of the original research [507] and draws on participants' unsolicited narratives to explore broader psychosocial dimensions of RPL and trial participation. Thematic analysis was used to analyse the interview data, following the six-phase framework outlined by Braun and Clarke [454]. The qualitative component was embedded within the pilot RCT, consistent with best practices for integrating interpretive qualitative approaches in clinical trials [508]. Not all trial participants contributed qualitative data; of the 22 women enrolled in the pilot trial, 11 consented to provide narrative information included in this analysis. The potential for selection bias is acknowledged, as participants with greater emotional burden or more complex reproductive histories may have been more inclined to share their experiences.

5.5.2 Participants and Recruitment

Participants in this study were individuals who had enquired and consented to be interviewed in the pilot phase of a clinical trial, and the interviews were conducted between March and October 2023. The sample included non-pregnant women of childbearing age between 20-49 years and their male reproductive partners, all of whom reported a history of two or more pregnancy losses. A total of 114 individuals engaged in the initial recruitment process, with 22 female participants and their male partners enrolling in the trial. Of these, eleven women consented to provide additional information

regarding their pregnancy loss experiences and were interviewed for this study. Initial recruitment for the trial was conducted via social media, and interviews were scheduled based on participant consent to be interviewed for trial eligibility screening. The sample included females residing in Australia with diverse reproductive histories, including those with recurrent miscarriages (two or more pregnancy losses), and differing levels of engagement with conventional and alternative healthcare systems. The study was distinctive in its inclusion of male reproductive partners, a group often underrepresented in research on RPL. For confidentiality, participants have been referred to using pseudonyms (Table 19). Table 19 presents characteristics of the eleven participants who consented to participate in the qualitative sub-study (Phase 1A) and agreed to the inclusion of their experiential and reproductive history data. Participants who did not consent to qualitative participation are not represented in this table, and no comparable data were collected from them. Accordingly, the information presented does not represent all participants enrolled in the pilot trial.

Table 19- Participant characteristics of the 11 participants that consented to participate in the sub-study.

Pseudonym	Age (Years)	Sex	Previous pregnancy losses
01	49	Female	>6
02	29	Female	5
03	38	Female	5
04	28	Female	2 ecto pic
05	31	Female	2
06	44	Female	2
07	36	Female	4
08	37	Female	5
09	31	Female	2
10	39	Female	2
11	32	Female	>6

5.5.3 Data Collection

Data for this qualitative sub-study were collected using an unstructured, multi-source approach consistent with the feasibility aims of the pilot RCT. Qualitative information was elicited through two open prompts administered at predefined points in the trial process: during initial eligibility screening, where participants were asked to describe their reproductive history, and at trial completion, where participants were invited to consent to further discussion or participation in future research.

While these interactions were not guided by a standardised interview schedule, they were intentionally open-ended to allow participants to raise issues of personal relevance. Qualitative data were derived from contemporaneous researcher notes taken during interviews,

participant-initiated and researcher-prompted written communications (e.g. emails), and structured follow-up records. Only information provided voluntarily by participants who consented to qualitative participation was included. Qualitative narratives included in this chapter were derived from participants who consented to qualitative participation during either the trial screening phase or following trial completion; this included some individuals who met initial eligibility criteria but did not ultimately enrol in the pilot trial but advised their consent for further research.

5.5.3.1 Researcher Notes

During the initial screening interviews, the researcher asked participants questions relevant to trial eligibility (e.g. history of two or more pregnancy losses). Although these interviews were designed to assess eligibility, many participants voluntarily shared personal information about their experiences of pregnancy loss. These conversations, while initiated for screening, often led to participants voluntarily sharing deeply personal experiences. The researcher took notes during the interview to accurately capture the participants' responses. All qualitative data were deidentified prior to analysis. Researcher notes and written communications were reviewed and coded using reflexive thematic analysis following the six-phase framework described by Braun and Clarke. Initial codes were generated inductively from the data and iteratively refined into candidate themes. Coding decisions were reviewed to ensure internal consistency and coherence across data sources.

5.5.3.2 Written Communication

In addition to verbal interviews, participants often engaged through written communication. This included email correspondence and responses to follow-up messages. Some of these communications were prompted by the

researcher (e.g. follow up questions), while others were participant initiated. In several cases, participants chose to elaborate on their experiences via email after the initial interview, to express their thoughts and emotions they had not shared verbally. These written exchanges provided rich, contextual insights and were treated as qualitative data alongside the interview notes.

5.5.3.3 Trial Records

Participants interactions were also documented in structured formats, including regular follow-up communications recorded in a centralised spreadsheet and monthly appointments logs recorded in REDCap trial data software [448]. This approach was chosen to accommodate the flexible and sensitive nature of participant engagement, particularly given the emotional context of recurrent pregnancy loss. These monthly appointments were primarily administrative; however, these records supported a deeper understanding of how participants interacted with the trial over time.

These materials provided rich, contextual insights and allowed for the inclusion of both direct quotations and reflective summaries. This approach aligns with Moore's (2007) emphasis on the value of recontextualising qualitative data and supports a more inclusive and participant-responsive methodology [459].

5.5.4 Ethical Considerations

The study was approved by the University of Technology Sydney Human Research Ethics Committee (ETH23-8644). Participants were informed of the study's purpose and their rights, including the voluntary nature of participation in the pilot study and the confidentiality of their responses. Once participants saw the social media posts and advertisements, they were directed to a consent form, outlining the trial information and asking their

consent to have an interview with the researchers to learn more about the trial. Upon receiving signed consent, the researcher contacted the potential participant and organised an online interview to answer any questions and outline further the specifics of the pilot study.

All participants were routinely invited to participate in interviews during the trial screening and consent process and were again invited at the conclusion of the pilot study to indicate whether they would consent to further discussion regarding their experiences with recurrent pregnancy loss. These interviews were conducted to explain trial procedures, confirm eligibility, and answer participant questions and were not guided by a structured interview schedule.

Although participants were systematically invited to interview, the sharing of experiential, emotional, or psychosocial information was voluntary and participant-initiated, arising in response to open prompts rather than directed questioning. Only information provided by participants who consented to qualitative participation was included in the analysis.

5.5.5 Data Analysis

Thematic analysis was conducted using an inductive approach, allowing themes to emerge from the data without imposing a pre-existing coding frame [454]. The analysis followed six key phases: Familiarisation where the researcher reviewed the dataset to gain an in-depth understanding of the content; searching for themes, where statements and responses were grouped into potential themes based on patterns of meaning; revision of themes which were discussed with Amie Steel and Vanessa Scarf, and refined to ensure internal coherence and distinctiveness; defining and naming of themes, where each theme was clearly defined and supported by representative quotes; and production of the report where the final themes

were integrated into a coherent narrative, supported by illustrative data extracts. The analysis was informed by Naeem et al.'s (2023) structured approach to thematic analysis [453]. Additionally, Moore's (2007) reflections on the reuse of qualitative data guided the integration of contextual notes and participant narratives into the thematic framework [459].

To visually explore the qualitative data from the participant responses, a word cloud was developed using NVivo 14. The dataset, compiled from coded quotes in an Excel spreadsheet, was imported into NVivo. A word frequency query was run, excluding common stop words. NVivo's built-in word cloud visualisation tool produced a word cloud in which the size of each word reflects its frequency across the dataset. This method was chosen to provide an overview of dominant themes and emotional language, supporting the identification of recurring concerns such as emotional distress, lack of support, and medical uncertainty.

5.5.6 Trustworthiness and Reflexivity

To enhance the trustworthiness of the findings, the researcher engaged in reflexive journaling throughout the analysis process, documenting analytic decisions, and emerging interpretations. Themes were reviewed iteratively to ensure they accurately reflected the data and were grounded in participants' lived experiences. The use of direct quotes and contextual summaries further supported the credibility of the findings.

The researcher's positionality as a practitioner who regularly works with women and couples experiencing RPL informed the approach to data collection and interpretation. This clinical background provided an understanding of the emotional and psychosocial dimensions of RPL, while also requiring a conscious effort to remain aware of how professional

experience might influence how they understood and interpreted what participants shared.

5.6 Results

Figure 16 represents a word cloud generated from the qualitative responses provided by participants in the study. The visual representation highlights the most frequently mentioned terms that related to their pregnancy loss experiences. The size of each word corresponds to its frequency and perceived importance within the dataset. Prominent words such as ‘miscarriages’, ‘miscarrying’, ‘support’, ‘feels’, ‘folic acid’ reflect the central themes that emerged from participant narratives. Other frequently mentioned words like ‘*MTHFR*’, ‘anxious’, ‘alone’, ‘isolated’ and ‘issues’ further highlight both the emotional struggle and the recurring themes of isolation, frustration, and the search for understanding and care. This figure visually summarises the key concerns and recurring topics raised by participants, offering insight into the emotional and systemic dimensions of their experience with pregnancy loss.

Figure 13 - Word cloud image of themes highlighted by participants

5.6.1 Themes Identified

Thematic analysis revealed four key themes reflecting the experiences, perceptions, and decision-making processes of participants who consented to participate in the qualitative sub-study (Phase 1A) regarding recurrent pregnancy loss and trial participation. These themes are outlined in Table 20.

As qualitative data were derived only from participants who voluntarily provided narrative information, the themes presented reflect the perspectives of this subgroup and are not intended to represent the experiences of all participants enrolled in the pilot trial.

5.6.1.1 Theme 1: Emotional Distress and Isolation

All participants described their experience as emotionally distressing and isolating. Many were exhausted by their grief, expressing feelings of being alone in their grief, with limited support from healthcare providers or social

networks. The first theme was emotional distress and isolation. One participant described a feeling of isolation after experiencing her fifth pregnancy loss and not being given any answers from her health practitioner, while a second said she felt like a 'pin bag' passed from doctor to doctor and test to test.

"I feel isolated and alone in my journey to have kids." – Participant 03 Interview notes following her 5th pregnancy loss with no answers.

"She and her husband have had 8 miscarriages. She just seems to miscarry all the time and it's so stressful. She feels like a 'pin bag' and wants it all to stop. She feels so sad and isolated" – Participant 11, Interview notes after more than 6 pregnancy losses being passed from Dr to Dr.

Table 20- Themes and subthemes identified from participant narratives

Themes	Subthemes
Emotional Distress and Isolation	- Exhaustion and grief - Feelings of loneliness - Anxiety around menstrual cycles
Lack of Medical Support or Understanding	- Dismissive clinical encounters - Absence of diagnostic clarity - Self education and unmet informational needs - Desire for control and informed choice - Concerns about folic acid allocation in the trial
Lack of *MTHFR* Acknowledgement	- - Perceived lack of clinician engagement and *MTHFR* test results

	-Participant reports of clinician uncertainty, dismissal or limited discussion regarding the relevance of *MTHFR* to RPL
Peer Support and Community Connection	- Online forums and shared experiences - Emotional validation through community - Validation from peers
Frustration with Systemic Healthcare Gaps	- Inappropriate clinical environments - Lack of continuity and coordinated care - Seeking personalised and integrative support

The participants reported that their experience of feeling alone was heightened by the fact that there was limited support for them when physically having the pregnancy loss and when trying to understand why it had happened. They described the pressure they felt to get pregnant each month which was heightened every time they experienced a pregnancy loss.

> *"She is very anxious and lonely and feels like she is alone in this". – Participant 02, researcher interview note following her fifth pregnancy loss dreading each menstrual cycle.*

The cumulative impact of repeated pregnancy losses led to emotional burnout and a sense of hopelessness, as shared by this participant who explained a feeling of reduced emotional resilience associated with her 6th pregnancy loss which was occurring at the time of study enrolment:

> *"My emotional resilience is somewhat overtaxed, especially as I'm currently miscarrying once again." – Participant 01, email correspondence*

5.6.1.2 Theme 2: Lack of Medical Support or Understanding

All participants reported frequent experiences of being dismissed or inadequately supported by medical professionals. One way in which this occurred was through dismissive clinical encounters, where many participants described receiving vague or dismissive responses when seeking guidance, and 'unhelpful' advice when they asked about 'prevention' or testing to stop a further pregnancy loss. For example, one participant described being characterised by their doctor as 'negative' because she asked for further investigations into the cause of repeated ectopic pregnancies:

> *After two ectopic pregnancies, she asked the Dr what she could do to prevent this happening again. She was told 'nothing', when she pushed further and said, 'surely there is some additional testing', She was told "you are very negative aren't you?". Participant 04 following her second ectopic pregnancy that resulted in the removal of one of her fallopian tubes, researcher interview note.*

This experience of being dismissed by medical doctors was reported consistently across all participants where they felt that the emotional toll of multiple pregnancy losses was not considered and often, they thought the doctors just did not care. This lack of consideration extended to inappropriate processes such as having women who were experiencing a miscarriage wait in an inpatient maternity area. For example, one participant described how she had joined a miscarriage trial at a major Sydney hospital and had been required to wait for her study follow-up appointments each week in a maternity ward.

> *"I was put into the maternity ward to wait for the appointments in the weight*

loss trial for miscarriages. It's just so distressing – did they not even think about what they were doing? It's so typical. People just don't care about women who are having miscarriages". Participant 08, following her fifth pregnancy loss. Researcher interview note.

Another participant described her miscarriage experience following her appointment in the Early Pregnancy Assessment Service (EPAS) unit of a major hospital, where she was placed in the maternity ward:

"I was put into the maternity ward to have my miscarriage. I had to wait there to have my ultrasound. It was total confusion. I was just put into the big category of the unknown. Just told to go home and try again" Participant 04, researcher interview note.

Another consistent sub theme within this theme was the absence of diagnostic clarity. There was a clear need for the participants to have a structured and thoughtful response when they asked their medical professional what they could do to prevent such an event happening again. This participant describes the lack of support in a small country town following her second pregnancy loss.

"Dr's come here infrequently, and they cannot give any reason as to why I'm miscarrying which is really frustrating". Participant 05, researcher interview note.

This participant recounted following her 6th pregnancy loss in 18 months.

"I have no idea why I keep miscarrying and neither do any of my Doctors", Participant 02, researcher interview note

5.6.1.3 Theme 3 – Lack of Awareness and Frustration Around MTHFR

Among participants who consented to the qualitative sub-study, a

recurring theme was perceived lack of awareness and frustration around *MTHFR* polymorphisms. Several participants described having undertaken their own research to understand possible contributors to recurrent pregnancy loss, including folate metabolism and *MTHFR* polymorphisms, and expressed a desire to discuss these findings with their healthcare providers. These participants reported frustration when they perceived limited clinical engagement, acknowledgement, or guidance regarding this topic. *She knows that she shouldn't take folic acid but none of the Drs seem to know anything about it. She's annoyed that she has had several miscarriages, and no one has pointed this out. "None of the Drs seem to know anything about it. Why?" Participant 10, following her second pregnancy loss. Researcher interview note.*

These encounters contributed to a broader sense of perceived medical disenfranchisement. One participant, interviewed during the trial screening phase but not enrolled in the pilot study, described feeling dismissed following multiple pregnancy losses and contrasted this with a subsequent live birth after independently changing folate supplementation from folic acid to 5-MTHF.

"I can't even tell you how many doctors have told me MTHFR doesn't cause issues while I sat there holding back the anger and tears knowing the truth. All of us with MTHFR have been completely gaslit by the medical community for years" Participant 07 following her fourth pregnancy loss. Email correspondence.

This participant describes her frustration that the doctors don't even know what it is.

"I don't understand why the Doctors don't know about MTHFR and methyl folate" Participant 01, researcher interview note.

The participants' perceptions about the impacts of *MTHFR* on RPL then created a barrier to trial participation as concerns about being allocated to the folic acid arm of the trial emerged as a significant barrier to participation. Participants expressed reluctance to risk receiving a supplement they believed could be harmful due to their genetic profile. There existed a broader desire to have control over treatment options, particularly the prescribed form of folate. This participant describes her reluctance to take the folic acid, given she had been taking it during her previous pregnancy losses.

"Given the clinical trial is randomised, the risk of being in the folic acid study weighs very heavy on us. The main anomaly we haven't tried is the removal of folic acid". Participant 01, email correspondence.

5.6.1.4 Theme 4: Frustration with Systemic Healthcare Gaps

Most participants described systemic failures in miscarriage care, including inappropriate clinical environments and lack of follow-up. Often the participants would turn to their general practitioner who would tell them to go to the hospital. The hospital would often tell them to go home until they were physically losing the baby because there was nothing they could do for them.

Repeatedly, participants explained that the system had failed them. These couples felt like they just don't fit into the current healthcare system. Following her fifth pregnancy loss, this participant describes her frustration with not getting the support she thought she should have:

"It's so typical. People just don't care about women who are having

miscarriages". Participant 08, researcher interview note and direct quote.

And another participant who lived in a small country town, describes the lack of support provided in rural areas:

"I'm in a small country town and find there is no support. Doctors come here so infrequently" Participant 05, researcher interview note and direct quote.

This participant described how she felt shortly after having her 5th pregnancy loss, she described feeling her distress at the lack of healthcare support generally:

"I've had no support or no help at all. It's so distressing" Participant 08, researcher interview note.

5.6.1.5 Theme 5: Peer Support and Community Connection

Often participants referenced online forums and peer networks as sources of emotional support and shared understanding. It was clear that when participants were not getting the support they needed from their medical practitioner, they would turn to online forums to speak to other couples that were going through the same experience and emotions. This participant describes their desperation to have a child and felt that the online forums were the only areas they could receive support because the health professionals were not providing it:

"We are desperate to have a child and just want support." – Participant 05 researcher interview note.

Participants felt it was important to speak to others who could share their experiences and understand what they were going through, when others around them could not. This participant described how important the forums

were to her to share her experiences with other women experiencing the same emotions as her:

> *"They met through the miscarriages they have both had on an online forum. The forum is where she can meet with like-minded people going through the same issues" – Participant 04 researcher interview note.*

Often, they reflected how good it was to speak to someone who not only understood the mental turmoil but could offer thoughtful advice and suggestions for prevention.

These connections provided validation and a sense of belonging often absent in clinical settings.

> *"She felt so isolated and alone in her journey to have kids that she reached out to a miscarriage forum where she could talk to meet other women just like her" Participant 04, researcher interview note.*

5.6.2 Limitations

This qualitative sub-study has several limitations. Data were collected through unstructured interviews and participant-initiated communications rather than a standardised questionnaire, which limits consistency across participants and introduces potential selection bias. Only participants who consented to qualitative participation and voluntarily shared their experiences were included; therefore, the findings may overrepresent individuals with greater emotional burden or more complex reproductive histories.

As not all trial participants contributed qualitative data, the findings are not intended to be representative of the full trial cohort. Instead, they provide exploratory, contextual insights into lived experiences of recurrent pregnancy loss and trial participation. These limitations are inherent to the

feasibility-focused and ethically constrained design of the sub-study and are acknowledged to ensure appropriate interpretation of the results.

5.7 Discussion

This exploratory study offers insight into the lived experiences of women and couples navigating RPL, highlighting patterns and perspectives that may inform future research and clinical reflection. It reveals a persistent gap between evolving clinical guidelines and the realities of care. While institutional practices have made strides toward more empathetic and inclusive care, my findings underscore that systemic, informational, and emotional support remains inconsistent, particularly in early pregnancy loss.

Participants consistently voiced a need for their grief to be acknowledged, regardless of gestational age, challenging the entrenched tendency within clinical practice to downplay the significance of early pregnancy loss and telling patients to simply ‘keep trying’, despite its profound emotional impact. This aligns with prior research indicating that the emotional impact of early pregnancy loss is often underestimated [11, 12, 489, 509]. The findings reinforce calls for trauma-informed and empathetic care models that validate grief and provide structured emotional support for both parents [510]. Studies have shown that women and their partners frequently report dissatisfaction with care around the time of miscarriage and healthcare providers often feel ill-equipped to offer emotional support [509, 511]. The lack of continuity of care and absence of structured follow up, reflect systemic gaps that leave families feeling unsupported, echoing concerns raised in earlier studies which advocate for individualised, empathetic, and inclusive care that addresses the needs of both parents [12, 503, 504]. A recent mixed methods study conducted in an Australian private maternity hospital found that

acknowledgement of loss was the most important element identified among the ten key elements of emotional care that contributed to high satisfaction among patients and partners experiencing pregnancy loss [512]. Although RPL is considered to be common, people experiencing it just want recognition that for them, it is a significant life event that deserves acknowledgement. These insights suggest that future care pathways should prioritise acknowledgement, continuity of care, clear communication, and structured follow-up to ensure that the emotional impact of pregnancy loss is not overlooked.

Despite the existence of clinical practice guidelines [498], this study reveals several systemic shortcomings. Firstly, a consistent and deeply troubling pattern of systemic failure in the care of individuals experiencing RPL. The Five Trimesters Codesign Report [513] identified widespread gaps in continuity, communication, and emotional support across all stages of maternity care, suggesting that the challenges faced by those with RPL may be amplified versions of the systemic shortcomings. Participants described being dismissed by clinicians, denied access to testing, and left without psychological support, experiences that compounded their grief and frustration. These narratives align closely with existing literature that emphasises the lack of national bereavement care guidelines and the minimisation of miscarriage in clinical settings [499], and found that less than half of individuals felt adequately supported by the medical community following miscarriage [514]. Further research highlights the fragmented nature of RPL care and advocates for a graded model of support that begins after the first loss, not the third [162, 515]. Others critique the global tendency to offer only superficial reassurance rather than comprehensive care [504].

These needs align with longitudinal findings, which emphasise that couples are “never pregnant in the same way again” after a loss and require tailored support throughout subsequent pregnancies [500]. Together, these studies corroborate the lived experiences captured in this research and highlight the urgent need for standardised, trauma-informed, and multidisciplinary care pathways for RPL. Our health professionals survey on RPL, highlighted that they too require more defined protocols and support for couples who experience RPL and suggest that dedicated appointments should be created to support couples [511], reinforcing the need for systemic reform across the maternity care continuum.

Participant feedback from this study highlights how pervasive the sense of unsupported care is in the management of RPL. Participants consistently described their experience as fragmented, lacking defined protocols, and emotionally traumatic. There was a sense that the system did not really care about them. Beyond the need for trauma-informed care and psychological support, there is a pressing requirement for defined protocols and standardised testing procedures in the management of RPL. This includes the integration of preconception care for men, which has been largely overlooked in current practice [479, 511]. Men’s reproductive health plays a critical role in pregnancy outcomes, and preconception assessments should include screening for genetic risks, lifestyle factors, and environmental exposures that may affect sperm quality and fertility [516]. Furthermore, emerging research highlights the importance of personalised approaches to folate supplementation and genetic screening. Studies demonstrate significant associations between methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms, particularly C677T and A1298C, and

increased risk of unexplained recurrent pregnancy loss and male infertility [287, 517-519]. These polymorphisms are linked to elevated homocysteine, which can impair reproductive outcomes. Despite widespread folic acid supplementation, individuals with *MTHFR* variants may require alternative forms such as 5-MTHF and tailored dosing strategies. Incorporating genetic testing and biomarker assessment into RPL care pathways would improve diagnostic accuracy and enable more effective, individualised treatment plans.

To better support couples and families experiencing RPL, several changes are necessary. First, standardised, trauma-informed care protocols should be implemented across all stages of pregnancy loss, including early miscarriage. Healthcare professionals must receive mandatory training in empathetic communication and grief sensitivity to ensure compassionate care. Routine psychological follow-up should be offered to both parents, with midwifery-led models providing a less stigmatising entry point for support. Finally, public health campaigns that aim to improve societal understanding of pregnancy loss and normalise conversations around this often-silenced experience would contribute greatly to the awareness of RPL and its impact on individuals and couples experiencing it. These findings mirror international critiques of RPL care, including the lack of standardised follow-up, inconsistent implementation of guidelines, and insufficient attention to psychosocial needs [498, 500].

While this study is based on a small sample, the consistency of participant experiences, particularly regarding emotional distress, lack of medical support, and systemic gaps, mirrors findings from international research and policy critiques. These recurring themes suggest that individual narratives,

even in limited qualitative studies, can offer valuable perspectives on broader systemic shortcomings in RPL care.

5.8 Conclusion

While clinical recognition of RPL has improved, this study demonstrates that participants continue to experience fragmented, uncertain, and inconsistent care, particularly in relation to preconception support and folate supplementation. Confusion regarding folate form, dose, and rationale for prescription was a recurrent feature of participant narratives, reflecting a disconnect between emerging biomedical evidence, clinical guidance, and lived experience.

These findings indicate that improving care for individuals affected by pregnancy loss requires not only compassionate communication and psychosocial support, but also clearer translation of evidence into practice, including transparent discussion of folate formulations and personalised supplementation strategies. By foregrounding participant perspectives within the context of a folate-based clinical trial, this chapter highlights the importance of aligning biomedical innovation with patient-centred implementation to improve both acceptability and clinical relevance.

5.9 Chapter Conclusion

This chapter presented findings from the qualitative sub-study embedded within a pilot randomised controlled trial comparing FA and 5-MTHF in the context of recurrent pregnancy loss. By examining participants' reproductive histories, care pathways, and understanding of folate supplementation, the chapter provides critical contextual insight into how personalised folate interventions are experienced in real-world reproductive care.

Key themes included confusion surrounding folate prescribing, limited

understanding of differences between folate forms, inconsistent communication from health-care providers, and a perceived lack of coordinated preconception support. These findings are directly relevant to the overarching aims of this thesis, as they illuminate implementation and acceptability considerations that cannot be captured through biochemical or clinical endpoints alone.

Importantly, these findings highlight that the clinical relevance of personalised folate supplementation in recurrent pregnancy loss depends not only on biological plausibility, but also on effective communication, practitioner knowledge, and patient trust. By integrating participant experience with clinical trial data, this chapter contributes to a more comprehensive evaluation of feasibility and informs the design of future folate-based trials and care models.

The following chapter builds on these insights by examining the perspectives of health professionals involved in RPL care, providing a complementary systems-level view of current practice and readiness for change in the implementation of personalised folate strategies.

Chapter 6 Results - Health Professionals Require More Defined Protocols, Better Funding and Patient Resources to Support Couples with Recurrent Pregnancy Loss (RPL)

6.1 Chapter Introduction

This chapter presents the findings from Phase 2 of the thesis: a national survey of health professionals who support couples experiencing RPL. Building on the clinical feasibility outcomes from the pilot RCT (Chapter 4) and the emotional and systemic insights from participant interviews (Chapter 5), this phase explores the implementation landscape, specifically, how folate supplementation is currently prescribed and what barriers and enablers exist for adopting more personalised approaches.

The decision to include a practitioner survey was informed by the broader aim of the thesis, to evaluate not only the clinical relevance of 5-MTHF supplementation, but also its acceptability and feasibility within real-world practice. The literature reviews in Chapter 2 highlighted widespread overexposure to FA and adverse health outcomes due to this excess.

These concerns were echoed across multiple phases of the thesis. In the pilot trial, UMFA concentrations were observed in participants receiving FA compared with those receiving 5-MTHF, while the qualitative interviews highlighted gaps in practitioner knowledge and inconsistent folate prescribing. These findings were further contextualised by the practitioner

survey, which demonstrated that the majority of clinicians reported routine prescribing of FA, with limited use of alternative folate forms, providing important insight into current folate prescribing patterns in routine clinical care.

This chapter investigates practitioner knowledge, attitudes, and behaviours related to RPL care including folate prescribing, *MTHFR* polymorphisms, and preconception care. It also explores the inclusion of male partners in treatment planning, the availability of clinical resources and protocols, and the types of evidence practitioners rely on to guide decision-making. These insights are essential for assessing the readiness of the health system to support personalised folate interventions and for identifying opportunities to improve clinical practice and policy.

By examining the perspectives of those delivering care, this chapter contributes to a more comprehensive understanding of feasibility and helps bridge the gap between research findings and clinical application. The final chapter integrates findings across all phases to identify key implications for practice, education, and policy reform.

6.2 Publication Details

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https://pubmed.ncbi.nlm.nih.gov/40775429/

6.3 Background

The rate of pregnancy loss (defined as the spontaneous loss of pregnancy before 24 weeks' gestation [520]) in Australia is similar to that of other countries with 10% to 19% of women experiencing a pregnancy loss. Recurrent pregnancy loss (RPL) is estimated to affect 1% of couples [158, 521], however it is now presumed to be closer to 5% [7]. RPL has traditionally been defined as three or more pregnancy losses in a row [522], however it is more commonly defined as two or more pregnancy losses prior to 24 weeks' gestation by the American Society for Reproductive Medicine (ASRM) [523] and the European Society for Human Reproduction and Embryology (ESHRE) [157], and in Australia it is defined as two or more pregnancy losses prior to 20 weeks' gestation [161]. The pooled risk of pregnancy loss has been found to be 15.3% of all recognised pregnancies, which equates to around 23 million pregnancy losses before 24 weeks' gestation each year [8], however, it is believed to be much higher if preclinical pregnancy losses are included [514]. These numbers are hard to pinpoint because pregnancy loss is not typically reported [8, 445, 499, 524]. According to the Australasian Recurrent Pregnancy Loss Clinical Management Guideline 2024, the evaluation and management of RPL may include assessment of several risk factors. These include: maternal age at conception, antiphospholipid syndrome (an acquired thrombophilia), environmental and lifestyle factors (such as chemical exposures and stress), male factor issues, uterine abnormalities, endocrine disorders (such as thyroid dysfunction, hyperprolactinemia, polycystic ovarian syndrome (PCOS), obesity, glucose intolerance and progesterone deficiency), autoimmune conditions and inflammatory causes (such as endometritis and adenomyosis). Additionally,

a significant proportion of cases are classified as “unexplained loss” with chromosomal aneuploidy being the most common cause) [158, 161].

Until recently, there were no Australian guidelines or protocols for health professionals (HPs) to support couples with RPL, and clinical practice was largely informed by international recommendations [174]. The earlier guidelines advised evaluation of chromosomal and anatomical anomalies, thrombophilia’s, endocrinological disorders, infection, immune system disorders and environmental and lifestyle factors [174]. This gap in the Australian landscape was addressed in June 2024 with the release of the Australasian Recurrent Pregnancy Loss Clinical Management Guideline 2021, Parts 1 and 2, developed by the Australian Certificate of Reproductive and Endocrinology and Infertility (CREI) consensus Expert panel [160, 161]. Part 1 focuses on chromosomal factors and anatomical factors of the uterus and/or cervix, endocrine risk factors including thyroid disorders and hyperprolactinemia, polycystic ovarian syndrome (PCOS), obesity, glucose intolerance, and progesterone deficiency [161]. Part 2 addresses acquired thrombophilia, autoimmune conditions, inflammatory and endometrial causes (endometritis), environmental and lifestyle factors (for example, environmental exposures, stress), male factor issues (such as sperm aneuploidy) and unexplained RPL [160]. Unexplained pregnancy loss (UPL) accounts for an estimated 50-75% of all RPL cases [7]. These new guidelines do not include any recommendations regarding multivitamins, FA or 5-MTHF dose or the form of folate, despite folic acid being considered one of the most important nutrients for women to take during the periconception period (encompassing three months before and three months after conception) [525].

Folate is required for DNA synthesis [521] and is regulated by the gene methylenetetrahydrofolate reductase (*MTHFR*) [526]. Research suggests a potential association between *MTHFR* gene polymorphisms and unexplained infertility [79, 113]. Several studies found increased frequencies of *MTHFR* variants in individuals with unexplained infertility compared to controls [79, 527, 528]. The *MTHFR* C677T polymorphism has been associated with unexplained infertility in women [527-529], and the A1298C polymorphism has been linked with male infertility [80, 113, 192, 530]. Some studies also observed connections between *MTHFR* mutations and RPL [531-534]. Studies involving men [178] and women with *MTHFR* polymorphisms have shown improved pregnancy outcomes when the sample population are supplemented with 5-methyltetrahydrofolate (methyl folate) rather than folic acid [535, 536]. A recent case series identified that women with infertility had better pregnancy outcomes when folic acid was replaced with methyl folate and the dose of 5-MTHF was increased beyond the recommended 500 mcg [447]. New evidence suggests that not everyone requires the same form and dose of folate, and a more nuanced approach may be appropriate [32, 86, 88-90, 92-94, 537].

Preconception health care is crucial for women and men who are planning to conceive. It involves a variety of recommendations and practices to ensure the best possible health outcomes for both the mother and the baby. Previous interviews with health professionals (HPs) in Australia indicate low awareness of preconception health care, missed opportunities and confusion about responsibility for preconception care [538]. Many health professionals have a role in supporting women and couples in preconception and pregnancy. General practitioners (GPs) are considered ‘the first line’ contact

and are well placed through their primary care role to deliver the majority of preconception care, yet a 2013 study of Australian GPs reported that GPs lack the necessary time, resources and training to effectively provide adequate preconception care [539], with a later study identifying that only 54% of GPs were aware of the preconception guidelines even though they consider it their responsibility to give preconception care advice [540]. The findings of these previous studies were supported by further research in which women who wanted to become pregnant reported a lack of information about preconception care, including the use of preconception multivitamins [540]. Approximately three quarters (74%) of women report asking preconception advice from their GP [541], yet research suggests that a lack of knowledge about safety of multivitamins and nutrients may stop the GP prescribing these multivitamins [542]. Women see their obstetrician or gynaecologist once they are pregnant unless they have RPL or infertility issues, in which case they may receive preconception advice from their obstetrician or from a multidisciplinary health professionals network [543]. Midwives also provide care to women, typically once they are pregnant, but they do not routinely offer preconception care [543] despite having interest in providing pre- and interconception care, claiming that low prioritisation in service planning is the key barrier to providing such care [544]. A recent Australian study found a high degree of variability in the health professionals' recommendations regarding preconception care, and evidence that health professionals lacked awareness of existing preconception practice guidelines [545]. The stated they require more patient information, more training and resources, more consultation time, and improved referral networks [545]. Fertility specialists face challenges in providing evidence-based

investigations and treatments due to a lack of high-quality clinical trials [546]. The complexity of RPL research has resulted in calls for improved methodologies and consideration of epidemiological factors [546, 547]. Therefore, it is important to understand the barriers and readiness of health professionals to adopt different interventions and explore the strategies they employ to support couples with RPL.

6.4 Methods

6.4.1 Aim of the Study

The aim of this study was to explore the practice behaviours, knowledge, and attitudes of health professionals who see women and their partners with RPL, including their prescribing practices of FA or 5-MTHF and testing recommendations for either partner. The study also investigated health professionals' interest in and requirements for change to existing recommendations should they be required.

6.4.2 Study Design

This study was a national cross-sectional survey which assessed Health professionals' recommendations, testing, prescribing, and referral practices when providing care to women with RPL and their reproductive partners. The survey questions were structured around the existing recommendations for health professionals by Australian [221, 548, 549] and international guidelines [550, 551]. The health professionals were asked about their recommendations to women and men in relation to preconception health using a scale of 'never', 'sometimes' or 'always'.

6.4.3 Data Collection and Participant Recruitment

Data were collected from 11th October 2023 until March 2024. The survey link was emailed to members of the following associations for distribution –

Royal Australian College of General Practitioners (RACGP), the Australian College of Midwives (ACM), the Fertility Society of Australia (FSA), and the Perinatal Society of Australia and New Zealand (PSANZ), and was administered online using Qualtrics software, Version XM October 2023 [552]. Health professionals were eligible to participate if they were able to read English and were an Australian-based health professional in current clinical practice and provided care to women and their reproductive partners with RPL. The survey consisted of thirty-three questions with adaptive questioning for sixteen of the questions. The time frame to complete the survey was approximately ten minutes. Respondents were able to review and change their answers (through a back button). A prize draw for a $200 book voucher was offered to participants should they wish to participate and if they opted in, they were directed to a second survey that collected their email address and name to ensure response anonymity was maintained.

6.4.4 Development and Testing

Pilot testing and consultation of the survey questions was conducted with association representatives from the RACGP, ACM and PSANZ.

6.5 Survey Design

The survey design applied the RE-AIM framework [463] to evaluate the potential for public health and population impact. The RE-AIM framework includes five dimensions: *reach*, *effectiveness*, *adoption, implementation,* and *maintenance* [463]. These dimensions assist researchers in programme planning, implementation, evaluation, and reporting for health research [464]. The framework was employed to understand the views of health professionals towards the use of multivitamins and the recommendations and testing procedures they follow for patients with RPL. The key outcomes were

to: determine who are considered to be the front-line professionals that treat and support patients with RPL; evaluate the issues that are meaningful to the practitioner, i.e.: do they feel they have the resources they need to assist women who experience RPL; and to understand that if changes were warranted, would they participate and implement the changes. If so, are there any barriers to implementing these changes?

The survey instrument included five key sections: practitioner characteristics; preconception health care protocols (prescribing and testing); folate prescribing (form, dosing and use); RPL protocols (prescribing and testing); and resources and guidelines for clinical management of RPL.

6.6 Ethics and Informed Consent

Ethics approval was received from the University of Technology Sydney Human Ethics Committee (Approval no. ETH23-8411). This study was conducted in full accordance with the ethical principles outlined in the Declaration of Helsinki, including its most recent amendments, which emphasise respect for individuals, the right to self-determination, and the importance of informed consent. All participants were provided with detailed information about the study's purpose, procedures, and their rights, and gave explicit informed consent before participating. No personally identifying data were collected during the survey, except in instances where participants voluntarily provided contact details for the sole purpose of entering a prize draw. Such information was stored separately and was not linked to survey responses in any way.

6.7 Data Analysis

Stata software version 18 (2023) [553] was used to complete the analysis. The data were cleaned and coded in Excel prior to input into Stata. Missing

responses were excluded from analysis, and frequencies and percentages for all categorical data were prepared.

6.8 Results

The survey was distributed nationally via professional associations and practitioner organisations with established Australia-wide membership networks. As survey dissemination was undertaken by these organisations, the research team was not provided with information on the total number of invitations distributed, and a precise response rate could not be calculated.

A total of 175 responses were received. Following data cleaning, eight respondents did not commence the survey, 19 responses were incomplete, and nine respondents did not meet the screening criteria, resulting in 139 valid responses included in the final analysis. Respondents were geographically distributed across multiple Australian states and territories, supporting the classification of this study as a national survey.

While the sample is not intended to be statistically representative of all practitioners, it provides insight into the perspectives of clinicians practicing across diverse geographic regions and clinical settings.

6.8.1 Characteristics of Survey Respondents

The characteristics of the respondents are outlined in Table 21. The health professionals that responded to the survey were from QLD (n=44; 32%), NSW/ACT (n=40; 29%), VIC (n=24; 17%), SA (n=12; 9%), WA (n=13; 9%), and TAS (n=2, 1%) (data not shown). Over half the respondents (55.4%) were aged between 40-59 years. The respondents were predominantly female (86.3%). GPs comprised the largest professional group (25.9%) followed by midwives (23.5%).

Table 21- Characteristics of participants

Participant Characteristics		All (n =139)	
		N	%
Age in years	20-39	40	28.8
	40-59	77	55.4
	60+	22	15.8
Gender	Male	15	10.8
	Female	120	86.3
	Non-binary / Prefer not to say	4	2.9
Years in clinical practice	Less than 5 years	9	6.5
	6-10 years	29	20.9
	11-15 years	30	21.6
	16-20 years	23	16.5
	More than 21 years	48	34.5
Profession	General Practitioner	44	25.9
	Gynaecologist	27	15.9
	Fertility or IVF specialist (reproductive, endocrinology & infertility)	23	13.5
	Obstetrician	27	15.9
	Midwife	40	23.5
	Other, please specify	9	5.3
Frequency of care	Less than once per year	13	9.35
	Several times per year	47	33.8
	Once a month	19	13.7
	Two or more times per month	29	20.9
	At least once per week	31	22.3

Frequency of care" refers to the frequency with which respondents reported providing fertility-related or recurrent pregnancy loss–related care within their clinical practice.

Gynaecologists and obstetricians each accounted for 15.9%, while fertility or IVF specialists comprised 13.5% of the respondents. The largest grouping of respondents had been practicing for more than 21 years (34.5%) and over half the respondents (56.8%) provided care for women with more than two pregnancy losses at least once a month. Only 22.3% of health professionals reported providing care for RPL (i.e., three consecutive pregnancy losses), yet over half of those reported providing care for RPL once a month or more (56.8%).

6.8.2 Preconception Health Care

6.8.2.1 Recommendations and Testing for Women in Preconception Health

Participants' practice behaviours for women with regard to preconception health are presented in Table 22. The majority of health professionals (91.4%) indicated that they 'always' recommend a prenatal multivitamin containing folate to their female patients. Less commonly, but still reported by more than half of respondents, was 'always' recommending patients achieve an ideal body mass index (BMI) (59%), and blood sugar or diabetes control (64.8%). Most health professionals also reported 'always' recommending their patients follow a healthy diet (80.6%), follow a regular exercise routine (72.7%), and avoid cigarettes, alcohol and recreational drugs (89.9%). They also commonly reported (90.7%) reviewing women's current health status and medications as part of their assessment, while almost two thirds (63.3%) indicated 'always' referring to a specialist when necessary. The practice most identified by participants as 'never' being recommended was 'achieving an ideal BMI' (7.9%).

Respondents reported 'always' screening for STIs and other infectious diseases (59%), HPV cervical screening (71.9%) and evaluation of genetic issues (67.6%). When asked if there were any other practices they might recommend that were not listed in the survey, 54% of health professionals reported they made no other recommendations, however 46% said they recommended other practices to their preconception patients such as relaxation, checking Rubella vaccinations, mental health evaluations and cycle tracking.

Table 22- Frequency that health professionals provide recommendations to women and men during preconception

Recommendation	Provide recommendations to women (n=139)							Provide recommendations to Men (n=69)						
	All respondents	Never		Sometimes		Always		All respondents	Never		Sometimes		Always	
	N	*n*	*%*	*n*	*%*	*n*	*%*	*N*	*n*	*%*	*n*	*%*	*n*	*%*
Prenatal multivitamin containing folate	139	2	1.4	10	7.19	127	91.4	21	12	57.1	5	23.8	4	19.0
Achieving an ideal BMI	139	11	7.9	46	33.1	82	59	21	3	14.3	11	52.4	7	33.3
Blood sugar/diabetes control	139	8	5.8	41	29.5	90	64.8	21	4	19.0	7	33.3	10	47.6
Following a healthy diet	139	3	2.2	24	17.3	112	80.6	21	1	4.8	5	23.8	15	71.4
A regular exercise routine	139	4	2.9	34	24.5	101	72.7	21	1	4.8	4	19.1	16	76.2
Evaluation of personal/family history of genetic issues	139	6	4.3	39	28.1	94	67.6	21	1	4.8	6	28.6	14	66.7
Avoidance of cigarettes, alcohol, and recreational drugs	139	5	3.6	9	6.5	125	89.9							
Reduction/elimination of cigarettes								21	1	4.8	3	14.3	17	81.0
Reduction/elimination of alcohol								20	2	10.0	7	35.0	11	55.0
Reduction/elimination of drugs								20	1	5.0	5	25.0	14	70.0
Review of current health status and medications	139	2	1.4	11	7.91	126	90.7	21	1	5.0	6	30.0	13	65.0
Referral/correspondence with specialist if required	139	6	4.3	45	32.4	88	63.3	21	0	0	10	47.6	11	52.4
Screening for STIs and other infectious diseases	139	5	3.6	52	37.4	82	59.0	21	2	9.5	10	47.6	9	42.9
Cervical screening	139	4	2.9	35	25.2	100	71.9							

6.8.2.2 Recommendations and Testing for Men in Preconception

When asked about consulting with the male reproductive partner for a dedicated preconception visit (Table 22), 41% of health professionals reported that they do not include the male partner in a dedicated preconception visit, while only 11% reported they consistently do so. Nineteen percent of respondents reported 'always' prescribing a multivitamin containing folate to the male partner, while 57% of health professionals said they 'never' recommended one.

Achieving an 'Ideal BMI' for men is recommended 'sometimes' (52.4%), while 33.3% of health professionals stated they 'always' recommend an 'ideal BMI'. Blood sugar management was reported as 'always' prioritised by 47.6% of respondents, with an additional 33.3% said they 'sometimes' prioritise blood sugar management. So too, a healthy diet was stated as being strongly recommended, with 71.4% stating they 'always' recommend a healthy diet as well as a regular exercise routine (76.1%). Assessment for genetic issues was reported to be 'always' recommended by 66.7% of respondents. The recommendation to avoid cigarettes (80.9%), reduce alcohol (55.0%) and avoidance of drugs (70.0%) was not reported to be recommended as much for men as women, nor was the screening for STIs and other infectious diseases (42.9% for men versus 59.0% for women) (Table 22).

6.8.3 The Use of Multivitamins and Form of Folate Used

Most respondents (68.2%) reported recommending multivitamins only for women (Table 23), while 31.8% reported they suggest multivitamins for both partners. For those health professionals that do recommend a multivitamin, 59.9% reported they commenced the multivitamin three to four months before

attempting to conceive, while 12.1% recommended starting two months before, and 14.4% one month prior. A small percentage (5.30%) reported that they advise commencing the multivitamin when the woman is actively trying to conceive, with 8.3% reporting they provide other specific timings such as on cessation of contraception, once pregnant, or at their first consultation.

Table 23 - Prescribing behaviours regarding folate and multivitamin use for men and women experiencing fertility issues

			Participants (n=132)	%
Do you see male partners for a dedicated preconception visit?				
Yes (n=11%)	**No (n=41%)**	**Sometimes (n=48%)**	132	100
Patient group prescribed prenatal multivitamin				
Only to women			90	68.2
To both men and women			42	31.8
Stage for commencement of multivitamin				
Three to four months before trying to conceive			79	59.9
Two months before trying to conceive			16	12.1
One month before trying to conceive			19	14.4
When trying to conceive			7	5.3
Other, please specify			11	8.33
Length of time to take multivitamin				
For first trimester			37	18.9
Second trimester			3	1.5
Third trimester			4	2.0
Throughout the whole pregnancy			89	45.4
Breastfeeding			50	25.5
Other, please specify			13	6.6
Multivitamin products commonly prescribed				
Bayer Elevit			61	31.9
Bioceuticals In Natal			14	7.3
Blackmores Pregnancy & Breastfeeding Gold			48	25.1
Blackmores I-Folic			23	12.0
Patients Own Choice			16	8.4
Swisse Prenatal			2	1.0
Eagle Tresos B			2	1.0
Other			25	13.1
Form of folate in the multivitamin				
Folic acid			115	80.9

Folinic acid	13	9.2
Methyl folate	11	7.7
Don't know	3	2.1
Change of folate prescription in case of RPL		
No change	69	67.6
Increase the dose of folate	18	17.6
Reduce the dose of folate	1	1.0
Change the folate type	3	2.9
Stop the use of a folate supplement	1	1.0
Other, please specify	10	9.8
Awareness of the practice of prescribing 5mg of FA for women with RPL		
No	46	46.9
Yes	35	35.7
Not sure	17	17.4
Frequency prescribing 5mg of folic acid to a woman with RPL		
Never	3	8.6
Sometimes	12	34.3
About half the time	1	2.9
Most of the time	8	22.9
Always	11	31.4

For the duration of multivitamin intake, 45.4% of health professionals stated they recommend use throughout the entire pregnancy, and 18.9% recommend taking the multivitamin for the first trimester only. Additionally, 25.5% of health professionals responded they suggest continued use of multivitamins during breastfeeding, with few specifically advising the second (1.5%) or third (2.0%) trimesters. Bayer Elevit® was reported as the most commonly prescribed prenatal multivitamin (31.9%), followed by Blackmores Pregnancy & Breastfeeding Gold (25.1%), Blackmores iFolic (12.0%) and Bioceuticals In Natal (7.3%). The form of folate reported being prescribed most often was folic acid (80.9%).

Sixty-eight percent of health professionals reported not changing the folate

prescription when a woman experienced RPL, and 17.6% reported they increased the dose of folate if their patient had a pregnancy loss. A small percentage reported reducing the dose (1.0%) or changing the form of folate (2.9%) following RPL. Some respondents also reported recommending stopping the use of folate supplements (1.0%) or checking for *MTHFR* polymorphisms (<1.5%) and homocysteine.

6.9 Recurrent Pregnancy Loss Testing and Screening

health professionals were asked how frequently they undertake certain investigations when providing care to a woman with RPL (Table 24). The tests and screening reported most commonly by participants as 'always' requested for RPL were thyroid function testing (82.4%), antibody testing (76.9%), routine antenatal screening (70.3%), glucose metabolism testing (65.2%), Polycystic Ovarian Syndrome testing (PCOS) (52.4%), acquired thrombophilia (such as Antiphospholipid syndrome, anticardiolipin, lupus antigen) (53.9%), congenital thrombophilia (such as FVL, prothrombin, anti-thrombin, protein C/S) (32.5%), and bacterial vaginosis (25.6%). Referral to another practitioner was recommended by 73.0% of respondents. The tests and screenings most commonly reported as 'never' being requested by respondents were natural killer cell activity (85.1%), paternal sperm DNA fragmentation index (67.8%), and investigation of paternal BMI (47.13%). For the male partner, health professionals responded that the screenings they focused on were lifestyle practices such as smoking (53.9%), illicit substance use (47.9%), and alcohol intake (45.3%).

Table 24 - Investigations and screenings for women and their partners following RPL

	never		sometimes		always	
Investigations for women with RPL	*n*	*%*	*n*	*%*	*n*	*%*
Cytogenic analysis of products of conception	31	36.9	17	20.2	36	42.9
Peripheral maternal blood karyotyping	26	32.5	17	21.3	37	46.3
Peripheral paternal blood karyotyping	34	42	13	16.1	34	42.0
Leiomyoma	38	43.7	13	15.0	36	41.4
Mullerian anomalies	37	44.1	7	8.3	40	47.6
Uterine synechiae (Asherman's)	35	43.2	17	21	29	35.8
Thyroid function	7	8.2	8	9.4	70	82.4
Glucose metabolism	12	13.5	19	21.4	58	65.2
PCOS	11	13.1	29	34.5	44	52.4
Progesterone levels	23	29.9	23	29.9	31	40.3
Prolactin levels	26	30.2	25	29.1	35	40.7
HLA typing	64	82.1	11	14.1	3	3.9
NK cell activity	74	85.1	12	13.8	1	1.2
Paternal sperm DNA fragmentation index	59	67.8	20	23.0	8	9.2
Investigate paternal BMI	41	47.1	22	25.3	23	26.4
No tests - Refer to another practitioner	27	27.0	42	42.0	31	31.0
Screening tests for women with RPL						
Congenital thrombophilia	34	29.1	45	38.5	38	32.5
Acquired thrombophilia	19	16.2	35	30.0	63	53.9
Bacterial vaginosis	27	23.1	60	51.3	30	25.6

Chlamydia	12	10.3	51	43.6	54	46.2
Paternal smoking	23	19.7	31	26.5	63	53.9
Paternal alcohol intake	26	22.2	38	32.5	53	45.3
Paternal illicit substance use	27	23.1	34	29.1	56	47.9
Further tests for either males or females following RPL						
Male Karyotype	14	21.5	11	16.9	40	61.5
Female Karyotype	13	20.0	10	15.4	42	64.6
Ultrasonography	8	12.3	8	12.3	49	75.4
Sono hysterography	11	25.0	21	47.7	12	27.3
Thyroid function tests & Antibodies	6	9.2	9	13.6	50	76.9
Acquired thrombophilia screen	5	7.7	14	21.5	46	70.8
Antenatal screen	6	9.4	13	20.3	45	70.3
Products of conception: Karyotype & histopathology	10	15.9	15	23.8	38	60.3
Blood tests	7	11.1	7	11.1	49	77.8
Sperm testing	21	32.8	16	25.0	27	42.2
Blood testing	10	17.2	12	20.7	36	62.1
None, I refer	22	42.3	16	30.8	14	26.9
Referral Strategies for those that refer						
Refer to relevant support group	4	5.0	49	61.3	27	33.8
Refer to a psychologist/counsellor for further support	0	0.0	50	62.5	30	37.5
Speak to them myself to understand how they are feeling and lend support	0	0.0	18	22.5	62	77.5
Refer to another professional	5	6.3	61	76.2	14	17.5

6.9.1 Support for Patients with RPL and Referral Practices

In the survey, respondents were asked about their practices for managing RPL and the strategies they might employ to support patients (Table 25). The majority of health professionals reported that they would primarily review diet and lifestyle strategies like smoking and alcohol intake (82.5%), and caffeine intake (39.5%), ensure patients maintain a healthy BMI (75.4%), conduct further testing and investigations (77.2%), and prescribe prenatal multivitamins (70.2%). Psychological support was reported as being an important consideration with 82.5% of health professionals 'always' referring for emotional support. The health professionals reported that they predominantly speak to the patient themselves to understand how they are feeling and lend support where they can (77.5%) 'always' or 'sometimes' (22.5%). Some health professionals referred patients to a psychologist or counsellor for further support, 'sometimes' (62.5%) or 'always' (37.5%). The majority of health professionals 'sometimes' (76.5%) refer patients to other professionals, and some 'always' (17.5%) refer. Only 6.25% 'never' refer patients to other professionals (Table 25). Fifty-seven percent recommended continuing attempts to conceive (57%).

6.9.1 Resourcing

Health professionals were asked if they feel they have access to the necessary resources (Table 25) to support couples with RPL and 58.1% felt they either definitely or probably have access to sufficient resources. However, 34.4% felt they 'definitely' did not or 'probably' did not have the resources to support patients with RPL and 7.5% were unsure. Overwhelmingly health professionals identified meta-analysis of primary

research as considered extremely important (Table 26) (82.8%) or moderately important (10.8%) evidence for them to change their practice behaviours, with only (6.45%) rating it as not at all important.

Table 25 - Strategies used and resources required by health professionals to support couples with recurrent pregnancy loss (RPL)

	Participants (n=139)	
	n	%
Strategies employed by health professionals if patients experience RPL*		
Refer to another specialist/centre	74	64.9
Prescribe a prenatal multivitamin	80	70.2
Advise them to continue attempting to conceive	65	57.0
Further investigations & testing for either reproductive partner	88	77.2
Recommend medications, please specify	27	23.7
Review diet and lifestyle strategies (i.e., smoking and alcohol)	97	85.1
Ensure they maintain a healthy body mass index	86	75.4
Restrict caffeine intake	45	39.5
Referral for emotional support	94	82.5
Resources required by health professionals to best support individuals with RPL*		
Guidelines & protocols to follow	89	95.7
General Patient Resources	75	87.2
Handouts/information for men and women	71	76.3
Dosing charts and information for HPs	54	62.8
Referral information for support groups	52	55.9
Improved funding to ensure specialised preconception appointments	48	51.6
Recommendation of products and where they can be purchased	42	48.8
More specialised training in this area	44	47.3
Better referral networks	42	45.2
Pricing of the recommended products	36	41.9
Improved funding to ensure a timely appointment with a specialist	39	41.9
More access to midwives for women with RPL	33	35.5
Other, please specify	2	2.2

*Participants could select more than one response

Randomised clinical trials were the second most important research study type with respondents rating them as extremely important (77.4%), or moderately important (16.1%), with only 6.45% deeming them not at all

important. Observational studies were considered by participants to be moderately important (64.5%), not at all important (21.5%), and extremely important (14.0%), while case reports were rated moderately important (43.0%), not at all important (41.9%), and extremely important (15.1%).

Table 26 - Health professionals' perceptions regarding access, importance and influence of resources and research evidence for management of RPL

Perceived level of access to resources and research evidence required to amend protocols for management of RPL	**Definitely not *n* (%)**	**Probably not *n* (%)**	**Might or might not *n* (%)**	**Probably yes *n* (%)**	**Definitely yes *n* (%)**
	11 (11.8)	21 (22.6)	7 (7.5)	36 (38.7)	18 (19.4)
Types of research required by health professionals for them	**Not at all important *n* (%)**		**Moderately important *n* (%)**		**Extremely important *n* (%)**
Case reports	39 (41.9%)		40 (43.0%)		14 (15.1%)
Observational study	20 (21.5%)		60 (64.5%)		13 (14.0%)
Randomised control trial	6 (6.5%)		15 (16.1%)		72 (77.4%)
Meta-analysis of primary research	6 (6.5%)		10 (10.8%)		77 (82.8%)
Other	47 (50.5%)		30 (32.3%)		16 (17.2%)
Influential sources for RPL Management	**None at all *n* (%)**		**A moderate amount *n* (%)**		**A great deal *n* (%)**
International clinical guidelines	9 (9.8%)		38 (41.3%)		45 (48.9%)
National clinical guidelines	2 (2.3%)		25 (28.4%)		61 (69.3%)
Recommendation from a peer	15 (16.3%)		62 (67.4%)		15 (16.3%)
Feedback about response to treatment from a patient	21 (22.8%)		49 (53.3%)		22 (23.9%)
New research evidence	4 (4.4%)		43 (47.3%)		44 (48.4%)

Your own clinical observations	11 (12.0%)	59 (64.1%)	22 (23.9%)

When asked what resources health professionals would require to implement a change in recommendations to their patients, should the appropriate research be provided, the establishment of guidelines and protocols to follow was the most commonly chosen response (95.7%). Handouts and information for both men and women were reported as being crucial, with 87.2% of respondents advocating for patient educational resources for both men and women. Health professionals said they would like dosing charts and information on products like multivitamins (62.8%), what they cost (41.9%), and where they can be purchased (48.8%). Other recommendations health professionals reported as necessary included the need for more specialised training (47.3%), improved funding to ensure timely and specialised preconception appointments (51.6%) and specialised preconception appointments with a specialist (41.9%), and better referral networks (45.2%). Additionally, respondents expressed the need for increased access to midwives for the women with RPL (35.5%) and also mentioned improved referral information for support groups (55.9%). One practitioner commented that “research is vital” in this area and another stated that “this is a confronting issue for both health professionals and patients, and definitely more resources are required”.

For the clinical management of patients with RPL, the most influential source for decision making (Table 26) and management of these patients were national clinical guidelines with most reporting it had a great deal of influence (69.3%) and a moderate amount of influence (28.4%). International

guidelines (48.91%), and new research evidence (48.35%), were also reported as important with clinical observations (23.9%), feedback from a patient (23.9%), and recommendations from a peer (16.3%) less so.

6.10 Discussion

This survey of health professionals' practice behaviours in the management of RPL identified findings that have broad implications for reproductive health care as well as specific insights for RPL management. Firstly, males are often being excluded from preconception health care even though they provide 50% of the DNA to the embryo. Secondly, couples experiencing RPL require support, as do the health professionals supporting them. There is a need for improved and updated recommendations and protocols that include folate prescribing and dose, improved funding and training for health professionals, and better resources for couples experiencing RPL. Thirdly, folate is an integral part of preconception care, not only to prevent neural tube defects but to support DNA methylation critical for oogenesis and spermatogenesis. Recommendations for RPL lack folate and genetic testing for genes that affect folate metabolism, possibly due to a position held by leading bodies that *MTHFR* is unimportant in fertility [554].

This study highlights that health professionals require more defined protocols for RPL. They feel that they require further education, more funding, and more patient resources to help them better support couples experiencing RPL. Although the health professionals are following the typical testing procedures as defined by the Australian RPL recommendations [174], they do require more detailed clinical guidelines and protocols to follow, which is consistent with a variety of reviews that have evaluated clinical practice guidelines and found them conflicting, lacking in good quality evidence and

the involvement of those who experience RPL [162, 498, 555, 556]. A lack of resources for both patients [500, 501] and health professionals constrains how RPL is defined and treated [505, 557]. The current guidelines for RPL are insufficient and there is a call for more evidenced-based studies [8, 162, 504, 515]. Research highlights that over 75% of pregnancy losses are deemed 'unexplained' [7, 555], essentially meaning that no cause can be found. How can clinical guidelines be effective if they do not explore the possible cause of up to 75% of pregnancy losses? Reasons for these idiopathic RPLs may be associated with HLA immune factors [558, 559], thrombophilia [560] , endocrine and chromosomal abnormalities in greater than 50% of cases [559, 561, 562]. Epigenetics plays an important role in RPL through the regulation of key genes influencing DNA methylation and epigenetic modifications [562]. The causes of RPL therefore, such as chromosomal disorders, immune and endocrine dysfunction may well be related to disturbed methylation because of its critical role in maintaining the epigenetic modifications that affect these processes during spermatogenesis and embryonic development [111, 172, 563-567]. Studies show that DNA methylation is critically important for the epigenetic patterns and expression of genes that affect spermatogenesis and male reproductive organ development, as it is for women and oogenesis [110, 111, 171, 172, 563, 566, 568, 569]. *MTHFR* genes that affect methylation contribute significantly to spermatogenesis, or rather lack thereof [32, 34, 80, 114, 570-576], embryo implantation, growth and development, and abnormal immune balance [562], and it may be time to investigate this further [563, 567, 577]. This study highlighted the lack of testing for *MTHFR* polymorphisms. Numerous reviews highlight the importance of this gene in folate metabolism and fertility,

particularly in males [78-82, 84, 92, 111, 113, 114, 201]. The lack of association between *MTHFR* and fertility is due to the fact that much of the research only considers the *MTHFR* gene in relation to hyperhomocysteinemia [578-580]. The discussion should focus on *MTHFR* in relation to its ability to produce methyl folate and the influence this has on DNA methylation which affects oogenesis and spermatogenesis. Consistent with the views of the health professionals in this study, there is a united call for good quality evidence-based research and new strategies for unexplained RPL [8, 162, 504, 515, 555].

The survey highlighted that only one third of health professionals felt they had the necessary resources to support couples with RPL. Over half the health professionals reported requiring support groups for their patients which reflects one of the biggest issues patients with pregnancy loss face, the lack of emotional support and the minimisation of the psychological effect and instead a focus on the physical aspect of the loss [499]. Although four out of five health professionals in this study stated they refer their patients who experience RPL for emotional support, they predominantly speak to patients themselves to understand how they are feeling and lend support where they can. A significant number recommend continuing attempts to conceive. This is consistent with research that shows in the medical model, support is aimed at women's physical recovery and not emotional support [499, 581]. Women who experience pregnancy loss are left with feelings of grief for some time and do not feel supported enough by social and health care networks [499] and feel 'rushed' to get over it [501], when in reality it may take up to a year for anxiety and depression to be resolved [11] and the woman to recover from the grief [489]. Men also require psychological

support, but given they are not included in this aspect of fertility care, they often feel left out and the impact on them of RPL is unacknowledged [12]. Doctors and specialists may be willing to provide initial emotional support, but they do not have the training, resources or time to provide ongoing care [581] and often it is the next pregnancy and the first 20 weeks of gestation where patients feel unsupported [500]. The health professionals in this study clearly acknowledge the need for more support for their patients experiencing RPL, asking for more timely appointments with a specialist, dedicated preconception appointments, and greater access to midwives. Midwives do not necessarily see patients until they reach 20 weeks' gestation [582], and if women do have early pregnancy problems, they are more likely to present to their general practitioner or the Emergency department where support is limited [581]. Some hospitals have early pregnancy assessment services staffed by a midwife or nurse, but these are not in many locations and there is a lack of awareness amongst couples experiencing RPL that these exist [581, 583]. Therefore, the call for more access to midwives seems warranted as research shows that midwife support is important in the psychological health of women and their partners with RPL [584]. Following a pregnancy loss, couples want to understand why it happened, what they can do to prevent miscarriage happening again, and what tests can be done to find the cause [12, 514]. A delay or inability to find a cause increases trauma [556]. For a couple to be told their RPL is 'unexplained' is not helpful and very disempowering. There is a need to better identify ways to evaluate the 'unexplained' RPL and offer support to these couples.

This study highlights that there is little consistency in the prescribing of folate for women and most health professionals do not prescribe folate to males in

preconception or in the context of RPL. Although women are prescribed a FA-containing multivitamin most of the time, there is no consistency in the amount given, and rarely does the prescription change if a woman miscarries. This is reflective in the results of the survey that highlight the health professionals' need for prescribing and dosage recommendations for folate to support couples experiencing RPL. The worldwide recommendation for preconception for women ranges from 400-600 mcg in Australia [221, 585], 800 mcg in the United States [5, 25, 26], and 1,000 mcg in Canada [141-143]. The health professionals predominantly recommended Bayer Elevit® (800 mcg folic acid), Blackmores Pregnancy and Breastfeeding Gold® (500 mcg FA), and Blackmores I-Folic® (500 mcg FA), with over 80% recommending FA over 5-MTHF. Even though emerging research is showing that women who take a prenatal multivitamin containing FA in preconception and pregnancy in countries that have folic acid-fortification programmes are exceeding the upper dose deemed safe [48], health professionals are not heeding this warning. Excess FA has been linked with endocrine and hormonal issues [41], gestational diabetes [325, 347, 350, 353], and placental developmental issues [329]. Guidelines for FA need to discuss the potential for excess intake, particularly in countries like Australia, with both mandatory and voluntary fortification programmes. Health professionals in this study are largely unaware that alternate forms of folate exist outside folic acid, even though research highlights that other forms of folate may be more effective in cases of RPL, particularly when *MTHFR* polymorphisms are present [287].

An important finding of this study is the lack of attention males are given in preconception health planning. The data highlights that no dedicated

appointments for the male partner are being undertaken, even though Australian statistics report an estimated 40% of all infertility can be attributed to male reproductive function [586]. Other regions around the world report a similar finding [587], and male infertility may be contributing to as much as 70% of all infertility cases [110]. Research regarding men and infertility is scarce due to a lack of research in this area. Most papers on RPL focus on the female [176, 198, 479, 480], which was highlighted in a 2016 scoping review that reported only 11% of papers included male participants [480]. This is reflected in the fact that Australian [221, 548, 549] and international [550, 551] recommendations and guidelines put sole emphasis on the female. This is despite research highlighting that sperm concentration and quality, and pregnancy rates are improved by folate [181, 182] and micronutrient supplementation [183-185]. It was evident from the survey that even after RPL, health professionals conduct little testing for the male partner, with sperm testing conducted by less than half the practitioners, which is consistent with the clinical guidelines that largely ignore the impact of male factors on RPL [588]. Recent research highlights the importance of including male factor issues like DNA fragmentation testing into RPL guidelines [165]. Based on the above, recommendations for males need to be standard care to improve pregnancy outcomes and all guidelines and assessment tools for health professionals to support male fertility are warranted [516, 589].

6.10.1 Limitations

The strength of this study lies in the national approach inviting participation from fertility health professionals across all sectors of the fertility landscape in Australia. The key limitation is the sample size. The inability to calculate a

response rate is acknowledged as a limitation inherent to surveys disseminated via third-party professional organisations.

Convenience sampling techniques have limitations in that responses from individuals with distinct views on this topic may have been obtained and may not be reflective of the target population, limiting the generalisability of these findings. Those that responded may feel stronger towards this topic than those that did not, and therefore there is the potential for non-responder bias. However, the heterogeneity of responses across all fertility specialists suggests that this may have been limited.

6.11 Conclusion

Recurrent pregnancy loss needs to be a key priority. Men provide 50% of the DNA to the embryo, yet no assessment of sperm quality or the nutrients that support spermatogenesis like folate are prescribed. Men are largely omitted from the preconception and RPL picture. Practitioners require better resources, more funding for appointments to support couples with RPL, and protocols with dosing recommendations for folate. They require more support for couples physically and emotionally. There is a need for revised recommendations that include men and folate prescribing for both men and women. Unexplained infertility needs to be explained.

6.12 Chapter Conclusion

This chapter presented the findings from a national survey of health professionals who support couples experiencing RPL. The results highlighted significant variability in folate prescribing practices, limited use of alternative forms such as 5-MTHF, little or no *MTHFR* polymorphism testing, and a general lack of inclusion of male partners in preconception care. Despite

these gaps, practitioners expressed openness to change, particularly if supported by clear protocols, evidence-based guidelines, and improved funding for specialised services.

These findings complement and extend insights from earlier phases of the thesis. The pilot trial (Chapter 4) demonstrated clinical feasibility, while the qualitative interviews (Chapter 5) highlighted emotional and systemic challenges faced by patients. This chapter adds a third dimension by examining practitioner perspectives, thereby providing a more complete picture of feasibility, one that includes clinical outcomes, patient experience, and implementation potential within healthcare systems.

The survey also identified key barriers to practice change, including limited access to training, lack of standardised protocols, and insufficient resources to support couples with RPL. These insights are critical for informing future interventions and policy reform aimed at improving reproductive care and supporting the adoption of personalised folate strategies. The next chapter synthesises findings across all phases to explore their implications for clinical practice, education, and public health policy.

Chapter 7 Discussion

7.1 Chapter Introduction

This chapter synthesises findings from all phases of the thesis to provide a cohesive interpretation of the feasibility, relevance, and potential impact of personalised folate supplementation in couples experiencing RPL. Drawing together insights from the systematic literature reviews (Chapter 2), the pilot RCT (Chapter 4), qualitative participant interviews (Chapter 5), and the practitioner survey (Chapter 6), this integrated discussion explores how each phase contributes to the overarching research aim and informs future directions for clinical practice, education, and public health policy.

The mixed method design of the thesis was intentionally structured to reflect the complexity of RPL and the multifaceted nature of feasibility. The literature reviews highlighted the rationale for investigating alternatives to FA and identified systemic gaps in current supplementation guidelines. The pilot trial demonstrated clinical feasibility and biochemical relevance of 5-MTHF, while the qualitative interviews highlighted emotional and systemic barriers to care. The practitioner survey extended these findings by revealing variability in prescribing practices and highlighting both challenges and opportunities for implementation within the health system.

This chapter moves beyond individual results to examine how the findings align, reinforce, and challenge existing assumptions about folate use in RPL care. It considers the implications for clinical decision-making, practitioner

education, and policy reform, and identifies key opportunities to improve reproductive outcomes through more personalised, inclusive, and evidence-informed approaches.

7.2 Addressing the Research Objectives

The overarching aim of this thesis was to investigate the role of folate metabolism in RPL and evaluate the feasibility of personalised folate supplementation through an RCT pilot study, particularly in individuals with *MTHFR* polymorphisms. The research was designed to examine the feasibility of an RCT comparing 5-MTHF and FA to prevent pregnancy loss in couples with RPL (Research Objective 1), to explore the impact of different forms of folate and how it reduces unmetabolised folic acid (Research Objective 2), to understand the occurrence of *MTHFR* polymorphisms among couples with RPL (Research Objective 3), to explore reproductive couples' experiences of current RPL treatments and their perspectives regarding the acceptability and need for novel treatments of RPL (Research Objective 4), to explore the current practices of health professionals who commonly treat couples with RPL with regards to RPL management for both reproductive partners with a particular focus on folate prescription (Research Objective 5), and to understand health professionals' needs to support practice behaviour change regarding folate supplementation among couples with RPL (Research Objective 6) (Table 27). By addressing these research objectives, the thesis aimed to answer the broader research questions: how can we better support couples with RPL? By integrating findings from five distinct but complementary studies, the overall objective is to inform evidence-based clinical guidelines and improve outcomes for couples experiencing RPL.

7.3 Main Findings Summary

7.3.1 Informational Phase – Systematic Reviews

The first review assessed folic acid exposure in women during preconception and pregnancy, revealing that most women exceed the upper tolerable intake (UL) of 1,000 mcg/day due to supplementation and fortification, despite inadequate dietary folate intake. The second review examined the health effects of elevated folic acid intake and biomarkers, identifying associations with gestational diabetes and neurodevelopmental disorders such as autism in the offspring. These findings directly addressed Research Objective 1 by identifying the need for a trial comparing folate forms due to widespread excess intake, Research Objective 2 by demonstrating the impact of different folate forms on UMFA accumulation, and Research Objective 3 by highlighting the biological relevance of *MTHFR* polymorphisms in folate metabolism and the need for more personalised folate prescriptions. Other findings of these reviews included that women in preconception and pregnancy are not achieving their recommended folate intake from diet alone and require supplementation to achieve optimal intake levels, however it was found that 'excess' intake is always driven by supplement intake, highlighting the need for alternate forms of folate to folic acid that do not build up and cause 'excessive' levels. One important conclusion is that there is an urgent need to define what 'excessive' folic acid intake is and be cognisant of mandatory versus voluntary FA.

7.3.2 Phase 1 – Pilot RCT

The pilot RCT tested the feasibility and acceptability of comparing 5-MTHF and folic acid supplementation in couples with RPL. All participants carried *MTHFR* polymorphisms, with a higher prevalence among males. Biochemical

analysis showed that 5-MTHF improved folate biomarkers and reduced UMFA, while folic acid increased UMFA. These findings addressed Research Objective 1 by confirming the feasibility of a larger trial, Research Objective 2 by validating the metabolic advantages of 5-MTHF over folic acid, and Research Objective 3 by demonstrating the high prevalence of *MTHFR* polymorphisms in couples with RPL and the need for genetic screening. Another key finding was the instability issues of 5-MTHF in multivitamin supplements, which was an important confounding factor that needs to be taken into consideration for any further trials.

7.3.3 Phase 1A – Qualitative Interviews with Trial Participants

Interviews revealed that couples with RPL experience emotional distress, disenfranchised grief, and frustration with fragmented care. Participants felt unsupported and desired greater control and informed choice. These insights addressed Research Objective 4 by exploring the lived experiences of couples with RPL and their perspectives on current and novel treatments. There is a clear disconnect between clinicians and couples with RPL around *MTHFR* polymorphism, and couples want clarity and validation that *MTHFR* does matter.

7.3.4 Phase 2 – Practitioner Survey

The national survey of health professionals revealed inconsistent prescribing practices, limited inclusion of male partners, and a lack of standardised protocols when dealing with couples experiencing RPL. Practitioners expressed a need for clearer guidelines, more education on folate metabolism, and better resources. These findings addressed Research Objective 5 by identifying current gaps in RPL management practices, particularly around folate prescription, and Research Objective 6 by

highlighting the need for professional education, clinical tools, and systemic support to enable practice behaviour change.

7.1 Integrated Discussion

This thesis challenges the long-standing assumption that folic acid is universally safe and effective. While the primary outcome of feasibility was largely supported, the findings should be interpreted with appropriate caution. The study offers valuable insights into trial processes, intervention acceptability, and contextual considerations that can inform the design of a future trial. Together, these findings contribute to a growing body of evidence supporting 5-MTHF as a potentially safer, more biologically-compatible alternative to FA, particularly in cases of unexplained RPL, where standard approaches may be insufficient.

7.1.1 Integration Across Phases

The findings across all phases support a shift toward personalised folate supplementation strategies, particularly for individuals with *MTHFR* polymorphisms. They also highlight the need to redefine folic acid safety thresholds, integrate genetic screening into preconception care, and develop standardised, inclusive, and trauma-informed RPL management protocols (Table 28). This research provides a robust foundation for future clinical guidelines and public health policy reform aimed at improving outcomes for couples experiencing RPL.

Table 27- Summary of each research phase and how it relates to the research objectives

Informational Phase – Reviews FA exposure in preconception & pregnancy	Informational Phase – Review- what is the problem with excess folate?	Phase 1 – Pilot RCT trial	Phase 1A Qualitative Sub-Study of Participant Interviews	Phase 2 Practitioner Interviews
Chapter 2	Chapter 2	Chapter 4	Chapter 5	Chapter 6
Women in preconception and pregnancy are exceeding the UL (1,000 mcg) of folic acid intake. Driven by supplementation [RO1, RO2]	There are implications for both maternal and offspring health in exceeding the dose of FA/blood concentrations: GDM in pregnancy and neurodevelopment issues in the offspring [RO1, RO2, RO3]	*MTHFR* must be considered in RPL [RO3]	Couples with RPL feel 'abandoned' and forgotten [RO4]	HPs need more help in addressing RPL. [RO5, RO6]
Women in preconception and pregnancy do not achieve enough folate in their diet, however due to fortification these women are exposed to 'excessive' FA intake [RO1, RO2]	Excess FA and blood concentrations must be defined to ensure safety of pregnant women and their offspring [RO2]	A large trial comparing folic acid and 5-MTHF would be feasible and acceptable to participants [RO1, RO2]	Couples suffer great emotional distress and isolation [RO4]	Practitioners need more defined protocols and resources to support patients with RPL [RO5, RO6]
Folate metabolism of the individual is an important consideration [RO3]	Neurological development of the offspring is affected by high FA supplementation [RO1, RO2]	All couples had *MTHFR* polymorphisms, so could this be a potential risk in couples with RPL? [RO3]	Couples with RPL are frustrated with the systemic healthcare gaps [RO4]	HPs lack information on folate forms, dosing requirements, and product options and pricing [RO5, RO6]
Bioavailability is very different to metabolism. FA causes an accumulation (UMFA). 5-MTHF does not [RO1, RO2, RO3]	Significant variability in how folate intake is assessed and measured. We need consistency and we urgently need to define what defines 'excess' [RO1, RO2]	5-MTHF participants showed much lower UMFA in blood and higher 5-MTHF which is more favourable for fertility [RO2]	Couples with RPL desire control and informed choice [RO4]	Males are largely ignored in preconception care [RO5]
UMFA causes an *MTHFR* deficiency	UMFA –studies show adverse outcomes across	Stability of a product is key, and the 'instability' of	There is a disconnect with clinicians around *MTHFR*	There is little consistency in folate

which affects DNA methylation, and a lack of 5-MTHF which affects oocytes and spermatogenesis issues [RO2, RO3]	various populations and >0.51nmol/L increases risk of ASD [RO2]	the trial product likely affected the outcome [RO1]	[RO2, RO5]	prescribing, and it tends to be up to the HP's discretion as to how much folate is given and when [RO5]
Upper tolerable limit – all women who take a supplement with FA are exceeding the UL in countries with fortification [RO2]		Males need to be included in preconception care and checked for *MTHFR* [RO2, RO3]	Couples with RPL turn to peer support and community connection [RO4]	HPs require more funding and dedicated RPL appointments [RO6]
RO1:Examine feasibility or an RCT comparing 5-MTHF and FA; RO2: explore the impact of different forms of folate and its effect on UMFA; RO3: Understand the occurrence of *MTHFR* polymorphisms among couples with RPL; RO4: Explore experiences of RPL treatment in those with RPL; RO5: Explore current practices of health professionals (HPs) who support couples with RPL; RO6: Understand HPs' needs to support practice behaviour change regarding folate supplementation in couples with RPL				

7.1.2 Feasibility, Acceptability and Outcomes of a Randomised Controlled Trial Comparing 5-MTHF and Folic Acid

This feasibility pilot was a double-blind RCT that included both partners within reproductive dyads in the context of RPL. Recruitment, randomisation, and masking procedures were feasible, and participant engagement with study requirements was high. Overall, the study demonstrated feasibility and supports the potential for a larger, multicentre study. (Chapter 4). Recruitment yielded 766 enquiries, with 22 couples enrolled. Adherence to supplementation, dietary protocols, and abstinence guidelines was high, and participant feedback indicated strong satisfaction with the trial format. The trial demonstrated high feasibility and strong participant engagement, validating the potential for a larger, multicentre study. These findings lay the foundation for a scalable, patient-centred model of reproductive research.

However, a critical insight from this trial was the emotional resistance to FA randomisation, particularly among participants with *MTHFR* polymorphisms as outlined in the trial sub-study (participant interviews) as discussed in Chapter 5 of this thesis. This reflects a growing awareness of FA's potential limitations, particularly among individuals with *MTHFR* polymorphisms and RPL. This highlights the importance of trauma-informed trial design, and the need to incorporate patient preferences into future protocols. Trauma-informed research acknowledges that participants, particularly those experiencing RPL, may carry psychological distress, grief, or prior medical trauma that can influence their engagement with research [590, 591]. The Trauma and Resilience-informed Research Principles and Practices (TRIRPP) framework offers a structured approach to designing trials that promote psychological safety, avoid re-traumatisation and improve participation and engagement among individuals facing adversity [590].

Table 28 - Integrated Discussion points and evidence from Research Phases

Integrated Discussion Points	Evidence from Research Phases	Implications
1. Feasibility and acceptability of a randomised controlled trial comparing 5-MTHF and FA	The pilot RCT (Phase 1) demonstrated strong feasibility and acceptability of a double-blind trial comparing 5-MTHF and folic acid. Participants adhered to protocols and expressed satisfaction, though some were reluctant to be randomised to the folic acid arm.	Confirms the viability of a larger, patient-centred trial. Highlights the importance of trauma-informed design and the need for stable formulations of 5-MTHF. Supports RO1.
2. Genetic screening (*MTHFR*) and male inclusion in preconception care	All couples in the pilot RCT (Phase 1) carried *MTHFR* polymorphisms, with a higher prevalence among males. The practitioner survey (Phase 3) revealed limited inclusion of male partners in preconception care and low awareness of *MTHFR* implications. The qualitative interviews (Phase 2A) of participants revealed a disconnect between their *MTHFR* knowledge vs their practitioners.	Supports the integration of genetic screening (specifically *MTHFR* polymorphisms) into routine RPL assessment. Emphasises the need for male-inclusive care models and practitioner education. Addresses RO3 and RO5.
3. Emotional burden and systemic gaps in RPL management	Qualitative interviews (Phase 1A) revealed that couples with RPL experience disenfranchised grief, emotional distress, and frustration with fragmented care. Practitioners (Phase 2) acknowledged the emotional toll but lacked resources and protocols.	Highlights the need for trauma-informed, patient-centred care and better coordination across services. Supports RO4 and RO6.
4. Rethinking folic acid safety thresholds and the case for 5-MTHF	Systematic reviews (Informational Phase) showed that most women exceed the UL for folic acid, with emerging evidence linking excess intake to gestational diabetes and neurodevelopmental disorders. The pilot RCT (Phase 2) showed 5-MTHF reduced UMFA and improved biomarkers, and couples with RPL had a preference for 5-MTHF because of their own research and if they had *MTHFR* polymorphisms.	Supports redefining folic acid safety thresholds based on biological metabolism. Justifies personalised supplementation strategies using 5-MTHF, especially for those with *MTHFR* polymorphisms. Addresses RO2.

RO1.To examine the feasibility of an RCT comparing 5-MTHF and FA treatment to prevent pregnancy loss in couples with RPL.
RO2.To explore the impact of [a different version of folate reduces unmetabolised folic acid] for couples experiencing RPL, to achieve successful pregnancy.
RO3.To understand the occurrence of *MTHFR* polymorphisms among couples with RPL, and consider the potential impact of any differences on pregnancy outcomes
RO4.To explore reproductive couples' experiences of current RPL treatments and their perspectives regarding the acceptability and need for novel treatments of RPL.
RO5.To explore the current practices of health professionals who commonly treat couples with RPL with regards to RPL management for both reproductive partners, and with a particular focus on folate prescription.
RO6.To understand health professionals' needs to support practice behaviour change regarding folate supplementation among couples with RPL.

Future trials should prioritise participant-centred approaches that foster trust, reduce anxiety, and promote inclusivity. In this study (Chapter 4) several of these strategies were implemented, including: transparent communication about study procedures, risks and randomisation; flexible consent processes that allowed participants to reconsent or withdraw at any stage; and sensitivity to emotionally-triggering language in recruitment and survey materials. Participants were provided with a supportive environment through access to counselling and regular ongoing meetings with the researcher, which helped to identify and address any potential concerns early. The inclusive recruitment strategies engaged disenfranchised populations via trusted community channels like Pink Elephant, Red Nose and the Gidget Foundation. These measures were instrumental in enhancing participant experience and engagement, underscoring the importance of their continued use in future research, a point which is well supported in the literature highlighting that inclusive strategies that engage disenfranchised populations through trusted community channels (such as EPAS units, GPs and fertility clinics) have been widely advocated [590-592].

7.1.2.1 Biochemical Outcomes

Within the constraints of an under-powered feasibility study, descriptive analyses suggested differences in folate-related biomarkers between arms, including patterns in UMFA. (Chapter 4), the latter being a metabolic concern increasingly linked to adverse outcomes [45-47, 593-595]. These findings are particularly relevant when considered against the historical context of folic acid-fortification programmes, initiated in the 1990s in the United States [31, 289]. Although these programmes successfully reduced NTD prevalence [28, 596], widespread mandatory folic acid-fortification programmes were

implemented in the United States (1998), Canada (2000) [30, 291], and later in Australia, with the aim of ensuring a minimum intake of 400 mcg among women of reproductive age [596, 597] as described in Section 1.4. Initial projections estimated that fortification could increase FA intake by 72 to 130 µg per day [225], a level considered safe based on the metabolic capacity of the *DHFR* enzyme in metabolising FA [86, 87, 204, 226]. Today, voluntary fortification and widespread supplement use have led to FA intakes that frequently exceed recommended levels, often among populations not originally targeted by these interventions [27, 44-46, 225, 304-306]. One of the most important protocol components of the trial was the avoidance of folic acid-fortified foods by the participants (both males and females). Compliance with this dietary restriction was 100% for both arms and participants did not report significant difficulty in adhering to this restriction. This aspect of the protocol is particularly noteworthy, as folic acid intake from fortified foods introduces variability in circulating folate concentrations and contributes to the accumulation of UMFA, which can confound the interpretation of folate metabolism and its relationship to reproductive outcomes [41, 43, 598, 599]. By controlling this variable, the trial was able to more clearly observe the biochemical effects of 5-MTHF supplementation, as reflected in changes to folate biomarkers and reductions in UMFA. This level of dietary control is rarely implemented in clinical trials but is critically important for accurately assessing folate related interventions.

7.1.2.2 Reviewing Folate Biomarkers and the Need to Define 'Excess Folate'

Findings from this feasibility study do not redefine or challenge current "optimal" folate thresholds. Rather, combined with the literature, they highlight uncertainties around cumulative FA exposure in fortified settings

and motivate further research to clarify exposure thresholds and biomarker interpretation.

The WHO red blood cell folate threshold of 906 nmol/L is a population-level metric derived to reduce neural tube defect (NTD) risk and was not developed for RPL contexts. Our discussion of “overexposure” refers to cumulative folic acid intake in fortified settings and the potential for UMFA accumulation, a concept distinct from the NTD-focused RBCF sufficiency threshold. This study does not adjudicate optimal RBCF cut-offs for RPL, rather, it highlights the need for research that disentangles RBC folate sufficiency for NTD prevention from potential risks associated with cumulative FA exposure and UMFA in different clinical contexts.

As demonstrated in Chapter 2, my review identified that folate intakes below this threshold may still be associated with adverse outcomes, particularly in genetically susceptible individuals. Furthermore, this data supports growing concerns in the literature regarding the potential risks of FA overexposure in fortified populations [39, 44, 46, 48, 87, 326, 537]. This is particularly relevant given the high prevalence of *MTHFR* polymorphisms in my sample study, which may exacerbate overexposure risks. These results reinforce the urgent need to define biologically relevant upper limits for folate exposure, especially in populations with impaired folate metabolism or those undergoing treatment for RPL.

7.1.2.3 Biological Risks of Excess Folic Acid

The systematic review (Chapter 2) identified consistent associations between excessive FA intake and elevated blood folate concentrations with adverse maternal and offspring outcomes. These included a reported increased risk of gestational diabetes mellitus which has since been supported by another

Australian study [600], large for gestational age infants and neurodevelopmental concerns such as Autism Spectrum Disorder and attentional deficits. Notably these effects were observed at FA intakes above 400 mcg/day and at blood folate concentrations considered within the 'optimal' range, challenging the current assumptions about safety thresholds. The review also highlighted the lack of a biologically defined upper limit for folate status, particularly in countries with mandatory fortification and widespread supplement use.

Building on these findings, the pilot study observed a rise in UMFA among participants receiving FA (Chapter 4) with some exceeding the 1-2 nmol/L threshold considered beyond physiological capacity for metabolism. In contrast, UMFA decreased in the 5-MTHF groups, suggesting a more favourable metabolic profile. These results are particularly concerning given the high prevalence of *MTHFR* polymorphisms in the cohort, which impair folate metabolism and may exacerbate the risks associated with both folate insufficiency and overexposure. Participants with the C677T homozygous genotype (a small subgroup), lower serum folate and higher homocysteine were observed. These observations warrant confirmation in larger studies. These human data are further supported by animal studies which have shown that excess FA disrupts neurogenesis, induces anxiety-like behaviours [37, 106], and impairs learning and memory [151]. FA overexposure has been shown to alter neurotransmitter profiles and gene expression in the developing brain [601], increasing susceptibility to neurodevelopmental disorders such as ASD and attention deficit hyperactivity disorder [602]. Beyond neurodevelopment, excess FA may induce epigenetic modifications associated with impaired fertility, reduce

gamete quality, and increase embryonic loss [315].

Taken together, these findings may support the need for personalised supplementation strategies that consider genetic, epigenetic and sex-specific differences, particularly in fortified populations where cumulative FA exposure from diet and supplements may exceed metabolic capacity, particularly in individuals with impaired folate metabolism. They also highlight the need to define biologically relevant upper limits for folate exposure to ensure safety across diverse reproductive populations which is echoed in the literature [39, 44, 46, 48, 87, 326, 537].

7.1.2.4 Evaluating 5-MTHF as an Alternative to Folic Acid: Opportunities and Barriers

The biochemical observations of this RCT trial are consistent with prior evidence that 5-MTHF does not lead to elevated UMFA, particularly in individuals with *MTHFR* polymorphisms (Obeid et al., 2013; Ferrazzi et al., 2020). We observed that participants in the 5-MTHF arm showed folate biomarkers different to those who here in the FA arm and had reduced UMFA, consistent with earlier research [89, 94, 121, 123, 603]. These findings are particularly relevant in fortified populations where cumulative FA exposure may exceed metabolic capacity [48, 325, 328, 331, 417].

Pregnancy outcomes are presented descriptively so interpretation is constrained by small sample size and the documented 5-MTHF stability issue.

Research indicates that a minimum daily intake of 800 mcg of 5-MTHF is required to effectively bypass the metabolic limitations associated with *MTHFR* polymorphisms [86, 89-94]. Participants were instructed to take three capsules per day. The nominal formulation targeted ≥800 mcg 5-MTHF

per capsule, however, stability testing during the study indicated a mean content of approximately 199 µg per capsule, yielding an estimated delivered dose of ~600 µg/day. This degradation likely resulted in sub-therapeutic dosing and constrains interpretation of both biochemical and clinical observations.

The feasibility of implementing 5-MTHF more broadly in clinical and public health settings is constrained by several barriers. Firstly, while mechanistic and comparative studies suggest that 5-MTHF is more bioavailable and metabolically efficient than FA at raising folate concentrations [89, 94, 121, 123], it lacks equivalent large-scale population level evidence that underpins FA's established role in NTD prevention. A recent review by Samaniego-Vaesken (2024) concluded that 'despite the potential value of 5-MTHF as an alternative to FA, clinical studies would be urgently needed to support the efficacy, dosage, timing and/or safety of its use as a supplement [604]. However, 5-MTHF is more expensive to produce and formulate than FA, is less commonly available in standard prenatal supplements and often sold at a premium price [604]. Formulation stability emerged as a critical confounder in this pilot study and therefore future trials must prioritise formulation integrity, including validated stability testing and moisture-controlled packaging to ensure accurate interpretation of nutrient efficacy.

Another barrier is the limited awareness and training among health professionals. Many clinicians remain unfamiliar with the metabolic differences between FA and 5-MTHF, and few are equipped to interpret genetic screening results or prescribe personalised folate regimes [447]. The protocol employed in the trial was adapted and developed by a researcher with extensive clinical experience in administering 5-MTHF, which positively

influenced the trial's feasibility. Looking ahead, a key challenge for future trials and clinical interventions is to design protocols that accommodate individual genetic variability such as *MTHFR* polymorphisms. It is essential that health professionals are provided with the necessary resources and training to interpret individual responses to 5-MTHF effectively. The practitioners surveyed in my study (Chapter 6) clearly expressed the need for more structured protocols for patients with RPL, as well as patient handouts and educational materials. This aligns with previous research highlighting the critical role of such resources in supporting couples affected by RPL [12, 197, 511, 547, 605-607].

While the practitioner survey revealed that 82.8% of clinicians were open to changing practice based on meta-analyses, only 77.4% prioritised RCTs. Fifty-eight percent rated case reports as moderately or extremely important, and 78% thought that observational studies were moderately or extremely important. This suggests that clinicians may be increasingly receptive to more diverse forms of evidence, including mechanistic and biochemical evidence and clinical experience, when making prescribing decisions. This shift may reflect a growing recognition that complex interventions, such as personalised folate supplementation, may require more than traditional RCT evidence to inform practice, particularly in the area of RPL, where a large percentage of cases are 'unexplained'. However, implementation science literature consistently shows that even strong evidence does not automatically translate into practice change and that clinicians often require multiple forms of evidence including clinical guidelines, peer consensus, cost effectiveness data and practical tools before altering prescribing [608] and that engagement is required by multiple partners, practitioners and

researchers [609].

Finally, the feasibility of using 5-MTHF in future trials or clinical settings is also influenced by the accessibility and cost of testing for relevant biomarkers. Testing for UMFA and *MTHFR* polymorphisms is not routinely available in many clinical settings and may be prohibitive for large scale screening. These practical constraints must be considered when designing future research or proposing policy changes.

7.1.2.5 Proposed Revisions to Trial Design

From an implementation perspective, the trial demonstrated strong participant engagement with complex protocols (Table 29), including blood collection and dietary restrictions. However, several logistical and methodological challenges emerged that would inform future trial design. Recruitment strategies would be revised to include more sensitive and targeted approaches through GPs, hospitals, and EPAS units allowing for better support and screening of participants. Blood collection protocols would need to be expanded to include follow-up samples for both male and female participants, ideally conducted in clinical settings to overcome geographic and logistical barriers. While the dietary protocol to avoid folic acid was well adhered to and successfully reduced confounding, future trials would benefit from including participants of diverse nationalities to ensure generalisability. Genetic screening, which only included *MTHFR* polymorphisms, would be broadened to include other folate and vitamin B12-related genes such as *Transcobalamin 2 (TCN2), Methionine synthase reductase (MTRR), and Serine hydroxymethyltransferase 1 (SHMT 1)*. Finally, given the observed instability of the 5-MTHF formulation, future studies would incorporate detailed stability testing of supplements prior to, and throughout the trial

period, to ensure biochemical integrity and accurate interpretation of outcomes.

Table 29 - Trial success and revisions

Trial characteristic	What worked	Future revisions
Recruitment		More sensitive recruitment strategy via GPs, hospitals, and EPAS units
Blood collection	Baseline results were ideal	Conduct via clinics and hospitals at the time of follow-up visits. Would include follow-up bloods for men as well as women (not conducted due to financial restraints)
Dietary protocol	There was good engagement with the protocol of avoiding FA which eliminated a major confounder	
Nationalities		Include different nationalities
Genetics	*MTHFR* was an important inclusion	Would extend this to include other folate and vitamin B12-related genes like TCN2, MTRR, SHMT 1
Stability of 5-MTHF and FA supplements		Conduct detailed stability trials of the product prior to and during the trial.

In summary, the pilot RCT demonstrated the feasibility of a scalable, patient-centred trial model in the context of RPL. The study showed high participant engagement with complex protocols, including blood collection and dietary restrictions, and successfully implemented an FA-avoidance strategy to reduce potential confounding. Collectively, these findings lay the groundwork for future, adequately powered studies that could inform clinical guidelines and public health policy related to personalised folate

supplementation. The trial underscores the importance of inclusive, scientifically rigorous, and patient-informed approaches to reproductive health research.

7.1.3 *MTHFR* Polymorphisms May be Common in Couples with RPL

One finding of this research was that all participating couples in the pilot cohort carried at least one *MTHFR* polymorphism.. All couples carried at least one variant and over half of male partners were homozygous for either the C677T or A1298C variants (Chapter 4). This may open the way for more research to be conducted regarding *MTHFR* polymorphisms which may challenge prevailing clinical view that *MTHFR* polymorphisms are inconsequential, particularly in the context of fertility and pregnancy outcomes.

The C677T and A1298C variants reduce *MTHFR* activity by 30-70% depending on zygosity [77] impairing the conversion of 5,10 methylene tetrahydrofolate to 5-MTHF [80, 113], the only form of folate that supports the production of S-adenosylmethionine (SAM) and methylation [79]. Methylation is essential for oogenesis [167] and spermatogenesis [92, 114, 280, 571, 610-617] and is required for epigenesis and imprinting [170]. The biochemical significance of *MTHFR* in folate metabolism and methylation has been established [78, 85, 92, 201, 287, 447, 506, 536, 576, 613-616, 618-630]. Up to 75% of pregnancy losses are deemed ‘unexplained’ [7, 555] with over fifty percent of those due to chromosomal abnormalities [563]. Increasingly, research is pointing to epigenetic dysregulation, especially DNA methylation, as a contributing factor in these unexplained losses [562]. Methylation is being increasingly investigated for its impact in RPL because it has been shown to be closely linked to fertilisation [563], embryo development [111,

172, 563-567] and placental development [631]. Methylation, because of its critical role in maintaining the epigenetic modifications that affect these processes during embryonic development and spermatogenesis [8, 162, 504, 515, 555], may explain a significant portion of what is currently deemed 'unexplained' [563, 567, 577].

In the trial, the females with homozygous genotype exhibited significantly lower serum folate and higher homocysteine, consistent with impaired folate metabolism. Those receiving 5-MTHF supplementation showed greater improvements in serum 5-MTHF and reduction in UMFA, while UMFA rose markedly in the FA group. This is supported by prior research showing that individuals with the *MTHFR* polymorphisms consistently have lower folate and higher homocysteine concentrations, even when folate intake is adequate [123, 632-634]. These findings reinforce the metabolic advantage of 5-MTHF in genetically susceptible individuals and highlight the limitations of a uniform folate supplementation strategy.

Despite these findings, medical bodies such as the Royal Australian College of General Practitioners (RACGP), continue to advise against routine *MTHFR* testing [554], a position that has shaped clinical attitudes for over a decade. This has led to a widespread lack of clinical engagement with *MTHFR* screening, reflected in the RPL practitioner survey (Chapter 6), where fewer than 1% of respondents reported routinely testing for *MTHFR* polymorphisms. In contrast, the participant interviews (Chapter 5) revealed a clear disconnect between what patients want and what clinicians provide, with many participants expressing frustration at the dismissal of their concerns and a desire for *MTHFR* polymorphisms to be acknowledged, discussed and addressed. This mismatch is consistent with prior research

highlighting the gap between patient expectations and clinical practice in the context of the expanding role of genetic and biochemical testing in clinical care [197, 605-607]. Importantly, such gaps in clinician knowledge and engagement can undermine patient trust, particularly when individuals are told there is ‘nothing you can do’ to prevent further pregnancy loss. Several participants described feeling frustrated, disempowered and angry when told by health professionals there was nothing they could do to prevent another pregnancy loss despite their own research or lived experience suggesting otherwise. It also reflects a broader issue in evidence-based medicine, the tendency to dismiss emerging biochemical insights when they do not fit neatly into existing diagnostic frameworks [635]. As Fernandez and colleagues argue, evidence-based medicine often prioritises internal validity and RCTs as the gold standard, while sidelining mechanistic, biochemical, and context-sensitive evidence [635]. This rigid framework can delay the integration of emerging molecular evidence into clinical practice, even when such evidence has clear biochemical plausibility and relevance to patient outcomes. This perceived dismissal not only contributes to emotional distress but may also discourage patients from seeking further care or participating in future research. It is also important to clarify that the RCT study did not selectively recruit individuals with *MTHFR* polymorphisms, but rather all couples with RPL were found to carry at least one variant. This underscores the potential under-recognition of these polymorphisms in populations experiencing RPL and reinforces the need to revisit their clinical relevance in reproductive health and specifically in relation to RPL.

To bridge this gap, clinical guidelines must evolve to incorporate emerging biochemical and genetic evidence alongside traditional RCT data, particularly

in complex conditions like RPL where a large percentage of cases are 'unexplained'. Practitioner education should also prioritise upskilling in nutrigenomics, methylation, and personalised strategies enabling health professionals to interpret genetic findings such as *MTHFR* polymorphisms with greater confidence. Integrating patient-centred approaches, such as shared decision making and transparent communication about the limitations and potential of genetic testing, will be essential in aligning clinical care with both the evidence base and patient values and expectations.

7.1.4 5-MTHF as a Consideration for RPL

Given the high prevalence of *MTHFR* polymorphisms among the trial participants (Chapter 4), as discussed in 4.8.1, the continued reliance on FA as the primary form of folate supplementation warrants careful consideration. Individuals with *MTHFR* polymorphisms exhibit reduced enzymatic activity, impairing the conversion of FA into its biologically active form, 5-MTHF. This metabolic constraint can lead to suboptimal folate status, compromised DNA methylation, and fertility challenges.

This concern is further substantiated by the biochemical outcomes observed in the pilot study. Participants in the FA arm demonstrated significantly elevated UMFA, indicating saturation of the *DHFR* pathway and inefficient metabolism of FA. In contrast, those in the 5-MTHF arm demonstrated improved folate biomarkers, including higher serum and red blood cell folate concentrations. These findings are consistent with 5-MTHF having a biomarker profile that may be relevant in individuals with reduced *MTHFR* activity, warranting further investigation.

7.1.4.1 Emerging Evidence of Folic Acid Risks

Recent evidence suggests that FA supplementation, even at intakes below the established upper limit, may contribute to adverse outcomes, affecting fertility [630, 636]. My review identified associations between FA intake and conditions such as gestational diabetes [636, 637], neurodevelopmental disorders in the offspring including learning difficulties, memory issues, and autism spectrum disorders [300, 572, 638]. Animal studies further support these findings [33, 34, 114, 171, 172, 565, 566, 571], which highlight the epigenetic effect that excess FA has on offspring development [182, 192, 639]. These effects are not due to the masking of a vitamin B12 deficiency as originally assumed by the Institute of Medicine [202], but rather the saturation of *DHFR* which limits the body's ability to metabolise the larger FA dose [226].

Despite these concerns, government guidelines including from Food Standards Australia New Zealand (FSANZ), continue to recommend 400 mcg/day of FA for those individuals planning a pregnancy [585]. However, many prenatal supplements, including Elevit®, contain 800 mcg, double the recommended dose [640]. Elevit's formulation, registered in 2013 [641], has not been publicly re-evaluated in light of recent safety data.

This discrepancy highlights regulatory and clinical inconsistencies in FA supplementation. In countries with mandatory and/or voluntary fortification, women may already consume significant FA through enriched foods. FSANZ estimates that three slices of fortified bread alone provide approximately 120 mcg FA and cereals may contribute an additional 147-564 mcg (per 100g) [642]. Thus, women taking Elevit® may easily exceed the 1,000 mcg per day [643].

In 2016, a study suggested that FA intake ≤400 mcg does not lead to UMFA [337], however other studies found that fortified foods alone can contribute to UMFA [45, 46, 48, 196, 203-205, 225, 273, 406, 644] even at levels of intake at 200 mcg [150]. My review highlighted that some women in preconception may consume up to 630 mcg DFE from food alone [48]. The second review paper investigating excess FA (Chapter 2) supports this as evidenced by research showing that levels of FA intake above 400 mcg may produce adverse events (Chapter 2). These findings suggest that 400 mcg/day may represent the upper threshold for FA supplementation in diet-fortified populations. This thesis argues for a redefinition of 'excess' FA based on metabolic and genetic thresholds, not reported historical limits. The implications for clinical practice and public health policy are profound. Current guidelines fail to account for cumulative FA intake from fortified foods and supplements. Regulatory bodies must urgently reassess supplement formulations and update public health recommendations to reflect current science and protect maternal and fetal health.

7.1.4.2 5-MTHF

Unlike FA, 5-MTHF is the biologically active form of folate that bypasses the *DHFR* dependent conversion pathway and is immediately available for cellular uptake and methylation processes [86, 88, 90, 94, 121, 472]. The pilot RCT (Chapter 4) confirmed that 5-MTHF improves folate biomarkers without increasing UMFA, findings consistent with previous clinical trials [86, 88]. While the extent to which methylation issues affect couples with RPL remains unclear, the safety profile of 5-MTHF and its ability to overcome methylation challenges makes it a promising option. Moreover, 5-MTHF does not interfere with vitamin B12 metabolism [121, 225, 472], and effectively

raises serum folate concentrations necessary to prevent NTDs and support pregnancy health [121, 645, 646].

Unlike FA, which shows diminishing safety at higher doses, 5-MTHF has demonstrated a strong safety profile even at elevated concentrations. Clinical studies in various fields, including psychiatry and reproductive medicine, have reported no adverse effects at doses up to 15 mg/day [124, 128] and previous research shows no adverse safety profiles at doses up to 3,800 mcg per day for preconception and pregnancy [287].

In this pilot, no serious adverse events were observed with 5-MTHF at 2,500 mcg, however larger studies are needed to characterise safety and efficacy. It may be suitable for use in preconception and pregnancy care, especially among individuals with *MTHFR* polymorphisms, who may have impaired folate metabolism and are more vulnerable to the effects of excess FA.

In conclusion, this thesis contributes a new perspective to the discourse on folate supplementation by integrating feasibility, biochemical and experiential insights. it raises questions about current assumptions, highlights areas of uncertainty and identifies priorities for further research into personalised folate strategies. By re-examining FA safety within the context of cumulative exposure and exploring more personalised approaches, future research may better inform strategies to support maternal and fetal health in couples experiencing RPL. In particular, 5-MTHF may be considered as an alternative to folic acid for fertility and pregnancy supplementation, pending confirmation in adequately powered studies. Its biological activity, lack of observed UMFA accumulation in several studies, and non-interference with vitamin B12 metabolism have been reported in the literature, suggesting that 5-MTHF warrants further evaluation as an alternative folate form. Calls for its

use have been made by several researchers [86, 90, 94, 210, 476, 477, 647, 648], highlighting the need to re-examine supplementation practices through adequately powered studies.

7.1.5 Emotional Burden and Systemic Gaps in RPL Management

Findings from the qualitative sub-study indicated emotional and systemic challenges among participants who consented to share their experiences of RPL (Chapter 5). These participants described emotional distress, disenfranchised grief, and frustration with inconsistent clinical advice. Some reported feeling unsupported and described self-advocating for care, including seeking information through online communities and complementary practitioners. Collectively, these narratives point to perceived gaps in RPL management and underscore the relevance of trauma-informed, patient-centred approaches to care.

The national survey of health professionals (Chapter 6) corroborated these findings, revealing wide variability in folate prescribing practices and a lack of standardised protocols. While practitioners acknowledged the emotional toll of RPL, they reported limited resources and training to support affected couples. Folate prescribing practices varied widely, and few health professionals routinely considered folate metabolism or *MTHFR* polymorphisms in their assessments. Male partners were rarely included in care discussions and psychosocial support was limited in standard protocols. Most protocols focused on diagnostic testing contributing to patient confusion, distress, and disengagement from conventional care pathways.

The emotional vulnerability of patients during the preconception period and early pregnancy is well-documented [11, 489]. Yet, few clinical settings offer dedicated support services, and awareness of existing resources, such as

Early Pregnancy Assessment Services (EPAS), remains limited among both patients and providers [581, 583]. Several systemic barriers contribute to this gap. Fragmentation between state and federal health systems, particularly the division between primary care (largely federally funded) and hospital services (state administered) [649-651], creates ambiguity around responsibility for RPL management. This separation often results in inconsistent care pathways and unclear referral processes. Additionally, there is a lack of clarity regarding the roles of different health professionals in RPL care [499, 557]. Participants of the RCT in their interviews (Chapter 5) frequently described confusion and uncertainty about where to seek care when experiencing a pregnancy loss. Many were unsure whether to contact their general practitioner, present to an emergency department, or seek support from a specialist or midwife. This lack of clarity reflects broader systemic issues in the management of RPL, where roles and responsibilities across the healthcare system remain poorly defined. There is currently no consensus on which health professionals should lead care coordination for RPL [607]. General practitioners, obstetricians, fertility specialists, midwives and community nurses may all be involved at different stages, yet their roles overlap or remain ambiguous, particularly during the emotionally vulnerable preconception and early pregnancy periods [557].

These challenges are further compounded by the fact that approximately 50% - 75% of RPL cases remained unexplained, even after thorough clinical evaluation [555, 652]. This diagnostic uncertainty leaves clinicians without clear therapeutic targets and contributes to a reliance on empiric treatments such as progesterone, aspirin, corticosteroids and immunomodulatory agents, many of which lack robust evidence for efficacy in idiopathic RPL

[157, 160, 161, 652]. It also leaves patients feeling as though their suffering is minimised or medicalised without resolution [11, 158, 489, 499, 503, 504, 607]. The psychological burden of repeated unexplained losses, combined with this fragmented care and unclear pathways to prevent RPL, highlights the need for integrated, trauma-informed and patient-centred models of care. These models must clearly define professional roles, improve resource allocation, and bridge the administrative gap within the healthcare system to better support individuals with RPL.

Consistent with the views of the practitioners in the survey (Chapter 6) there is a growing call for new strategies to address unexplained RPL [322, 325, 328, 348, 387, 391], and given research demonstrates that couples experiencing RPL are highly engaged in their care and actively seek structured, evidence based guidance [456, 457], it highlights the need for a more integrative framework, one that values biochemical plausibility and patient specific genetic data alongside traditional population level evidence. Until such frameworks evolve, patients with RPL may continue to be underserved by current reproductive care models.

7.1.6 Male Inclusion in Preconception Care

This thesis highlights a gap in current preconception care protocols, namely the limited inclusion of men in folate-related discussions and interventions. Despite contributing half of the genetic material to the offspring, men are infrequently included in clinical conversations about preconception care or supplementation. In the national survey of health professionals (Chapter 6), only 11% of respondents reported routinely including male partners in preconception care discussions, with even fewer indicating that they prescribe folate to men. This observation is noteworthy given existing

evidence linking folate metabolism to DNA methylation, sperm quality, and embryonic development, as discussed in earlier chapters.

In this pilot study, male participants were included in a folate intervention trial and demonstrated high levels of engagement and adherence to study protocols. These observations support the feasibility of involving men in preconception research and highlight opportunities for future studies to more systematically incorporate male partners into preconception care and research frameworksIn this pilot cohort, homozygous C677T and A1298C *MTHFR* variants were observed among male participants, and these genotype data are reported descriptively to characterise the sample.

These genotypes are known to impair folate metabolism [317, 319, 327, 370, 393, 394], reduce enzyme activity [106, 401-403, 601, 653-656], and elevate homocysteine [297, 657], factors linked to oxidative stress, sperm DNA fragmentation and methylation [33, 34, 114, 171, 172, 565, 566, 571], and impaired spermatogenesis [226] affecting fertility [177, 566].

Despite these findings, broader systemic barriers continue to hinder male inclusion. A recent systematic review found health professionals face persistent challenges in delivering preconception care, including limited training and limited time, unclear role responsibilities, and a lack of coordinated service models [545]. Research emphasises the importance of paternal health, showing that paternal exposures and behaviours significantly influence pregnancy outcomes and offspring health, yet remain under-addressed in clinical practice [177, 178, 589, 658, 659]. A recent website audit revealed that online resources for preconception care are inconsistent and often lack male-specific recommendations and male-focused content which limits public awareness of male importance in preconception care

[660]. Gender dynamics also play a significant role in shaping care preferences and experiences. While both men and women are affected by RPL, there is an under-recognition of men's own emotional and psychological needs in reproductive care settings [197, 479].

Taken together, these findings highlight opportunities to further develop preconception care models that more systematically include men and consider individual variation in folate metabolism. Future research may explore how personalised folate strategies could be evaluated for both partners, alongside efforts to strengthen professional education, public awareness, gender-sensitive care models, and accessible digital resources.

7.2 Implications for Professional Practice

The integrated findings of this thesis highlight areas of uncertainty and variation in reproductive health and nutrition, particularly in relation to folic acid exposure, genetic diversity, and the psychosocial experience of RPL. Considered together, these findings provide a framework for discussion across practice, education, leadership, and public health, rather than immediate implementation.

7.2.1 Practice

In clinical practice, the evidence presented in this thesis raises considerations around the suitability of uniform folic acid supplementation, particularly in the context of individual variation and RPL. Findings from the pilot RCT and the systematic reviews suggest that 5-MTHF warrants further evaluation as an alternative folate form, including investigation of its relationship with UMFA accumulation, within adequately powered studies.Although these findings are preliminary and require validation in larger, adequately powered studies, they identify areas of further investigation regarding cumulative FA exposure

in fortified settings and for clearer definitions of what constitutes 'excess' intake. Furthermore, the inclusion of male partners in the RCT demonstrated the feasibility of collecting genotype data in men and highlighted the relevance of considering paternal factors in future preconception research. Existing literature links folate metabolism to sperm quality and DNA integrity, supporting further investigation into the role of folate-related pathways in male reproductive health.

These findings raise considerations regarding how genetic variability in folate metabolism is discussed and managed in clinical contexts, without establishing recommendations for routine testing. In this pilot study, *MTHFR* genotypes were reported descriptively to characterise the cohort, and no inferences regarding prevalence or screening policy can be drawn.

While current guidelines do not recommend routine *MTHFR* testing, the findings of this thesis, together with existing literature, support further research to determine whether genetic information may assist in tailoring folate supplementation strategies in selected clinical or research settings. Such approaches require validation in adequately powered studies before any implications for clinical practice can be considered.

7.2.2 Education

The practitioner survey indicated that many health professionals feel underprepared to manage RPL, reporting a need for clearer protocols, additional resources, further training, and more dedicated time for affected couples. These findings suggest variability in practitioner knowledge regarding folate metabolism, the different forms of folate, and the psychosocial dimensions of RPL, and are consistent with broader literature reporting gaps in confidence and education in this area. Considered together,

these observations point to the potential value of further evaluation of educational approaches for health professionals, including training in trauma-informed care, patient-centred communication, and the psychosocial impact of pregnancy loss. Continuing professional development may also benefit from addressing interpretation of folate biomarkers and the complexity of folate metabolism in RPL, particularly in cases that remain unexplained.

The findings also highlight opportunities to explore more inclusive educational models that recognise the contribution of both partners in reproductive health. Future research could examine how nutritional counselling, lifestyle support, and folate-related education might be more consistently extended to male partners, alongside females, within preconception care frameworks. Any consideration of specific folate forms, including 5-MTHF, requires further investigation in adequately powered studies before implications for practice can be determined.

Firstly, school-level education for boys and girls where reproductive biology is integrated into the curriculum, and a clear message is given to both sexes that they contribute equally to the genetic make-up of a child, is needed. This should also include modules on nutrition, genetics, and fertility. Second, a public awareness campaign where media is used to promote the shared reproductive responsibility in couples and highlight the role of male health factors, including folate status, in fertility and pregnancy outcomes. This includes the normalisation of male participation in preconception care. Thirdly, curricula in medical and allied health training should be updated to include male fertility. In conclusion, the exclusion of men from preconception care is a missed opportunity for intervention. By integrating genetic screening

and personalised supplementation into male reproductive healthcare, outcomes for couples experiencing RPL could be improved and there would be a move toward a more equitable model of care. The evidence is clear: male fertility is not optional, it is foundational.

7.2.3 Leadership

The practitioner survey indicated interest among health professionals in clearer guidance, improved access to resources, and greater multidisciplinary collaboration in the management of RPL. These findings suggest opportunities for leadership to support the development and evaluation of guidance frameworks that reflect emerging evidence, while incorporating perspectives from clinicians, patients, midwives, and advocacy organisations.

Considered alongside the broader literature, these observations highlight areas where further research and stakeholder consultation may inform future discussions around folate supplementation, cumulative exposure, and variability in individual metabolism. Any consideration of genetic factors, folate safety thresholds, or regulatory approaches require careful evaluation and robust evidence before implications for guidelines or policy can be determined. The findings of this thesis therefore provide context for ongoing dialogue and future investigation, rather than definitive recommendations for practice or regulation.

7.2.4 Public Health Policy

The findings of this thesis have relevance for public health discussions in countries with mandatory folic acid fortification, particularly with respect to cumulative exposure from fortified foods and supplements. Current guidelines are largely predicated on population-level benefits for neural tube

defect prevention, yet the excess folic acid review (Chapter 2) identified reported associations between higher folic acid intake, UMFA, and selected maternal and offspring outcomes. These findings do not establish causality but highlight areas of uncertainty that warrant further investigation.

Importantly, the WHO red blood cell folate threshold and existing supplementation recommendations were developed specifically for neural tube defect prevention and were not designed to address RPL or cumulative exposure in fortified populations. Considered together, the literature and feasibility findings underscore the need for future research to better characterise biologically relevant exposure ranges for folic acid, particularly in settings where intake may be derived from multiple sources.

These observations point to several priorities for future policy-focused research, including evaluation of supplement formulations, assessment of cumulative exposure across population subgroups, and exploration of how public health messaging distinguishes between folic acid and other folate forms. Any consideration of genetic variability, alternative folate forms such as 5-methyltetrahydrofolate (5-MTHF), or adjustments to fortification strategies require robust evidence, stakeholder consultation, and careful assessment of equity and unintended consequences before policy implications can be determined.

Findings from the qualitative component of this thesis (Chapter 5) indicate that some participants perceived inconsistency in clinical advice and limited emotional support within current RPL care pathways. While these narratives are not representative of all patients, they highlight the importance of transparent communication and supportive care models when public health recommendations intersect with clinical practice.

Taken together, this thesis contributes to ongoing dialogue regarding FA fortification and supplementation by identifying areas of uncertainty, variability, and patient concern that merit further investigation. Rather than prescribing policy change, the findings support continued research and multidisciplinary engagement to ensure that future public health guidance is biologically informed, evidence-based, and responsive to diverse reproductive health contexts (Table 30).

Table 30 - Future implementation policies

Domain	Implication
Practice	Introduce personalised folate supplementation protocols, especially for individuals with *MTHFR* polymorphisms. Limit folic acid intake to biologically safe thresholds (≤400 mcg/day) and consider 5-MTHF as a safer alternative. Expand preconception care to include male partners, given the impact of folate metabolism on sperm quality and DNA integrity.
Education	Train health professionals on folate metabolism, *MTHFR* implications, and the risks of excess folic acid. Include modules on trauma-informed care and patient-centred communication in RPL. Enhance curricula with guidance on interpreting folate biomarkers and personalised supplementation strategies.
Leadership	Lead the development of national guidelines that incorporate genetic screening, folate safety thresholds, and stakeholder input. Advocate for regulatory reform in supplement content and fortification policies. Engage multidisciplinary teams and patient advocacy groups in guideline development and public health messaging.

Public Health	Update folate terminology to distinguish between FA and 5-MTHF. Educate on an upper safe limit and the potential risks of exceeding it. Freeze voluntary fortification programmes until more research is conducted. Limit FA supplementation to 400 mcg. Recognise *MTHFR* polymorphisms in folate metabolism and incorporate into screening programmes for RPL patients.

7.3 Future Research Directions

The findings of this thesis identify several areas of uncertainty in reproductive and recurrent pregnancy loss (RPL) research, particularly in relation to folate metabolism, genetic variability, and the experience of RPL. This pilot study, which included reproductive dyads and male partners, provides a feasibility framework for future investigations rather than definitive conclusions.

To build on the current evidence base, future research would benefit from being systematically structured across a range of complementary methodological domains, including clinical trials, mechanistic studies, policy analysis, observational research, qualitative inquiry, and implementation science. Together, these approaches address distinct aspects of the evidence required to better understand folate use in reproductive care and to inform future research directions for couples affected by RPL.

7.3.1.1 Clinical Trials

Future clinical research should prioritise large-scale, multi-centre RCTs comparing 5-MTHF and FA, stratified by genetic polymorphisms and folate biomarkers in RPL and infertility participants. These trials must assess not only UMFA, pregnancy outcomes, and long-term child health, but also

directly evaluate whether 5-MTHF is as effective as FA in preventing NTDs. So too, establishing the optimal dose and timing for supplementation is important and whether it is safe across diverse populations and genetic profiles. Establishing this safety profile is critical for regulatory acceptance and clinical confidence in 5-MTHF as a viable alternative to FA in all reproductive couples. Crossover trials are needed to compare individual responses to different forms and doses of folate, particularly in *MTHFR* genetically stratified groups. These designs would allow researchers to observe differences in biomarker response, methylation status, and clinical outcomes. Additionally, research should quantify the prevalence of *MTHFR* polymorphisms in RPL affected populations to better inform screening protocols and targeted interventions.

Dose response studies are required to determine the optimal timing and dose of 5-MTHF, especially in individuals with different *MTHFR* polymorphisms so as to inform clinical prescribing of 5-MTHF. Longitudinal studies should follow participants over time to assess developmental, reproductive, and metabolic outcomes associated with 5-MTHF supplementation. The pilot study demonstrated the feasibility of including male partners and reported *MTHFR* genotypes descriptively within the cohort. These observations support consideration of paternal factors in the design of future preconception and RPL research.

7.3.1.2 Basic Science and Mechanistic Studies

To deepen our understanding of the biological mechanisms underlying folate metabolism, future research must include mechanistic studies that investigate the underlying biochemical and molecular pathways involved in folate metabolism and methylation. These studies will explain why and how

UMFA accumulates, how methylation is impaired, why there is an epigenetic effect caused by excess FA, and how these alter reproductive outcomes. Studies should aim to define biological upper limits for folic acid intake and explore the implications of cumulative exposure from fortified foods and supplements. Mechanistic research may explore how *MTHFR* polymorphisms affect DNA methylation in gametes and embryos. It should also assess the role of cofactors such as vitamin B12 deficiencies, which may confound results in populations with nutritional deficiencies. These studies are essential to clarify the 'biological effects' of cumulative FA exposure. By better understanding the biological mechanisms underlying folate metabolism, these studies will help develop *MTHFR* gene specific supplementation strategies. By linking mechanistic studies with clinical evidence, future research can generate a more nuanced understanding of how folate metabolism influences fertility outcomes. We need to gain consensus around *MTHFR* polymorphisms and their effect on folate metabolism. Several key methodological issues have contributed to the lack of consensus. Firstly, there has been a failure to account for FA exposure. Many studies investigating the impact of *MTHFR* polymorphisms do not control for participants' intake of synthetic FA [80, 109, 661, 662]. This is a critical oversight, as FA can elevate serum folate concentrations without improving intracellular methylation, thereby masking the true impact of *MTHFR* variants on fertility and pregnancy outcomes [280]. Second, geographic and nutritional confounders: a significant portion of *MTHFR* research has been conducted in regions with low vitamin B12 status like India. Since B12 is a cofactor in the methylation cycle, deficiencies can confound results and obscure the effects of *MTHFR* polymorphisms [80, 113,

663].

Third, while this pilot study included both partners and reported *MTHFR* genotypes descriptively within the cohort, most existing studies continue to focus primarily on women. This is notable given literature indicating that male *MTHFR* status has been associated with sperm quality, DNA integrity, and reproductive outcomes [81, 109, 200, 201] . The limited inclusion of men in prior research highlights an area for further investigation rather than establishing conclusions from the present study.

In addition, methodological limitations in the existing literature include inadequate stratification by genotype, with many studies combining heterozygous and homozygous carriers. Given that enzymatic activity differs substantially by zygosity, such grouping may obscure genotype-specific effects and contribute to inconsistent findings [664]. Future research would benefit from more rigorous genotype stratification to clarify the potential relevance of *MTHFR* variants in reproductive health contexts.

7.3.1.3 Policy Analysis and Public Health Research

Policy focused research is needed to evaluate the safety, equity, and effectiveness of current folic acid-fortification programmes and supplement recommendations, ensuring that they equitably serve all population groups, including those such as children, the elderly, and those not intending to conceive, who may be disproportionately affected by cumulative exposure. This includes reassessing both voluntary and mandatory fortification policies to prevent overexposure. Economic modelling should be conducted to assess the cost-effectiveness of personalised supplementation, genetic screening, and the fortification of foods with 5-MTHF to avoid overexposure. Studies must explore whether marginalised populations are

disproportionately affected by current FA policies. Research should also investigate regulatory barriers to approving 5-MTHF as a standard supplement form and identify pathways for its inclusion in standard care. This research will help ensure that public health policy is aligned with emerging science and responsive to the variation in genetic differences across different populations.

7.3.1.4 Observational and Population Studies

Large scale observational studies are required to validate the role of 5-MTHF in preventing NTDs and improving reproductive outcomes across genetically diverse populations, particularly in instances of RPL. These studies should be tailored to individual genetic profiles to assess the responses to folate supplementation and evaluate real world outcomes of personalised folate protocols. Additionally, research should quantify the prevalence of *MTHFR* and related polymorphisms in RPL-affected populations to inform clinical screening strategies and clinical decision making. A critical area for investigation is the development of a definitive referral pathway for couples experiencing RPL. Currently there is no consistent protocol guiding whether patients should first consult a general practitioner, an EPAS unit or fertility specialist. Mapping these referral pathways and identifying gaps in access to specialised RPL care, such as EPAS units or dedicated miscarriage clinics, will help guide improvements in service delivery and ensure timely and appropriate care.

Findings from the practitioner survey and the qualitative sub-study indicated participant and practitioner reported experiences of fragmented care pathways, inconsistent clinical advice, and limited emotional support. These observations point to potential gaps in care coordination and suggest that

further evaluation of documentation practices and referral processes across healthcare settings may be warranted.

7.3.1.5 Qualitative and Trauma-informed Research

Qualitative research can further contribute to understanding the lived experiences of individuals affected by recurrent pregnancy loss (RPL), particularly among underserved or marginalised populations. In the qualitative sub-study, participants who consented to share their experiences described emotional distress associated with randomisation and prior pregnancy loss, including concerns about potential allocation to the folic acid arm. These accounts suggest that trauma-sensitive approaches may be relevant when designing future research involving individuals with a history of pregnancy loss.

Future studies could evaluate how trauma-informed trial designs influence recruitment, retention, and participant wellbeing, and explore the development of validated tools and support frameworks for individuals navigating grief, trauma, and anxiety related to pregnancy loss. In addition, the practitioner survey indicated that some health professionals perceive a need for clearer referral pathways and more RPL-specific appointments. Considered together, these insights may inform the design of emotionally responsive research and care models that take into account both patient and practitioner perspectives.

7.3.1.6 Implementation Science

Concerns about excess FA have been evident since 1998, when animal studies first demonstrated potential adverse effects, yet neither clinical practice nor government agencies have adequately responded. The excess FA review (Chapter 2) of human studies further reinforces these concerns,

highlighting associations between excessive FA and gestational diabetes, and neurodevelopmental issues in the offspring.

The growing body of evidence highlights ongoing challenges in translating emerging research into clinical practice. Implementation science offers a useful framework for examining how new evidence is adopted and integrated, including the evaluation of guidelines, protocols, and educational interventions over time.

Specifically, research should evaluate how practitioner education of *MTHFR*, folate metabolism, and personalised supplementation influences clinical decision-making and improves patient outcomes in RPL patients. The development of clinical decision aids, based on genetic and biochemical blood profiles, will support practitioners in prescribing appropriate forms and dosages of folate.

The practitioner survey indicated interest among respondents in clearer protocols, additional training, and more dedicated appointments for RPL, suggesting opportunities for implementation research to examine how care processes might be improved and made more consistent.

7.3.1.7 Conclusion

The findings of this thesis bring together feasibility, biochemical, and experiential insights that contribute to ongoing discussion about how reproductive and recurrent pregnancy loss (RPL) care might better account for individual variability. Rather than supporting a single uniform approach, the work highlights areas where biochemical individuality, genetic diversity, and patient-centred considerations may be relevant topics for further investigation. The future research directions outlined in this thesis span

clinical trials, mechanistic studies, policy analysis, and implementation science, each contributing complementary perspectives toward a more nuanced understanding of RPL care.

Considered collectively, these findings suggest opportunities for future work to explore how more coordinated models of RPL care might be developed in consultation with key stakeholders, including patients, midwives, and reproductive health organisations. Such work could examine referral pathways, the role of Early Pregnancy Assessment Services (EPAS), and the development of guidance tools that reflect both emerging evidence and patient priorities, without presupposing specific models or outcomes.

The thesis also highlights the potential value of integrative decision-making frameworks that draw on biochemical, genetic, and contextual information alongside traditional evidence-based approaches. Further research is required to determine whether and how such information can be used to support individualised care, particularly in conditions such as RPL where many cases remain unexplained. Importantly, this thesis does not propose changes to clinical standards or screening practices, but instead identifies areas where further investigation may be informative.

Finally, the inclusion of male partners in this pilot study underscores the feasibility of involving men in reproductive research and highlights paternal factors as an area for future study. Existing literature suggests that paternal health and folate-related pathways may influence reproductive outcomes; however, the present findings are descriptive and do not support prescriptive changes to care. Future research may further explore how male partners can be appropriately incorporated into both research designs and preconception

care discussions. Finally, 5-MTHF may be considered as an alternative folate form to folic acid, particularly in contexts where concerns exist about cumulative folic acid exposure and unmetabolised folic acid accumulation. Its biological activity and biomarker profile warrant further investigation, especially in genetically susceptible populations.

However, regulatory inertia and outdated fortification policies remain significant barriers. Future reforms and public health messaging must evolve to reflect these insights and support informed choices for maternal and fetal health. Importantly, these reforms should be guided by robust evidence and clinical insights, with stakeholder consultation involving researchers, clinicians, midwives, patients, and advocacy groups. This collaborative approach will help develop biologically informed public health campaigns and safer alternatives to FA, like 5-MTHF. In summary, we can move toward a more equitable and effective model of care for individuals affected by RPL and infertility.

7.4 Strengths and Limitations

This thesis demonstrates several methodological and conceptual strengths. The pilot randomised controlled trial (RCT) employed a double-blind design and utilised digital platforms for recruitment and data collection, supporting the feasibility of conducting remote clinical research in a sensitive population. Participant retention and adherence to study protocols were high, indicating good acceptability of the study procedures and relevance of the research question to participants.

The integration of multiple methodologies, including systematic literature reviews, a feasibility RCT, qualitative interviews, and a national practitioner survey enabled a broad exploration of recurrent pregnancy loss (RPL) from

biochemical, clinical, and psychosocial perspectives. This triangulation provides complementary insights and strengthens interpretation by contextualising findings across different data sources, while remaining within the limitations of each methodological approach.

The systematic reviews synthesised evidence relating to folic acid exposure and folate metabolism, identifying areas of uncertainty and variability in existing literature. Descriptive biochemical data generated in the pilot RCT contributed additional context regarding folate-related biomarkers within the study cohort; however, these findings were not powered to establish safety thresholds or clinical efficacy. Observations regarding unmetabolised folic acid (UMFA) accumulation and folate biomarkers are therefore presented descriptively and interpreted cautiously.

Inclusion of male partners in the pilot RCT demonstrated the feasibility of engaging men in preconception research and allowed genotype data to be reported descriptively for cohort characterisation. These data do not support inferences regarding prevalence, screening, or clinical practice, but highlight areas for future investigation.

Despite these strengths, the study has several limitations. The pilot RCT was not powered to detect statistically significant differences in clinical or biochemical outcomes, limiting generalisability. The small sample size and short intervention duration constrain conclusions regarding effectiveness. Formulation instability, specifically degradation of 5-MTHF associated with moisture-retaining excipients, emerged as an important confounder and may have influenced observed biomarker patterns, underscoring the need for validated stable formulations in future trials.

While the online study design facilitated recruitment, it introduced logistical challenges in blood sample collection, particularly for participants in remote locations. Additionally, the qualitative interviews and practitioner survey provide valuable insights into experiences and perceptions but are subject to response bias and limited representativeness. Further research with larger and more diverse samples is required to confirm and extend these findings.

7.5 Chapter Conclusion

This chapter integrated findings from all phases of the thesis to synthesise insights into the feasibility, contextual relevance, and areas of uncertainty surrounding personalised folate supplementation in couples experiencing recurrent pregnancy loss (RPL). Drawing on evidence from the literature reviews, pilot trial, qualitative interviews, and practitioner survey, the synthesis identified variability in current practice, differences in folate exposure across settings, and limited incorporation of personalised approaches within existing care models.

Considered together, these findings indicate that personalised folate supplementation is feasible to investigate within a patient-centred research framework and highlight priorities for future, adequately powered studies. They also underscore the importance of multidisciplinary, inclusive, and evidence-informed approaches that consider both biological and psychosocial dimensions of reproductive health, without establishing clinical or policy recommendations from this feasibility programme alone.

Chapter 8 Thesis Conclusion

This thesis presents a mixed method investigation into recurrent pregnancy loss, with a particular focus on the role of folate metabolism, *MTHFR* polymorphisms, and the feasibility of personalised supplementation strategies. The overarching aim was to evaluate the feasibility and clinical relevance of using 5-MTHF as an alternative to FA in couples experiencing RPL, especially those with impaired folate metabolism due to *MTHFR* polymorphisms.

To achieve this aim, six research objectives were addressed through two systematic reviews, a pilot RCT trial, qualitative interviews with trial participants, and a national practitioner survey. The research addressed each objective through distinct methodological phases, yielding meaningful insights across all areas.

The first research objective was to examine the feasibility of conducting an RCT trial comparing 5-MTHF and FA supplementation in couples with RPL. This was addressed in Phase 1 through the pilot RCT, which demonstrated that couples were willing to engage in a structured intervention involving dietary restrictions, genetic screening, and delayed conception. The trial confirmed that a larger study would be both feasible and acceptable, with high adherence and positive participant feedback.

The second objective of this thesis was to explore the relationship between 5-methyltetrahydrofolate (5-MTHF) supplementation and unmetabolised folic acid (UMFA) levels. This objective was addressed in Phase 1 through descriptive comparison of folate-related biomarkers in participants receiving

either 5-MTHF or folic acid (FA).

Within the constraints of this feasibility study, descriptive analyses indicated differing patterns of UMFA between the two intervention arms, with UMFA levels appearing lower at follow-up among participants receiving 5-MTHF and higher among those receiving FA. These observations are presented descriptively only and were not supported by inferential statistical testing. They are considered in the context of mandatory folic acid fortification, where baseline folic acid exposure may already be elevated.

An important methodological limitation identified during the trial was instability of the 5-MTHF formulation, with stability testing revealing degradation of the active ingredient during the intervention period. This formulation issue likely influenced observed biomarker patterns and limits interpretation of both biochemical and clinical outcomes.

Taken together, these feasibility-level findings do not establish clinical efficacy or safety differences between folate forms. However, they identify UMFA as a relevant biomarker for further investigation and support the need for larger, adequately powered trials using validated, stable formulations to examine folate metabolism, UMFA dynamics, and reproductive outcomes across different folate forms in fortified populations.

The third objective of this thesis was to characterise *MTHFR* polymorphisms within the pilot cohort and to explore their relevance to folate metabolism in the context of recurrent pregnancy loss (RPL). This objective was addressed through genetic testing conducted as part of the pilot study, with genotype data reported descriptively to characterise the sample. No inferences regarding population prevalence or sex-based differences were undertaken.

In parallel, findings from the qualitative sub-study (Chapter 5) indicated that some participants were aware of their *MTHFR* status prior to trial enrolment and described frustration regarding perceived inconsistency or dismissal of these concerns in clinical settings. These participant-reported experiences highlight gaps in communication and alignment between patient expectations and clinical practice, rather than establishing conclusions about guideline adequacy or clinical appropriateness.

Taken together, these findings do not support conclusions regarding the clinical utility of *MTHFR* testing or the comparative appropriateness of specific folate forms. Instead, they identify areas of uncertainty and variability that warrant further investigation in adequately powered studies, particularly in the context of unexplained RPL where standard diagnostic pathways often provide limited explanatory insight.

The fourth objective was to explore couples' experiences of current RPL care. This was addressed through a qualitative sub-study embedded within the pilot trial (Phase 1A), which captured the lived experiences of couples navigating RPL. Participants described significant emotional distress, disenfranchised grief, and frustration with fragmented and inconsistent care pathways. A recurring theme was the lack of acknowledgement of *MTHFR* polymorphisms by health professionals. This gap between patient expectations and clinical practice contributed to emotional distress and in some cases, reluctance to engage with conventional care and turning to online groups for support. These findings highlight the need for trauma-informed, patient-centred models of care that validate grief, support informed decision making, and integrate emerging biochemical and genetic evidence into RPL management.

The fifth objective was to explore the current prescribing practices of health professionals who support couples with RPL, with a particular focus on folate supplementation. This was addressed in Phase 2 through a national survey of health professionals, including general practitioners, fertility specialists, obstetricians, and midwives. The findings revealed considerable variability in prescribing behaviours, with most practitioners routinely recommending FA-based prenatal multivitamins to female patients, with little prescribing of alternative folate forms like 5-MTHF. Fewer than 1% of surveyed health professionals reported routinely testing for *MTHFR* polymorphisms. The survey also highlighted a significant gap in the inclusion of male partners in preconception care. This is particularly concerning given the well-established role of folate in DNA methylation, sperm quality, and embryonic development. Male participants in the pilot trial demonstrated high levels of compliance and were disproportionately affected by homozygous *MTHFR* variants, further highlighting the need to include men in folate related interventions and genetic screening protocols. Addressing these gaps is essential to improving the quality and consistency of care for couples experiencing RPL and to support the transition toward more personalised, inclusive and evidence-based reproductive health strategies.

The sixth research objective was to understand the needs of health professionals in supporting practice behaviour change regarding folate supplementation among couples with RPL. This was addressed through the national practitioner survey which revealed that many health professionals require more support to manage RPL effectively. Practitioners expressed a need for updated protocols, better referral pathways, and more explicit guidance on folate, both in terms of distinguishing between different forms

and determining appropriate dosages for various patient profiles, alongside additional resources to support both clinical decision-making and patient education. To address these gaps, the thesis proposes a national framework for RPL care, including a multi-level education model that begins with school-based reproductive health education, continues through public awareness campaigns promoting shared reproductive responsibility, and concludes with updated medical curricula and practitioner training. Ultimately the practitioner survey revealed a clear appetite for change and an opportunity for implementation research to support the transition toward more personalised, inclusive, and evidence-informed reproductive care. Equipping health professionals with the tools, knowledge and resources to support couples experiencing RPL is essential to improving outcomes.

In conclusion, this thesis offers novel insights and practical solutions to a complex and under-addressed area of reproductive health. It calls for a shift toward personalised care and provides a roadmap for future research, clinical innovation, and policy transformation. These recommendations are timely, actionable, and essential to bridge the gap between emerging science and current practice.

Chapter 9 Appendices

Appendix 1 – Publication Literature Review 1

nutrients

MDPI

Review

Women Taking a Folic Acid Supplement in Countries with Mandatory Food Fortification Programs May Be Exceeding the Upper Tolerable Limit of Folic Acid: A Systematic Review

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Abstract: Background: In preconception and pregnancy, women are encouraged to take folic acid-based supplements over and above food intake. The upper tolerable limit of folic acid is 1000 mcg per day; however, this level was determined to avoid masking a vitamin B12 deficiency and not based on folic acid bioavailability and metabolism. This review's aim is to assess the total all-source intake of folate in women of childbearing age and in pregnancy in high-income countries with folate food fortification programs. Methods: A systematic search was conducted in five databases to find studies published since 1998 that reported folate and folic acid intake in countries with a mandatory fortification policy. Results: Women of childbearing age do not receive sufficient folate intake from food sources alone even when consuming fortified food products; however, almost all women taking a folic acid-based supplement exceed the upper tolerable limit of folic acid intake. Conclusions: Folic acid supplement recommendations and the upper tolerable limit of 1000 mcg set by policy makers warrant careful review in light of potential adverse effects of exceeding the upper tolerable limit on folic acid absorption and metabolism, and subsequent impacts on women's health during their childbearing years.

Keywords: folate; folic acid; 5-methyltetrahydrofolate; fortification; unmetabolized folic acid; preconception; pregnancy

check for updates

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1. Introduction

Governments worldwide encourage women of childbearing age to take folic acid (FA) prior to pregnancy to avoid neural tube defects (NTDs) [1–10] and some governments (e.g., Australia, Canada and United States) have implemented a FA food fortification program to ensure that women who unintentionally conceive or are not using a folic acid supplement have sufficient folate to prevent neural tube defects [11,12]. Since the mandatory folic acid fortification program began in 2009, Australian policy makers have recommended 400 mcg of FA in addition to fortification for individuals considering conception [6].

While the fortification policy has prevented NTDs, emerging research has documented the presence of unmetabolized folic acid (UMFA) in the serum in people in countries that have a FA fortification program and/or take a FA supplement [13–15]. While the effect of this is unclear, some have raised concerns regarding UMFA and adverse health effects [16–21].

1.1. Folate Metabolism

Folate-dependent biochemical processes are influenced by the form and bioavailability of folate [22]. Folate is a generic term for vitamin B9 that includes several forms, including that naturally found in food (natural folate). All folate forms have a common structure but

https:doi.org/10.3390/nu14132715

Appendix 2 - Search Strategy Literature Review 1

We conducted electronic searches for eligible studies within each of the following databases on 7th and 8th October 2021:

Health:

MEDLINE (OVID)

Embase (Excerpta Medica database) (OVID)

Multidisciplinary:

CINAHL (EBSCO)

Scopus

Public Health, health promotion and occupational health databases:

BiblioMAP (EPPI-Centre database of health promotion research) - https://eppi.ioe.ac.uk/cms/Databases/tabid/185/Default.aspx

Search strategy: Andorra OR Antigua and Barbuda OR Aruba OR Australia OR Austria OR Bahamas OR Bahrain OR Barbados OR Belgium OR Bermuda OR "British Virgin Islands" OR Brunei OR Canada OR "Cayman Islands" OR "Channel Islands" OR Chile OR Croatia OR Curacao OR Cyprus OR "Czech Republic" OR Denmark OR Estonia OR "Faroe Islands" OR Finland OR France OR "French Polynesia" OR Germany OR Gibraltar OR Greece OR Greenland OR Guam OR "Hong Kong" OR Hungary OR Iceland OR Ireland OR "Isle of Man" OR Israel OR Italy OR Japan OR Korea OR Kuwait OR Latvia OR Liechtenstein OR Lithuania OR Luxembourg OR Macao OR Malta OR Monaco OR Nauru OR Netherlands OR "New Caledonia" OR "New Zealand" OR "Northern Mariana Islands" OR Norway

OR Oman OR Palau OR Poland OR Portugal OR "Puerto Rico" OR Qatar OR "San Marino" OR "Saudi Arabia" OR Seychelles OR Singapore OR "Sint Maarten" OR "Slovak Republic" OR Slovenia OR Spain OR "St. Kitts and Nevis" OR "St. Martin" OR Sweden OR Switzerland OR "Trinidad and Tobago" OR "Turks and Caicos Islands" OR "United Arab Emirates" OR "United Kingdom" OR "United States" OR Uruguay OR "Virgin Islands"

AND

pregnancy OR preconception care OR periconceptional OR pre-pregnancy OR prenatal care OR perinatal Care OR pregnant woman

AND

Folic acid OR folvite OR pteroglutamic acid OR vitamin B9 OR vitamin M OR unmetabolised folic acid OR unmetabolized folic acid OR folate

AND

Food, fortified OR food supplemented OR dietary supplements OR biofortification OR food supplements OR food assistance OR nutrition surveys OR health Surveys OR Prenatal supplement

Appendix 3 – Quality Assessment of Studies – Literature Review 1

Study Name	1. Introduction - aims/objectives clear?	2. Methods - study design appropriate?	3. Sample size justified?	4. Target/reference pop. clearly defined?	5. Sample frame appropriate ?	6. Was the selection process appropriate?	7. Non responders categorised?	8. Risk factors/outcomes appropriate to the aims?	9. Risk factors measured correctly?	10. SI and/or precision estimates?	11. Methods described for repetition?	12. Basic data adequately described?	13. Response rate & non-response bias?	14. Non-responders described?	15. Were the results internally consistent?	16. Results presented for all the analyses?	17.Discussions conclusions justified by the results?	18. Limitations ?	19. Funding sources/ conflicts?	20. Ethical approval/consent?
Yang 2007	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	UN	Yes	Yes	Yes	No	Yes
Whitrow 2009	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	UN	Yes
Trivedi 2018	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Tinker 2012 (obesity)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	UN	Yes
Tinker 2012	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	UN	Yes	No	No	UN	Yes	No	Yes	Yes	No	Yes
Sotres-Alvarez 2013	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Shin 2016	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Roy 2012	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		No	UN	Yes	Yes	Yes	Yes	No	Yes
Rai 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	UN	UN	Yes	Yes	Yes	No	Yes
Plumptre 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Pick 2005	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Murphy 2021-Impact	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Murphy 2021 - Gestational	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	UN	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Mojtabai 2004	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	UN	Yes
Masih 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Martinussen 2012	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes

Marchetta 2016	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Livock 2017	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Jun-20	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Hure 2009	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes		Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Hromi-Fiedler 2012	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	UN	No	Yes	Yes	Yes	Yes	No	Yes
Hamner 2013	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	No	Yes
Gomez 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	No	Yes	Yes	Yes	Yes	No	Yes
Gaskins 2012	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	UN	UN	Yes	Yes	Yes	No	Yes
Gaskins 2019	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Gaskins 2014	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	UN	No	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Furness 2013	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	No	UN	Yes	Yes	Yes	Yes	No	Yes
Dubois 2017	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	No	Yes
Dorise 2020	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No	Yes
Dietrich 2005	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	UN	Yes
Crider 2018	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Cena 2008	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No	Yes
Boeke 2013	Yes	Yes	UN	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	UN	Yes
Beringer 2021	Yes	Yes	UN	Yes	Yes	Yes	UN	Yes	Yes	No	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	No	Yes
Bailey 2019	Yes	Yes	Yes	Yes	Yes	Yes	UN	Yes	Yes	Yes	Yes	Yes	UN	UN	Yes	Yes	Yes	Yes	Yes	Yes
French 2003	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	UN	No	Yes	Yes	Yes	UN	No	Yes

Legend: UN=unknown, SI= statistical significance

Appendix 4 – Publication Details, Literature Review 2

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REVIEW · Articles in Press, 105240, September 11, 2025 · *Open Access*

Health effects of excess folic acid use and high blood folate during preconception and pregnancy: a systematic review

Carolyn Ledowsky [1] · Vanessa Scarf [2] · Agata Sobczyńska-Malefora [3,4] · Tristan Carter [1] · Nathan E Timbrell [3] · Amie Steel [1]

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Abstract

Adequate folate is essential for preventing neural tube defects (NTDs), therefore supplementation with folic acid (FA), is widely recommended for women during preconception and early pregnancy. However, the potential risks associated with excessive FA intake and blood concentrations of folate, are not well recognised. The aim of this review was to evaluate the potential adverse health outcomes associated with excessive FA intake and/or blood folate concentrations during preconception and pregnancy. The review included studies that measured FA intake via dietary sources and supplements, and/or quantified serum/plasma and red cell (RBC) folate concentrations. Excessive FA intake and elevated blood folate concentrations were linked to several adverse outcomes. The most significant association was with gestational diabetes mellitus, where elevated FA intake and high blood folate concentrations were found to increase the risk. Excessive FA supplement intake and high folate concentrations were linked to neurodevelopment in offspring, as well as metabolic disruptions, including insulin resistance, large for gestational age, higher birthweight and increased adiposity. Widely accepted cut-off values for excess folic acid supplementation and/or blood concentrations do not currently exist and therefore it is important that a definition of 'excess' is defined based on biological risks to prevent adverse health outcomes.

Key words

folate · folic acid · gestational diabetes · preconception · pregnancy · maternal health

https://www.rbmojournal.com/article/S1472-6483(25)00447-X/fulltext

Appendix 5 – Search Strategy Literature Review 2

1	folic acid/
2	folate.mp
3	folic acid.mp
4	((excess* or over-supplement* or high* or elevat*) adj1 (folic acid or folate)).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
5	unmetaboli?ed folic acid.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
6	umfa.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
7	vitamin B9.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
8	pteroglutamic acid.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
9	folvite.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
10	Vitamin M.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
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12	Pregnancy/
13	matern*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
14	pregnan*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
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19	prenatal*.mp.
20	pre-natal*.mp.
21	perinatal*.mp.
22	peri-natal*.mp.
23	Pregnancy Complications/
24	"folic acid"/ae

25	miscarr*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
26	RPL.mp. [mp=title, abstract, heading word, drug tradename, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
27	Infertility/
28	Infertility, Male/
29	Infertility, Female/
30	Premature Birth/
31	Prenatal Exposure Delayed Effects/
32	Abortion, Habitual/ or Abortion, Spontaneous/
33	recurrent pregnancy loss.mp.
34	11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22
35	(adverse* or risk* or safe* or toxic* or harm* or complicat* or side effect*).mp.
36	1 or 2 or 3 or 7 or 8 or 9 or 10
37	4 or 5 or 6
38	36 and 37
39	23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 35
40	34 and 38 and 39
41	limit 40 to yr="1998 -Current"

Appendix 6 – Characteristics of Studies Relating to Pregnancy- Literature Review 2

Study ID	Country	Date	Study design	Time point of FA exposure	Type of FA exposure	Blood FA measurement	Population description	Total number of participants	Did an increase in FA increase risk?	Study Quality
Fertility & Pregnancy										
Murto 2014	Sweden	2005-2010	Case control study	Preconception	Supplement	Plasma	Women diagnosed with infertility Control group - randomly selected from the same geographic area as the infertile women. The controls were healthy, proven fertile, non-pregnant and nonlactating women.	368: 180 unexplained infertility and 188 control	Yes	Moderate
Polzikov 2020	Russia	2017	Randomised controlled trial	Preconception	Dietary	Serum	Women aged 20-35 years of age with a regular menstrual cycle (21-35 days) with infertility	110	Yes	Strong
VanUitert 2014	Netherlands	2009-2010	Cohort study	1st Trimester	Dietary	RBC/serum	Women 18 years of age with ongoing intrauterine single pregnancies of 6-8 weeks gestation.	77 pregnancies	Yes	Strong
Vila-Nova et al, 2013	Brazil	2001-2010	Randomised controlled trial	1st trimester	Supplement	NA	Women 16-45 years of age with previous cleft lip/palate	268	No	Moderate

Preeclampsia										
Corsi 2022	Canada	Apr 2011-Dec 2015	Randomised controlled trial	All pregnancy	Supplement	NA	Pregnant women with twins	462	Yes	Moderate
Hashemi 2016	Iran	Feb 2015-Feb 2016	Randomised controlled trial	Preeclampsia diagnosis until delivery	Supplement	NA	Pregnant women with documented preeclampsia	85	No	Strong
Jankovic-Karasoulos 2021	Australia	Nov 2004-Sept 2008	Case control study	Preconception/1st trimester	Dietary/supplement	Serum/plasma	Nulliparous women with singleton pregnancies attending antenatal clinics at multiple SCOPE centres at 15 +- 1 weeks of gestation	325 women	Yes	Moderate
Li 2020	China	Sep-13	Cohort study	1st/2nd trimester	Dietary/supplement	NA	Pregnant women	4853	Yes	Strong
Manizheh 2009	Iran	Apr 2005-Feb 2008	Randomised controlled trial	All pregnancy	Dietary/supplement	NA	Nulliparous pregnant women aged 20-30 years	200	No	Moderate
Gestational Diabetes										
Chen 2021	China	March 2016-Dec 2018	Cohort study	Preconception/1st trimester	Supplement	RBC/serum	Pregnant women with folate and vitamin B12 measurements examined at	1058	Yes	Strong

							recruitment between 9-13 gestational weeks GDM assessed at 24 to 28 weeks			
Cheng 2022	China	2019-2021	Retrospective case-control study	2nd trimester	Dietary	Serum	Pregnant women	Total 744 - 147 GDM patients, 597 controls	Yes	Strong
Lai 2018	Singapore	Jun 2009-Sep 2010	Cohort study	3rd trimester	Dietary	Plasma	Pregnant women 18-46 years <14 weeks gestation and their offspring	1247 pregnant women, final analysis for bloods 913	Yes	Strong
Li 2019a	China	Oct 2017-Jan 2018	Cross-sectional study	2nd trimester/3rd trimester	Dietary	Serum	Chinese pregnant women 24-28 weeks' gestation	406	Yes	Strong
Li 2022	China	2017-2018	Cohort study	2nd trimester/3rd trimester	Dietary	Serum	Pregnant women 24-28 gestational weeks	1388 pregnancies	Yes	Moderate
Li2019b	China	Jan 2013-May 16	Cohort study	Preconception/1st trimester	Dietary	NA	Pregnant women	4353	Yes	Strong
Liu 2020	China	Oct 2018-Dec 2018	Cohort study	1st trimester	Dietary/supplement	RBC	Chinese women in early pregnancy	366	Yes	Strong
Liu 2023	China	Apr 2013-	Cohort study	1st/2nd trimester	Dietary	Serum	Pregnant women up to 24 gestational weeks	42,478	Yes	Moderate

		Mar 2017								
Saravanan 2021	UK	2012-2018	Cohort study	1st/2nd trimester	Dietary/supplement	Plasma/UMFA	Pregnant women <16 weeks' gestation attending antenatal care in 10 study sites across the UK	4746	Yes	Strong
Wen 2018	Argentina, Australia, Canada, Jamaica and UK	Apr 2011-Nov 2015	Randomised controlled trial	All pregnancy	Supplement: FA vs placebo	NA	Pregnant women with at least one high risk factor for preeclampsia	2464	No	Moderate
Xie 2019	China	May 2013-May 2014	Cohort study	2nd Trimester	Dietary	RBC	Pregnant women 19-24 weeks (second trimester of pregnancy)	Total 2282 - 392 with GDM, 1890 controls	Yes	Strong

Appendix 7 – Characteristics of Studies Relating to Offspring – Literature Review 2

Study ID	Country in which the study conducted	Date	Study design	Pregnancy or prepregnancy	FA exposure	Blood FA measurement	Population description	Total number of participants	Did an increase in FA increase risk?	Study Quality
Allergy										
Best et al, 2021	Australia	Nov 2011-Dec 2016	Cohort study	3rd trimester pregnancy	Dietary & supplements	serum; UMFA;	Pregnant women >18 years of age whose unborn infant had a first degree relative with a history of medically diagnosed allergic disease. Recruited women >36 wks' gestation Infants at 1 year of age	561 mother-infant dyads	No	Strong
Magdelijns et al, 2011	Netherlands	Oct-2000	Cohort study	3rd trimester pregnancy	Supplements	RBC	Health pregnancy women week 10-14 gestation = conventional n=2343 Recruitment through organic food shops/midwives = alternative group n=491 Children . Part of the KOALA	2343 Conventional 491 Alternative pregnant women 2640 children	No	Strong

							Birth Cohort study			
Molloy et al, 2020	Australia	2010-2013	Cohort study	3rd trimester pregnancy	Dietary/Supplements	RBC	Pregnant women 28-32 weeks' gestation and infants 1 year of age	1074	No	Moderate
Parr et al, 2017	Norway	1999-2008	Cohort study	2nd trimester	Dietary/ supplements	Plasma	Women 18 weeks' gestation	95,248 mothers 114,761 children	Yes	Strong
Roy et al, 2018	United States	2006-2011	Cohort study	1st/3rd trimester	Dietary/ supplements	Plasma	Mother-infant dyads	858	No	Moderate
Trivedi et al, 2018	United States	1999-2002	Cohort study	1st/2nd trimester	Dietary/ Supplements	NA	Participants were 1279 mother-child pairs from Project Viva.	1279 mother-child dyads	No	Moderate
Vereen et al, 2019	United States	Dec 2006-Jun 2011	Cohort study	2nd Trimester	Dietary/ supplements	Plasma	Mother-infant dyads	676	No	Strong
Yang et al, 2015	China	March 2010-March 2011	Case control study	Preconception	Supplements	NA	Infants with asthma infant controls	Infant asthma cases n= 150 control n= 212 meta-analysis involving 14,438 participants	Yes	Strong
Congenital Malformations										
Bortolus et al, 2021	Italy	Jul 2009-Feb 2014	Randomised controlled trial	Preconception	Supplements	NA	Women of childbearing age (18-44 years) who planned a pregnancy	1060 randomised, 505 pregnant women	No	Moderate

							within 12 months.			
Husen et al, 2022	Netherlands	Oct-04	Cohort study	Preconception	Dietary/supplements	RBC; serum; plasma	Pregnant women 9+6 weeks of gestational age, depending on the time of the first ultrasound	166	Yes	Strong
Michels et al, 2012	Netherlands	2003-2007	Case control study	Pre/1st trimester	Supplements	NA	Women of children with positional plagiocephaly (PP) and mothers without it (CO)	7516 of which 80/94 questionnaires were given to mothers of patients with PP. Control group 55/74	Yes	Moderate
Michikawa et al, 2020	Japan	2011-2014	Cohort study	Preconception	Dietary	NA	Women in pregnancy were recruited @12th week of gestation.	89,235	Unsure	Weak
Large for Gestational Age										
Xie et al, 2018	China	May 2013-May 2014	Case control study	2nd trimester	Dietary	RBC	Pregnant women 19-24 weeks Cases: Mothers who delivered LGA infants (birth weight >90% for gestational age) control: randomly selected at 2:1	Cases: 542 Control : 1084	Yes	Strong

							ratio and matched for age and gestational weeks at enrolment among the health mothers.			
Methylation										
Plumptre et al, 2020	Canada	Nov 2010-Jan 2012	Cohort study	1st and 3rd trimester	Dietary/supplements	RBC; serum; UMFA	Pregnant women 18-45 yrs between 12-16 weeks' gestation	368 healthy women aged 18-45 years, 289 deliveries at full term. PREFORM Study	Yes, cord blood	Strong
Neurodevelopment & cognitive function										
Compan-Gabucio et al, 2021	Spain	2003-2008	Cohort study	1st trimester/ 2nd/3rd	Dietary/supplements	NA	Mother-child dyads available at 4-5 year assessment (665 girls, 664 boys)	1329 mother-child dyad	Yes	Moderate
Compan-Gabucio et al, 2022	Spain	2003-2008	Cohort study	1st/3rd trimester	Dietary/supplements	NA	Pregnant women who gave birth to a live newborn	2625	No	Strong
Raghavan et al, 2018	United States	1998-2013	Cohort study	Post birth	Supplements	Plasma	Mother-infant pairs. Mothers of newborns 24-72 hours post-partum.	1257 infant pairs (86 ASD cases and 1171 neurotypical children)	Yes	Moderate
Raghavan et al, 2020	United States	1998-2014	Cohort study	Post birth	Dietary	Other: cord blood UMFA	Mothers who delivered a singleton live	567 children (92 ASD, 475 neurotypical)	Yes	Strong

							birth at Boston Medical Centre			
SharmanMoser et al, 2019	Israel	2000-2013	Case control study	Preconception/pregnancy	Supplement	NA	Children diagnosed with ASD	2009 children with ASD diagnoses They were matched on a ratio of 1:10 to 19,886 ASD free children	No	Strong
Valera-Gran et al, 2017	Spain	Feb 2004-Jan 2008	Cohort study	1st/2nd trimester	Dietary/Supplement	NA	Pregnant women (10-13 weeks gestation) mother-child pairs were included in the final analyses.	1682 mother-child pairs	Yes	Strong
Preterm Birth										
Olapeju et al, 2019	United States	1998-2014	Cohort study	Preconception & pregnancy	Supplement	Plasma	Mothers who delivered at the Boston Medical Centre recruited 24-72 hours after delivery while still hospitalised.	7576 and 2313 blood tests	No	Moderate
Small for Gestational Age										
Chen et al, 2015	Singapore	Jun 2009-Sept 2010	Cohort study	3rd trimester	Dietary/supplement	Plasma	Pregnant women who were attending antenatal care <14 weeks of gestation	986	Yes - high levels did not reduce SGA babies	Strong

Li et al, 2019 (c)	China	Aug 2013-Nov 2013	Cohort study	Preconception/pregnancy	Dietary/supplement	NA	Women post pregnancy with a live birth	7307	No	Strong
Navarrete-Munoz et al, 2019	Spain	May 2004-Aug 2008	Cohort study	1st/3rd trimester	Dietary/supplement	NA	Mother-child dyads	2302 mother-child pairs 2644 Women agreed to participate with a total of 2302	Yes	Strong
Nguyen et al, 2017	Vietnam	Oct 2011-May 2012	Randomised controlled trial	Preconception/pregnancy	Supplements	NA	Women of reproductive age	5011 in which 1813 became pregnant with 1639 live births.	Unsure	Strong
Pastor-Valero et al 2011	Spain	Feb 2004-Jun 2005	Cohort study	Preconception & pregnancy	Dietary/supplement	NA	Pregnant Spanish women aged 16+ years +	1563 eligible, 840 participated (54% participant rate), 786 gave birth	Yes	Moderate

Appendix 8 – Quality Assessment of Studies with Offspring Outcomes

Author	Selection Bias Rating	Study Design rating	Confounders rating	Blinding rating	Data collection rating	Withdrawal and drop out rating	Overall Rating
Best et al, 2021	2 = Moderate	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Bortolus et al, 2021	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	2 = Moderate
Chen et al, 2015	1 = Strong	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Compan Gabucio et al, 2021	1 = Strong	2 = Moderate	1 = Strong	NA	1 = Strong	3 = Weak	2 = Moderate
Compan Gabucio et al, 2022	1 = Strong	2 = Moderate	1 = Strong	1= Strong	1 = Strong	2 = Moderate	1= Strong
Husen et al, 2022	1 = Strong	2 = Moderate	1 = Strong	2 = Weak	1 = Strong	NA	1= Strong
Li et al, 2019 (c)	1 = Strong	1 = Strong	1 = Strong	NA	1 = Strong	NA	1= Strong
Magdelijns et al, 2011	1 = Strong	2 = Moderate	1 = Strong	NA	1 = Strong	2 = Moderate	1= Strong
Michels et al, 2012	2 = Moderate	1 = Strong	2 = Moderate	NA	3 = Weak	NA	2 = Moderate
Michikawa et al, 2020	1 = Strong	2 = Moderate	1 = Strong	NA	2 = Moderate	1 = Strong	3 = Weak
Molloy et al, 2020	2 = Moderate	2 = Moderate	1 = Strong	NA	2 = Moderate	1 = Strong	2 = Moderate
Navarrete-Munoz et al, 2019	1 = Strong	2 = Moderate	1 = Strong	NA	1 = Strong	1 = Strong	1= Strong
Nguyen et al, 2017	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	2 = Moderate	1= Strong
Olapeju et al, 2019	2 = Moderate	2 = Moderate	2 = Moderate	3 = Weak	1 = Strong	NA	2 = Moderate
Parr et al, 2017	2 = Moderate	2 = Moderate	1 = Strong	2 = Moderate	1 = Strong	NA	1= Strong
Pastor-Valero et al, 2011	3= Weak	2 = Moderate	1 = Strong	NA	1 = Strong	NA	2 = Moderate
Plumptre et al, 2020	1 = Strong	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	NA	1= Strong
Raghavan et al, 2018	2 = Moderate	1 = Strong	1 = Strong	3 = Weak	1 = Strong	2 = Moderate	2 = Moderate

Raghavan et al, 2020	1 = Strong	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Roy et al, 2018	2 = Moderate	2 = Moderate	1 = Strong	2 = Moderate	1 = Strong	3 = Weak	2 = Moderate
Sharman Moser et al, 2019	2 = Moderate	2 = Moderate	2 = Moderate	NA	2 = Moderate	NA	1= Strong
Trivedi et al, 2018	1 = Strong	2 = Moderate	1 = Strong	3 = Weak	1 = Strong	3 = Weak	2 = Moderate
Valera-Gran et al, 2017	1 = Strong	2 = Moderate	1 = Strong	NA	1 = Strong	1 = Strong	1= Strong
Vereen et al, 2019	2 = Moderate	3 = Weak	1 = Strong	1 = Strong	1 = Strong	NA	1= Strong
Xie et al, 2018	2 = Moderate	1 = Strong	1 = Strong	2 = Moderate	1 = Strong	NA	1= Strong
Yang et al, 2015	2 = Moderate	2 = Moderate	1 = Strong	NA	2 = Moderate	NA	1= Strong

Appendix 9 – Quality Assessment of Studies with Pregnancy Outcomes

Author	Selection Bias Rating	Study Design rating	Confounders rating	Blinding rating	Data collection rating	Withdrawal and drop out rating	Overall Rating
Chen et al, 2021	2 = Moderate	2 = Moderate	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1= Strong
Cheng et al, 2022	1 = Strong	1=Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Corsi et al, 2022	2 = Moderate	1 = Strong	1 = Strong	3 = Weak	1 = Strong	1 = Strong	2 = Moderate
Hashemi, 2016	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Lai et al, 2018	1 = Strong	2 = Moderate	1 = Strong	2 = Moderate	1 = Strong	2 = Moderate	1= Strong
Li et al, 2019 (a)	1= Strong	2= Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong
Li et al, 2019 (b)	2 = Moderate	2= Moderate	1 = Strong	1=Strong	1 = Strong	1 = Strong	1= Strong
Li et al, 2020	1 = Strong	2 = Moderate	1 = Strong	1= Strong	1 = Strong	1 = Strong	1= Strong
Li et al, 2022	1 = Strong	2 = Moderate	1 = Strong	2 = Moderate	1 = Strong	2 = Moderate	1= Strong
Liu 2020	1 = Strong	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	2 = Moderate	1= Strong
Liu 2023	1 = Strong	2 = Moderate	1 = Strong	3 = Weak	1 = Strong	2 = Moderate	2 = Moderate
Manizheh et al, 2009	1 = Strong	1 = Strong	2 = Moderate	3 = Weak	1 = Strong	1 = Strong	2 = Moderate
Murto et al, 2014	2 = Moderate	2 = Moderate	2 = Moderate	3 = Weak	1 = Strong	2 = Moderate	2 = Moderate
Polzikov et al, 2020	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Saravanan et al, 2021	2 = Moderate	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Van Uitert et al, 2014	2 = Moderate	2 = Moderate	2 = Moderate	2 = Moderate	1 = Strong	1= Strong	1= Strong
Vila-Nova et al, 2013	2 = Moderate	1 = Strong	1 = Strong	1 = Strong	3 = Weak	1 = Strong	2 = Moderate
Wen et al, 2018	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1 = Strong	1= Strong
Xie et al, 2019	2 = Moderate	2 = Moderate	2 = Moderate	1 = Strong	1 = Strong	1=Strong	1= Strong

Appendix 10 – Trial Study – History of Recurrent Pregnancy Loss Form

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Confirmation of recurrent pregnancy loss history

Protocol Number	U1111-1283-5932
Study Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Study Site	University of Technology Sydney Campus
HREC Approval Number	ETH22-7218
PhD candidate	Carolyn Ledowsky
Lead Researcher and co-researcher	Dr Amie Steel, Dr Vanessa Scarf,
Participant's Name	
Medical practitioners' name	
Medical practitioner's phone number	
Medical practitioner's email	
Medical practitioner's Signature	
Date	

I confirm that _____ (participant name) has a history of recurrent pregnancy loss, in that they have had two or more pregnancy losses.

Signed: _____

Name of Medical Practitioner: _____

Date: _____

Appendix 11 – Trial Study – List of Folic Acid-fortified Foods to Avoid

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Folic acid Food and non-folic acid food

By law, commercial bread flour must be fortified with folic acid. Non-commercial and organic products do not have to fortify but can if they choose to. The list on the left are the products with folic acid to avoid. The list on the right are goods that are NOT fortified with folic acid and can be consumed freely.
Should you have any favourite products you eat, without folic acid, and they are not on this list please let your researcher contact know so they can be added to the list.

Flour	
Fortified with Folic Acid	**Without Folic Acid**
Note: all wheat-based flours are fortified with folic acid, which does not need to be listed on the ingredient list.	White Wings Gluten Free Flour Orgran Plain Flour

Bread, Rolls & Bagels	
Fortified with Folic Acid	**Without Folic Acid**
Tip Top - The one - Sunblest - 9 Grain - Fruit Bread - Cafe Texas Toast - Buns & Rolls - Berger Thins - Sandwich Thins - English Muffins Wonder White Lawson's Bread Bakers Delight Helgas Burgen bread Abbott's bakery Coles bread range Woolworths bread range Genius Bread, Rolls & Wraps	Bill's Bread • Multifruit • Ancient Grains • Wholemeal • Multigrain • 100% Spelt • Medium Rye • Khorasan Whole Green Bakery (All products) Naturis Bakery (All products) Pure Life (All products) Well and Good Bake at Home bread Genius Gluten Free Bagels Helga's Gluten Free bread Tip Top Gluten free bread Abbott's Bakery gluten free bread

Folate Trial for Recurrent miscarriage
Folic acid fortified foods list
Version 2: 24/7/22

Abes Bagels - Woolworths/Coles Sanoma breads	Coles Gluten Free bread Naturally Gluten Free Products

Wraps	
Fortified with Folic Acid	**Without Folic Acid**
Note: commercially produced wraps must be fortified with folic acid, which does not need to be listed on the ingredient list. Mountain Bread wraps Simson's Pantry wraps Nana Bakery Lebanese wraps Mission wraps Helga's wraps Woolworth's wraps Coles wraps	Note: organic products and wheat free products are exempt from mandatory fortification. However, they may choose to add it and must list it on the label. Mountain Bread Natural Wraps Mission Gluten Free Wraps Coles Gluten Free wraps

Crumpets & Pikelets	
Fortified with Folic Acid	**Without Folic Acid**
Note: commercially produced pikelets and crumpets must be fortified with folic acid, which does not need to be listed on the ingredient list. Golden Bakery crumpets Woolworths crumpets Coles crumpets Coles pikelets Golden Bakery pikelets	Note: organic products and wheat free products are exempt from mandatory fortification. However, they may choose to add it and must list it on the label. Genius Gluten Free crumpets Liberate Gluten Free crumpets Genius Gluten Free pancakes

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Cereal	
Fortified with Folic Acid	**Without Folic Acid**
Note: some manufacturers also voluntarily choose to fortify other foods with folic acid e.g., breakfast cereal. **Kellogg's** - All Bran - All Bran Fruit n Oats - Bran Flakes - Corn Flakes - Corn Flakes Gluten Free - Golden Wheats - Guardian - Just Right - Mini Wheats Apricot - Mini Wheats Blackcurrant - Mini Wheats strawberry - Mini Wheats Whole Wheat - Special K - Special K Gluten Free - Sultana Bran - Sultana Bran Gluten Free - Coco flakes - Coco Flakes Gluten Free - Weet-Bix - Weet-Bix Gluten Free Lowan Flake Medley with Wild Berries Sanitarium - Cornflakes - Fruity Box – Apricot - Fruity Box – Tropical - Fruity Box – Wild Berry - Good Start - Light n Tasty - Lite-Bix - Soy Tasty - Weet-Bix - Weet-Bix HiBran Soy & Linseed - Weet-Bix plus Oat Bran Uncle Toby's - Lite Start Breakfast Bars	Note: organic products and wheat free products are exempt from mandatory fortification. However, they may choose to add it and must list it on the label. **Carman's** - Fruit and Nut Muesli - Cranberry, Blueberry and Goji - Fruit Free - Bircher - Berry Bircher - Crispy Deluxe Almond & Maple - Crispy Deluxe Fruit & Nut - Berry Bircher - Low Sugar Toasted Almond & Cashew Sanitarium - Organic Weet Bix Macro - Organic Corn Flakes - Organic Rolled Oats - Organic Quick Oats Kialla Pure Organics - Overnight Oats Uncle Toby's - Quick Oats - Rolled Oats Freedom Foods - Active Balance Varieties - Maple Crunch - Corn Flakes - Rice Puffs - XO Crunch Berry Flavour - Maple Almond Clusters - Orgran Cereals - Corn Flakes - Rice Puffs

- Lite Start Breakfast Cereal Carman's - Low Sugar Raspberry & Vanilla Cereal - Sultana & Cinnamon Cereal - Almond & Coconut Cereal	

Baking	
Fortified with Folic Acid	**Without Folic Acid**
Note: products containing wheat flour must be fortified with folic acid, which does not need to be listed on the ingredient list. Green's cake mixes Betty Crocker cake mixes White Wings cake mixes Coles cake mixes Woolworth's cake mixes Noshu Sugar Free Cake/Biscuit Mixes	Note: organic products and wheat free products are exempt from mandatory fortification. However, they may choose to add it and must list it on the label. Kialla Pure Organic Pancake Mix Coles I'm Free From pancake mix, cake and brownie mixes Well and Good (all products) Orgran Products

Fruits and Vegetables	
Fortified with Folic Acid	**Without Folic Acid**
None are fortified	All fruits and vegetables are fine to eat

Protein	
Fortified with Folic Acid	**Without Folic Acid**
No meats/fish/chicken are fortified	All fresh beef, veal, chicken, lamb, offal All fish and seafood All fine to eat

Appendix 12 – Trial Study – Qualtrics Participant Screening Form

Qualtrics initial screening questionnaire

Thank you for enquiring about participation in the **Folate for Pregnancy Success** trial.

Please answer the questions below to check your eligibility for trial participation.

Recruitment for the trial will commence in September 2022 and aims to finish by April 2023. Participants will be involved in the trial for up to 10 months after they start.

This trial is testing two forms of folate – folic acid and methyl folate - to see if one is better than the other to assist women who have recurrent pregnancy loss achieve a successful pregnancy. If you are involved in the trial, you will be asked to take a prenatal multivitamin and make some dietary changes. More detailed information will be given to you as soon as we determine that you are eligible for this trial.

Below is a snapshot of the trial information:

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Folate Trial for Recurrent Miscarriage

Summary Participant Information Sheet

Title	Folate Trial for recurrent miscarriage
Short Title	Folate trial for recurrent miscarriage
Protocol Number	*[Protocol Number]*
Project Sponsor	None
Coordinating Principal Investigator/ Principal Investigator	Carolyn Ledowsky
Associate Investigator(s)	Dr Amie Steel Dr Vanessa Scarf
Location	Australia (online recruitment)

1 Introduction

You are invited to take part in this research project because you have had multiple miscarriages. The research project is testing a new treatment to help you successfully achieve pregnancy. The new treatment is a different form of folate called 5-methyl folate (5-MTHF).

This Summary Participant Information Sheet provides an introduction to the research project. Knowing what is involved will help you decide if you want to take part in the research. If you are eligible to participate and interested in participating, we will send you more detailed information and organise to meet with you to answer any questions.

Participation in this research is voluntary. You do not have to take part in this study if you do not wish. You will receive the best possible care whether or not you take part.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

- Understand what you have read
- Consent to take part in the research project
- Consent to have the tests and treatments that are described
- Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

2 What is the purpose of this research?

The aim of this study is to compare 5-MTHF with folic acid to improve the rate of pregnancy success in couples who have experienced recurrent pregnancy loss. Currently folic acid, is the main form of folate given to women who are considering falling pregnant. 5-methyl folate is an

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active form of folate which is metabolised differently but has not been clinically trialled in women with recurrent pregnancy loss. Our project will help to fill the gap in knowledge about the effectiveness of this form to help achieve pregnancy success.

This study may help inform new approaches to supporting couples with recurrent pregnancy loss, and the health professionals providing care to these couples.

This study will contribute towards the requirements of a Doctor of Philosophy for the principal investigator, Carolyn Ledowsky.

3 What does participation in this research involve?

To participate in this research, you will be asked to do the following:

1. Take three capsules of a prenatal multivitamin daily
2. Avoid foods with folic acid fortification (you will be given a list of what you can and cannot eat)
3. Have blood tests that include Vitamin B12, Folate, homocysteine
4. Check for a gene that affects the way you make biologically active folate
5. Meet with the lead researcher once every 6 weeks of the trial for the duration of the time you are in the trial
6. Your reproductive partner must agree to participate in this research as well

4 How long will the trial run?

There will be a recruitment stage that will run from September 2022 until April 2023. You will remain in the trial for 10 months from when you begin trial involvement.

5 You can withdraw consent at any time during the trial

You can withdraw your consent at any time throughout the trial.

6 To be eligible your partner must also consent to be involved in the trial

Your partner will also be invited to participate in the trial. This is because a baby is conceived with genetic material from both partners. We want to ensure that both partners are taking a form of folic acid. Should your partner not provide consent to be included in the trial, then you cannot participate.

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I consent for details of my previous pregnancies and some personal data to be collected to see if I qualify for this trial.

- ❑ I have read the Information Sheet above, or someone has read it to me in a language that I understand.
- ❑ I understand the data collected now will be to see if I qualify for the trial
- ❑ If I do qualify, I understand that a member of the research team will contact me to give me more information about the trial and I will have an opportunity to ask questions and decide if I want to participate in the trial.
- ❑ I understand that I will be given a signed copy of this document to keep.

Name of Participant (please print) _____

Signature _____ Date _____

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Please complete the following questions to assess your eligibility:

Question 1 – Have you had two or more miscarriages before reaching 22 weeks gestation?

Question 2 – Are you between 20 and 49 years of age?

Question 3 – Can you speak and understand English sufficiently to comprehend the purpose and risks of the study and provide consent?

Question 4 – Are you able to attend a pathology centre for collection of blood samples?

Question 5 – Are you pregnant or breastfeeding?

Question 6 – Are you undergoing any IVF or assisted reproductive technologies (i.e.: Intrauterine insemination (IUI), Intracytoplasmic Sperm Injection (ICSI) etc.)

Question 7 – Are you taking any **of the following medications?**

- phenytoin (e.g., Dilantin, Dilantin-125, Dilantin infatabs,)
- valproic acid (e.g., Sodium Valproate Sandoz, Valprease 200, Valpro EC, Valproate Winthrop EC 200, Epilim EC)
- carbamazepine (e.g., carbamazepine 100mg/400mg/200mg, oral liquid, modified release tablet)
- methotrexate (e.g., methotrexate Accord, Methotrexate Ebewe)
- trimethoprim hydrochloride (e.g., Malean, Myleran, Trimethoprim Mylan, Zyban)
- trimethoprim sulphate (e.g., Bactrim, Septra)
- trimethoprim (e.g., Triprim. Alprim)
- aminopterin sodium (e.g., Aminopterin)
- phenobarbital (e.g., Phenobarb)
- phenobarbital sodium (e.g., Phenobarbitone)
- primidone (e.g., Apo-amiodarone, Apo-clonidine, Apo-cyproterone, Apo-eplerenone, Apo-frusemide)
- divalproex (eg., Depakote, Depakote ER

Question 8 – Do you have a known sensitivity to any form of folate.

Question 9 – Do you or have you had any of the following health conditions:

- antiphospholipid syndrome
- blocked fallopian tubes
- Pelvic inflammatory disease
- Primary ovarian insufficiency
- endometriosis
- Factor V Leiden

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- Schizophrenia
- Bipolar disorder
- Psychosis
- Autoimmune disease
- Cancer

(Submit button)

Then Yes/No they are eligible

No:

Unfortunately, you are not eligible for this trial. Please contact your medical practitioner for support for your recurrent pregnancy loss or if you feel you need to speak to someone about how you are feeling, you could ring Lifeline on 131114 (local call).

Yes:

Thank you for your interest. You are eligible to participate in this trial. Please provide your preferred contact details below so we can send you detailed information on the trial and organise a time with the lead researcher to review the information and answer any questions

Please provide your Name. First/last

Please provide your best contact number.

Please provide your email address where we can send you detailed information on the trial.

Click this link to select your preferred day/time to meet with the lead researcher (link to acuity scheduling platform to select day/time

Appendix 13 – Trial Study – Participant Information Sheets – Females

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Folate Trial for recurrent miscarriage

Summary Participant Information Sheet

Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Short Title	Folate trial for recurrent miscarriage
Protocol Number	*[Protocol Number]*
Project Sponsor	None
PhD Candidate	Carolyn Ledowsky
Lead Researcher	Dr Amie Steel
Co researcher	Dr Vanessa Scarf
Location	Australia (online recruitment)

PART 1 - WHAT DOES MY PARTICIPATION INVOLVE?

1.1 Introduction

You are invited to take part in this research project because you have experienced recurrent pregnancy loss. The research project is a pilot study designed to test study procedures for a larger trial and is testing a new treatment to help you successfully achieve pregnancy. The new treatment is called 5-methyl folate (5-MTHF).

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Folate Trial for recurrent miscarriage

Summary Participant Information Sheet

Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Short Title	Folate trial for recurrent miscarriage
Protocol Number	*[Protocol Number]*
Project Sponsor	None
PhD Candidate	Carolyn Ledowsky
Lead Researcher	Dr Amie Steel
Co researcher	Dr Vanessa Scarf
Location	Australia (online recruitment)

PART 1 - WHAT DOES MY PARTICIPATION INVOLVE?

1.1 Introduction

You are invited to take part in this research project because you have experienced recurrent pregnancy loss. The research project is a pilot study designed to test study procedures for a larger trial and is testing a new treatment to help you successfully achieve pregnancy. The new treatment is called 5-methyl folate (5-MTHF).

This Detailed Participant Information Sheet tells you about the research project. It explains the tests and treatments involved. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you do not understand or want to know more about. Before deciding whether to take part, you might want to talk about it with a relative, friend or your local doctor.

Participation in this research is voluntary. If you do not wish to take part, you do not have to. You will receive the best possible care whether or not you take part.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

- Understand what you have read
- Consent to take part in the research project
- Consent to have the tests and treatments that are described
- Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

1.2 What is the purpose of this research?

The aim of this study is to conduct a pilot study, which is a mini trial in preparation for a larger trial to compare 5-methyl folate with folic acid to achieve successful pregnancies for couples who have experienced recurrent pregnancy loss. Folic acid is the main form of folate given to women who are considering falling pregnant. 5-methyl folate is an active form of folate that is metabolised by the body has not been clinically trialled in women with multiple miscarriage.

This study may help inform the researchers on how best to conduct a larger study of this nature, including what worked, what the results were and how we can produce this trial for a larger number of people. It may also help understand new approaches to support couples with recurrent pregnancy loss, and the health professionals providing care to these couples.

Many studies have evaluated the importance of folate in the development of the neural tube and to prevent neural tube defects. Folate is essential for gene health which ensures the integrity of

both the sperm and egg. In Australia, it is typically recommended that individuals take 500mcg of folate per day.

In Australia, medications, drugs, and devices have to be approved for use by the Australian Federal Government agency, the Therapeutic Goods Administration. *5-MTHF* is approved in Australia as a form of folate to be included in multivitamins and prenatal supplements.

This research has been initiated by the principal investigator, Carolyn Ledowsky, and will contribute towards the requirements of her Doctor of Philosophy.

This research has not been funded by a government body and is conducted by the University of Technology Sydney. There is no commercial sponsorship of this research study.

1.3 Who can participate in this Study?

Couples must meet the following criteria to participate in this study:

a) Have had two or more medically confirmed pregnancy losses before 22 weeks gestation
b) Be able to speak and understand English sufficiently to comprehend the purpose and risks of the study and to provide consent
c) Ability to attend a local blood collection centre for collection of bloods
d) You must be willing to take a prenatal multivitamin every day until your partner conceives or until you withdraw from the study
e) You must be willing to follow a diet that avoids folic acid-based foods. This does not mean you cannot eat certain foods, but rather will be given alternate brands to choose from
f) Your partner must be willing to be included in the trial (he can opt out if you conceive during the trial period)

You will be ineligible for this study if you:

a) Are currently pregnant or breastfeeding
b) Are currently undergoing any assisted reproductive technologies such as IVF, ICSI.
c) Are currently taking medication that might interfere with folate absorption such as:
   - Phenytoin (i.e., Dilantin, phenytek)
   - Valproic acid (i.e., Depakene, Stavzor)
   - Valproate sodium
   - Carbamazepine (i.e., Tegretol, Equetro, Epitol, Tegretol XR, Carbamazeipine chewtabs, carbamazepine CR, Carbatrol, Teril, Carnexiv
   - Methotrexate

- Trimethoprim hydrochloride (i.e., antibiotic under the name of Primsol, proloprim, TMP)
- Trimethoprim sulphate
- Trimethoprim
- Aminopterin sodium
- Phenobarbital
- Phenobarbital sodium
- Primidone (i.e., Mysoline)
- Divalproex (i.e., Depakote, Depakote ER, Depakote Sprinkles)

d) Have a known sensitivity to folic acid or methyl folate (5-methyltetrahydrofolate)

e) Have a history of a psychiatric disorders including severe anxiety and/or depression, autoimmune disease, cancer, antiphospholipid syndrome, endometriosis, Factor V Leiden, fallopian tube blockage, sexually transmitted infections, polycystic ovarian syndrome, or uterine fibroids.

1.4 What will happen first?

You will be randomly selected by a computer to enter one of the following groups:

a) Folic acid based prenatal multivitamin)

b) Intervention group (5-MTHF folate based prenatal supplement)

None of the researchers on this project and none of the participants will know which participants are in which group. Your chance of entering either group is 50%.

The trial will run for a total of 12 months: two reproductive cycles not attempting to conceive, and 6 months attempting to conceive or pregnant.

Once you have been initially screened for eligibility, the principal investigator will organise an online meeting to explain all the features of this trial to you and confirm your eligibility. During this first meeting eligible participants will be invited to enrol in the study. Individuals will also be provided with the opportunity to enrol at a later time if they wish to consider their involvement further.

Once enrolled, you will then be asked to complete the following tasks within the first week:

1. ***Visit a blood collection clinic near you to collect blood samples*** to allow us to test for:
   - *Homocysteine* - this is a marker that can indicate your body's use of folate and vitamin B12. This will only be tested in the first week of study involvement.

- *Vitamin B12* – this nutrient is required for the uptake of your folate. It will be retested at 12 weeks following your introduction of the prenatal.
- *Serum folate and red cell folate* – serum folate represents more recent folate intake, whereas red cell folate represents long-term intake
- *MTHFR gene test* – this gene makes an enzyme that is the last step in the folate cycle
- *Unmetabolised folic acid* – this is the amount of folic acid in your blood
- *Methylmalonic acid* – this is a marker for Vitamin B12 deficiency

2. ***Commence the prenatal multivitamin*** that has been randomly allocated to you and your partner. You will be required to take three capsules per day. Your researcher will give you instructions on how to introduce the supplements.
3. ***Avoid all folic acid-fortified foods***. This may mean you need to swap to brands that do not add folic acid to foods but should not mean you need to avoid a food group entirely. A detailed information sheet will be provided to you showing you which foods you should avoid and which foods to include. We are asking you to do this as we do not want any fortified foods to influence the level of folate you are taking affect the outcome of the trial. Please note this is an important component of the trial as folic acid can build up if you are taking both supplements and foods fortified with folic acid.
4. ***Avoid taking any other vitamin or mineral supplements unless advised by a medical practitioner***. You should notify the research team of any additional supplements used during the trial period.

We will advise you to take the prenatal for a 'pre-conception period' of 2 reproductive cycles, during which time we ask that you do not try to fall pregnant. This is because it can take some time for folate levels in the body to reach optimal levels. Following the two to three-month period, you will be free to attempt pregnancy.

1.5 What will happen during the trial?

During the trial, the following will be required:

1. ***Visit a blood collection centre upon enrolment and at specific time points throughout the trial*** to retest your serum folate, red cell folate, Vitamin B12, methylmalonic acid unmetabolised folic acid and MTHFR gene test.
    a. Week 6 – unmetabolised folic acid
    b. Week 12- red cell folate, serum folate

2. ***Take the prenatal every day (3 capsules)***
3. ***Meet with the lead researcher online at the start of the trial, Week 1, 2 and 3 to ensure a smooth introduction of the multivitamin and diet protocols and then every six weeks for the duration of the trial.*** This check in will take no longer than 30 minutes. During this time, the researcher will discuss:
   - Your adherence to the required prenatal vitamin intake
   - Your adherence to the dietary guidelines aimed at avoiding dietary folic acid
   - Any adverse events that may have happened
   - Any support you may need during the trial
   - Any feelings of anxiety or stress associated with the trial
   - Any issues you have had since the last meeting
4. ***Complete an online form before each meeting with your lead researcher.*** There will be a separate form for male and female partners. Female partner form will collect data such as:
   - Your menstrual cycle
   - Your period length
   - Your ovulation symptoms i.e.: cervical mucus and/or temperature
   - Your pregnancy status
   - Any issues you may be having with the prenatal multivitamin
   - Any issues you may be having with the diet
   - Any adverse effects
   - Any stress

Each meeting with your researcher will be via video conference software (e.g., Zoom) or telephone. You will be able to do this follow up from your home or office and does not require any travel.

The trial will run from January 2023 until December 2023. Depending on when you join the trial you will be in this trial for 12 months.

Participants will not be accepted after April 30, 2023.

In addition to the prenatal vitamin supplement, all participants will receive a $50 Visa card as a recognition of any inconveniences associated with your involvement in this trial.

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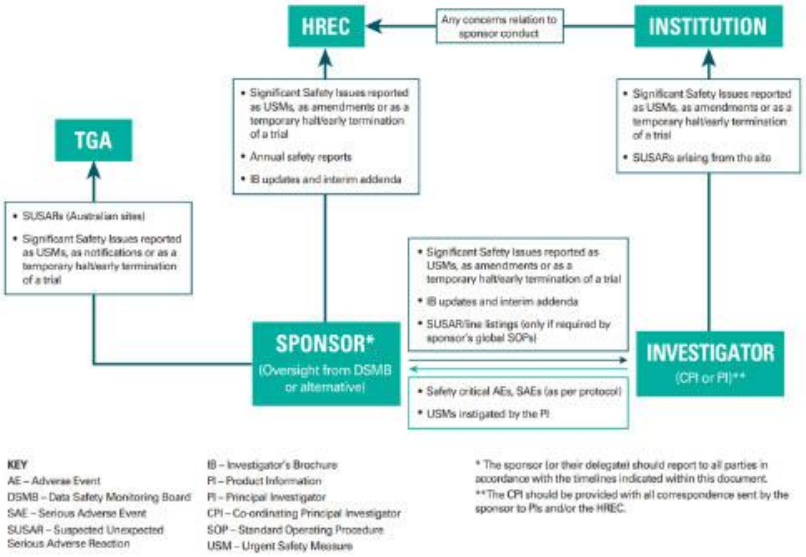
1.6 Human Research Rights

Human research is governed by Australian law that establishes rights for participants and imposes general and specific responsibilities on researchers and institutions. This research will be compliant with legal obligations and will be monitored by the whole research team at the University of Technology Sydney. At all stages of the research study, you and your partner will be monitored for safety. Any adverse events will immediately be reported to the safety committee which consists of a fertility specialist, a general practitioner, a clinical trial specialist and nutritionist, a psychologist, the lead researcher, and a senior member of the research team to decide if the adverse event is due to the prenatal product or not. Below is the reporting guidelines and flow chart for any adverse events during the trial as stated by the National Health and Medical Research council of Australia.

Appendix 1: Report Flowchart for Investigational Medicinal Product Trials



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1.7 Who receives the different products?

You will be participating in a pilot study for a further randomised controlled research project. Sometimes we do not know which treatment is best for treating a condition. To find out we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same, each participant is put into a group by chance (random). You will have a 50/50 chance of receiving the investigational product (5-MTHF) or the control (folic acid) product.

You will be participating in a double-blind study. This means that neither you nor your study researchers will know which treatment you are receiving. However, in certain circumstances one of the research team may need to find out which treatment you are receiving.

This research project has been designed to make sure the researchers interpret the results in a fair and appropriate way and avoids researchers or participants jumping to conclusions.

There are no additional costs associated with participating in this research project, nor will you be paid. All prenatal products, tests and medical care required as part of the research project will be provided to you free of charge.

If you have a local doctor, we strongly recommend that you inform them of your participation in this research project as confirmation of your history of recurrent pregnancy loss will be required by your GP.

1.8 What do I have to do?

There will be no physical or lifestyle restrictions placed on you during this study

You will be required to avoid folic acid-fortified foods. A list of foods fortified with folic acid and of foods not fortified with folic acid will be provided to you.

You will be able to continue with your regular medication as long as it does not interfere with folate metabolism.

The list of medications not allowed is included in the exclusion criteria above (see list under 'Section 1.4: Who can participate in this Study?')

You can still donate blood should you wish to during the trial.

It is your responsibility to ensure that you take the prenatal multivitamin each day and that you avoid folic acid fortified foods.

1.9 Other relevant information about the research project

There will be up to 60 couples taking part in this research project: 30 couples will be assigned the control product (folic acid-based multivitamin), 30 couples will be assigned the intervention product (5-MTHF based multivitamin). The research project will only be conducted in Australia.

1.10 Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part, you do not have to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

If you do decide to take part, you will be given this Detailed Participant Information Sheet and a Consent Form to sign. You will be given a copy of each to keep.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the University of Technology Sydney.

1.10 What are the alternatives to participation?

You do not have to take part in this research project to receive fertility support. Other options are available; these include consulting with your GP and/or seeing a fertility specialist. Your study researcher will discuss these options with you before you decide whether or not to take part in this research project. You can also discuss the options with your local doctor.

1.11 What are the possible benefits of taking part?

We cannot guarantee or promise that you will receive any benefits from this research; however, possible benefits may include having a successful pregnancy.

1.12 What are the possible risks and disadvantages of taking part?

The disadvantages of you joining this research may be:

- That you do not achieve a successful pregnancy
- That you may have another miscarriage

Supplements often cause side effects. You may have none, some or all of the effects listed below, and they may be mild, moderate, or severe. If you have any side effects, or are worried about them, talk with your study researcher. Your study researcher will also be looking out for side effects.

There may be side effects that the researchers do not expect or do not know about and that may be serious. Tell your study doctor immediately about any new or unusual symptoms that you get.

Many side effects go away shortly after treatment ends. However, sometimes side effects can be serious. If a severe side effect or reaction occurs, your study researcher may need to stop your treatment. Your study researcher will discuss the best way of managing any side effects with you.

Known possible side effects associated with ***folic acid*** supplement use are headaches, migraines, nausea, redness, tiredness, itchiness, rash, slight flushing, vitamin B12 deficiency, short term menstrual cycle changes.

Known possible side effects associated with ***5-MTHF*** supplement use are headaches, migraines, redness, rash, anxiety, depression, irritability, short term menstrual cycle changes. More details about these possible side effects are listed in the table below.

Side Effect	How often is it likely to occur?	How severe might it be?	How long might it last?
Folic acid			
Headaches	unknown	Mild to moderate	While taking folic acid
Nausea	unknown	Mild to moderate	While taking folic acid
Redness	unknown	Mild to moderate	While taking folic acid
tiredness	unknown	Mild to moderate	While taking folic acid
itchiness	unknown	Mild to moderate	While taking folic acid
Rash	unknown	Mild to moderate	While taking folic acid

Slight flushing	unknown	Mild to moderate	While taking folic acid
Vitamin B12 deficiency	After several months of taking folic acid or 5-MHTF	B12 anaemia	Until you stop the folic acid and take Vitamin B12
5-MTHF			
headache	Unknown	Mild to moderate	While taking 5-MTHF
migraine	Unknown	Mild to moderate	While taking 5-MTHF
redness	Unknown	Mild to moderate	While taking 5-MTHF
rash	Unknown	Mild to moderate	While taking 5-MTHF
anxiety	At any time taking 5-MTHF	Mild to moderate	While taking 5-MTHF
depression	At any time taking 5-MTHF	Mild to severe	While taking 5-MTHF
irritability	At any time taking 5-MTHF	Mild to moderate	While taking 5-MTHF
Menstrual cycle changes	Short term (2-3 months)	Mild	Usually only for the first 2-3 months of taking the 5-MTHF

If you become upset or distressed as a result of your participation in the research, the study researcher will be able to arrange for counselling or other appropriate support. Any counselling or support will be provided by qualified staff who are not members of the research project team. This counselling will be provided free of charge. Please advise your researcher immediately of any support you may require.

1.13 What will happen to my test samples?

We are testing the MTHFR gene to evaluate your body's ability to utilise various forms of folate. This gene makes an enzyme which converts folate into its active form.

These tests will be collected by your local pathology centre and no tissue will be banked or stored by the researchers for the purpose of this study.

You can choose whether you receive your MTHFR gene results or not. If you decide to receive your results your lead researcher will speak to you and help you understand the effect of this gene on your folate metabolism. Should you wish to speak to your GP or discuss this with a

genetic counsellor you can. For further information about https://www.pregnancybirthbaby.org.au/about-pregnancy-birth-and-baby near you, contact Pregnancy, Birth and Baby on 1800 882 436.

Genetic testing involves the study of genetic material (typically DNA) that is shared with your blood relatives. Genetic research in this study is to understand how your body might metabolise folate. This genetic test will not be used to diagnose or treat a disease.

Statutory or contractual duties may require you to disclose results of genetic tests or analysis to third parties (for example, insurance companies, employers, financial and educational institutions), particularly where results provide information about health prospects. If the results of your genetic tests are not available to you or you choose not to have the results given to you, then your future requests for insurance will not be affected by participating in this research.

If you do obtain the results of your genetic tests, you may then be obliged to disclose this on any future application for insurance or employment should it be requested.

1.14 What if new information arises during this research project?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your study researcher will tell you about it and discuss with you whether you want to continue in the research project. If you decide to withdraw, your study researcher will make arrangements for your regular health care to continue. If you decide to continue in the research project, you will be asked to sign an updated consent form.

1.15 Can I have other treatments during this research project?

Whilst you are participating in this research project, you may not be able to take some or all of the medications or treatments you have been taking for your condition or for other reasons. It is important to tell your study researcher and the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture, or other alternative treatments. You should also tell your study doctor about any changes to these during your participation in the research project. Your study researcher should also explain which treatments or medications need to be stopped for the time you are involved in the research project.

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1.16 What if I withdraw from this research project?

If you decide to withdraw from the project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to discuss any health risks or special requirements linked to withdrawing.

If you do withdraw your consent during the research project, the study researcher and relevant study staff will not collect additional personal information from you, although personal information already collected will be retained to ensure that the results of the research project can be measured properly and to comply with law. You should be aware that data collected by the researchers up to the time you withdraw will form part of the research project results. If you do not want them to do this, you must tell them before you join the research project.

1.17 Could this research project be stopped unexpectedly?

This research project may be stopped unexpectedly for a variety of reasons. These may include reasons such as:

- Unacceptable side effects
- The form of folate being shown not to be effective
- The form of folate being shown to work and not need further testing

1.18 What happens when the research project ends?

When the research project ends you will have the opportunity to review the results and ask any questions of your lead researcher. Information regarding the form of folate you were taking will be provided if requested and you will be provided with a list of products that you can purchase on an ongoing basis.

A summary of the results will be sent to you at the completion of the project once all data is analysed. We expect this update to be completed by mid-2024.

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PART 2 - HOW IS THE RESEARCH PROJECT BEING CONDUCTED?

2.1 What will happen to information about me?

The data collected in this research study will be re-identifiable (coded). This means that once you consent to be included in this study, only the immediate research team will know your name or personal details. You will be allocated a unique study ID for the duration of the trial so that no one outside the immediate research team has access to any of your personal data or results.

Your personal data will be stored on University of Technology Sydney records keeping systems (Content Manager) that are password protected. No hard copies will be kept for any personal data after the completion of the trial and will be destroyed as soon as these are uploaded to the external hard drive.

The data collected must be stored for at least 15 years from date of final publication in research involving clinical trials. After this time the data may need to be kept for a longer period should the research be re-analysed or re-published. At the end of the 15 years the data will be destroyed further to University of Technology records management program https://www.gsu.uts.edu.au/policies/recordsmgmt.html

Your coded data may be required for further research studies that build on the knowledge gained in this research study. No personal data will be kept on file for future research.

By signing the consent form, you consent to the study researcher and relevant research staff collecting and using personal information about you for the research project. Any information obtained in connection with this research project that can identify you will remain confidential. As discussed above any personal information will only be known by the immediate research team and for the duration of the study you will be given a unique study ID that only the lead researcher will have access to. Your personal information will be kept in the UTS securer recordkeeping software programme and will be password protected so no one outside the research team can access it. Your information will only be used for the purpose of this research project, and it will only be disclosed with your permission, except as required by law.

Information about you may be obtained from your health records held at this and other health services for the purpose of this research. By signing the consent form, you agree to the study team accessing health records if they are relevant to your participation in this research project.

Your health records and any information obtained during the research project are subject to inspection (for the purpose of verifying the procedures and the data) by the relevant authorities, the institution relevant to this Participant Information Sheet, University of Technology Sydney or as required by law. By signing the Consent Form, you authorise release of, or access to, this confidential information to the relevant study personnel and regulatory authorities as noted above.

It is anticipated that the results of this research project will be published and/or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your permission. No personal data will be presented in any form in the results. Only the Study ID may be published.

In accordance with relevant Australian privacy and other relevant laws, you have the right to request access to your information collected and stored by the research team. You also have the right to request that any information with which you disagree be corrected. Please contact the study team member named at the end of this document if you would like to access your information.

Any information obtained for the purpose of this research project and for the future research described in Section 16 that can identify you will be treated as confidential and securely stored. It will be disclosed only with your permission, or as required by law.

2.2 Complaints and compensation

If you have any complaints/issues, please make contact with your lead researcher at any time to notify them or discuss any issues with them at your monthly meeting. The lead researcher will attempt to resolve any issues for you at that meeting and if this cannot be resolved then the wider research team will be notified so that your complaint can be resolved.

Adverse Event

Any adverse event must be reported to the lead researcher immediately by phone. The lead researcher will notify the other investigators within 2-3 hours who will then meet with the Advisory Safety and Monitoring Board within 24 hours.

If the adverse event is not related to the investigational product, it will be recorded, and you will be advised to seek medical advice.

If the adverse event is related to the investigational product and deemed serious you will be advised to cease the product immediately and seek medical advice. This event will be reported to the TGA and relevant bodies within 24 hours.

If the adverse event is deemed non-serious you will be advised to either reduce the dose or cease the product.

If you suffer any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital.

2.3 Who is organising and funding the research?

This research project is being conducted by Carolyn Ledowsky as a contribution to her Doctor of Philosophy at the University of Technology Sydney.

No member of the research team will receive a personal financial benefit from your involvement in this research project (other than their ordinary wages).

Carolyn Ledowsky is the founder of MTHFR support Australia, which is a company aimed at supporting people with fertility issues associated with the MTHFR genetic polymorphisms.

2.4 Who has reviewed the research project?

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the HREC of the University of Technology Sydney.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research (2007)*. This statement has been developed to protect the interests of people who agree to participate in human research studies.

2.5 Further information and who to contact

The person you may need to contact will depend on the nature of your query.

If you want any further information concerning this project or if you have any medical problems which may be related to your involvement in the project (for example, any side effects), you can contact the lead researcher on [redacted] or email: carolyn.j.ledowsky@student.uts.edu.au for any queries.

Name	*Carolyn Ledowsky*
Position	*PhD Candidate*
Telephone	[redacted]
Email	*Carolyn.j.ledowsky@student.uts.edu.au*

Complaints contact person

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Reviewing HREC name	*Research Office, UTS Sydney*
HREC Executive Officer	*Emma Kirk, Senior Research Ethics office*
Telephone	*02 9514 2478*
Email	*Research.ethics@uts.edu.au*

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Consent Form

Title:	**The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial**
Short Title:	**Folate Trial for recurrent miscarriage**
Protocol Number	**(insert protocol number)**
Project Sponsor	**None**
PhD Candidate	**Carolyn Ledowsky**
Lead Researcher	**Dr Amie Steel**
Co Researcher	**Dr Vanessa Scarf**
Location	**Online**

Declaration by Participant

- I have read the Participant Information Sheet, or someone has read it to me in a language that I understand.
- I understand the purposes, procedures and risks of the research described in the project.
- I give permission for my doctors, other health professionals, hospitals, or laboratories to release information to University of Technology Sydney concerning my disease and treatment for the purposes of this project. I understand that such information will remain confidential.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the study without affecting my future health care.

I understand that, if I decide to discontinue the study treatment, I may be asked to attend follow-up visits to allow collection of information regarding my health status. Alternatively, a member

of the research team may request my permission to obtain access to my medical records for collection of follow-up information for the purposes of research and analysis.

Declaration by Participant for Blood and Tissue Samples

I consent to the use of blood and tissue samples taken from me for use, as described in the relevant section of the Participant Information Sheet, for:

- This specific research project
- Other research that is closely related to this research project
- Any future research.

By signing this consent section, I agree to the use of my tissue samples for genetic testing, as outlined in the relevant Section of the Participant Information Sheet.

I understand that I will be given a signed copy of this document to keep.

Name of Participant (please print) _____

Signature _____ Date _____

Declaration by Study Researcher†

I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Name of Study Researcher† (please print) _____

Signature _____ Date _____

† A senior member of the research team must provide the explanation of, and information concerning, the research project.

Note: All parties signing the consent section must date their own signature.

Appendix 14 – Trial study - Participant Information Sheets – Males

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Folate Trial for recurrent miscarriage

Summary Participant Information Sheet - Male

Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Short Title	Folate trial for recurrent miscarriage
Protocol Number	*[Protocol Number]*
Project Sponsor	None
PhD Candidate	Carolyn Ledowsky
Lead Researcher	Dr Amie Steel
Co researcher	Dr Vanessa Scarf
Location	Australia (online recruitment)

1 PART 1 - WHAT DOES MY PARTICIPATION INVOLVE?

1.1 Introduction

You are invited to take part in this research project because you and your partner have experienced recurrent pregnancy loss. The research project is a pilot study designed to test the study procedures for a larger trial testing a new treatment to help you successfully achieve pregnancy. The new treatment is called 5-methyl folate (5-MTHF). This is a form of folate.

This Detailed Participant Information Sheet tells you about the research project. It explains the tests and treatments involved. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you do not understand or want to know more about. Before deciding whether to take part, you might want to talk about it with a relative, friend or your local doctor.

Participation in this research is voluntary. If you do not wish to take part, you do not have to. You will receive the best possible care whether or not you take part.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

- Understand what you have read
- Consent to take part in the research project
- Consent to have the tests and treatments that are described
- Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

1.2 What is the purpose of this research?

The aim of this study is to conduct a pilot study, which is a mini trial in preparation for a larger trial to compare 5-methyl folate with folic acid to achieve successful pregnancies for couples who have experienced recurrent pregnancy loss. Folic acid is the main form of folate given to women who are considering falling pregnant. 5-methyl folate is an active form of folate that is metabolised by the body has not been clinically trialled in women with multiple miscarriage.

This study may help inform the researchers on how best to conduct a larger study of this nature, what worked, what the results were and how we can produce this trial for a larger number of people. It may also help understand new approaches to support couples with recurrent pregnancy loss, and the health professionals providing care to these couples.

Many studies have evaluated the importance of folate in the development of the neural tube and to prevent neural tube defects. Folate is essential for gene health which ensures the integrity of both the sperm and egg. In Australia, it is typically recommended that individuals take 500mcg of folate per day.

In Australia, medications, drugs, and devices have to be approved for use by the Australian Federal Government agency, the Therapeutic Goods Administration. *5-MTHF* is approved in Australia as a form of folate to be included in multivitamins and prenatal supplements.

This research has been initiated by the principal investigator, Carolyn Ledowsky, and will contribute towards the requirements of her Doctor of Philosophy.

This research has not been funded by a government body and is conducted by the University of Technology Sydney. There is no commercial sponsorship of this research study.

1.3 Who can participate in this Study?

Couples must meet the following criteria to participate in this study:

a) Have had two or more medically confirmed pregnancy losses before 22 weeks gestation
b) Be able to speak and understand English sufficiently to comprehend the purpose and risks of the study and to provide consent
c) Ability to attend a local blood collection centre for collection of bloods
d) You must be willing to take a prenatal multivitamin every day until your partner conceives or until you withdraw from the study
e) You must be willing to follow a diet that avoids folic acid-based foods. This does not mean you cannot eat certain foods, but rather will be given alternate brands to choose from

You will be ineligible for this study if you:

a) If your partner is currently pregnant or breastfeeding
b) If you or your partner are currently undergoing any assisted reproductive technologies such as IVF, ICSI.
c) Are currently taking medication that might interfere with folate absorption such as:
   - Phenytoin (i.e., Dilantin, phenytek)
   - Valproic acid (i.e., Depakene, Stavzor)
   - Valproate sodium
   - Carbamazepine (i.e., Tegretol, Equetro, Epitol, Tegretol XR, Carbamazeipine chewtabs, carbamazepine CR, Carbatrol, Teril, Carnexiv
   - Methotrexate
   - Trimethoprim hydrochloride (i.e., antibiotic under the name of Primsol, proloprim, TMP)
   - Trimethoprim sulphate

- Trimethoprim
- Aminopterin sodium
- Phenobarbital
- Phenobarbital sodium
- Primidone (i.e., Mysoline)
- Divalproex (i.e., Depakote, Depakote ER, Depakote Sprinkles)

d) Have a known sensitivity to folic acid or methyl folate (5-methyltetrahydrofolate)
Have a history of a psychiatric disorders including severe anxiety and/or depression, autoimmune disease, cancer, sexually transmitted infections,

1.4 What will happen first?

You will be randomly selected by a computer to enter one of the following groups:

a) Folic acid based prenatal multivitamin
b) Intervention group (5-MTHF folate based prenatal supplement)

None of the researchers on this project and none of the participants will know which participants are in which group. Your chance of entering either group is 50%.

The trial will run for a total of 12 months: two months not trying to conceive and 6 months attempting to conceive or pregnant.

Once you have been initially screened for eligibility, the principal investigator will organise an online meeting to explain all the features of this trial to you and confirm your eligibility. During this first meeting eligible participants will be invited to enrol in the study. Individuals will also be provided with the opportunity to enrol at a later time if they wish to consider their involvement further.

Once enrolled, you will then be asked to complete the following tasks within the first week:

1. ***Visit a blood collection clinic near you to collect blood samples*** to allow us to test for:
   - *Homocysteine* - this is a marker that can indicate your body's use of folate and vitamin B12. This will only be tested in the first week of study involvement.
   - *Vitamin B12* – this nutrient is required for the uptake of your folate. It will be retested at 12 weeks following your introduction of the prenatal.
   - *Serum folate and red cell folate* – serum folate represents more recent folate intake, whereas red cell folate represents long-term intake

- *MTHFR gene test* – this gene makes an enzyme that is the last step in the folate cycle

2. ***Commence the prenatal multivitamin*** that has been randomly allocated to you and your partner. You will be required to take three capsules per day. Your researcher will give you instructions on how to introduce the supplements.
3. ***Avoid all folic acid-fortified foods***. This may mean you need to swap to brands that do not add folic acid to foods but should not mean you need to avoid a food group entirely. A detailed information sheet will be provided to you showing you which foods you should avoid and which foods to include. We are asking you to do this as we do not want any fortified foods to influence the level of folate you are taking affect the outcome of the trial. Please note this is an important component of the trial as folic acid can build up if you are taking both supplements and foods fortified with folic acid.
4. ***Avoid taking any other vitamin or mineral supplements unless advised by a medical practitioner***. You should notify the research team of any additional supplements used during the trial period.

We will advise you to take the prenatal for a 'pre-conception period' of 2 reproductive cycles of your partner, during which time we ask that you do not try to fall pregnant. This is because it can take some time for folate levels in the body to reach optimal levels. Following the three-month period, you will be free to attempt pregnancy.

1.5 What will happen during the trial?

During the trial, the following will be required:

1. ***Visit a blood collection centre upon enrolment and every three months*** to retest your serum folate and red cell folate
2. ***Take the prenatal every day (3 capsules). You will not be required to follow any of the trial requirements once your partner conceives.***
3. ***Meet with the lead researcher online at the start of the trial, week 2 and then every six weeks.*** This check in will take no longer than 30 minutes. During this time, the researcher will discuss:
   - Your adherence to the required prenatal vitamin intake
   - Your adherence to the dietary guidelines aimed at avoiding dietary folic acid
   - Any adverse events that may have happened
   - Any support you may need during the trial

- Any issues you have had since the last meeting
- ***Complete an online form before each meeting with your lead researcher.*** Any issues you may be having with the prenatal multivitamin
- Any issues you may be having with the diet
- Any adverse effects
- Any stress

Each meeting with your researcher will be via video conference software (e.g., Zoom) or telephone. You will be able to do this follow up from your home or office and does not require any travel.

The trial will run from January 2023 until December 2023. Please note that you will not be required to follow any of the trial protocols above once your partner conceives.

Participants will not be accepted after April 30, 2023.

In addition to the prenatal vitamin supplement, all participants will receive a $50 Visa card as a recognition of any inconveniences associated with your involvement in this trial.

1.6 Human Research Rights

Human research is governed by Australian law that establishes rights for participants and imposes general and specific responsibilities on researchers and institutions. This research will be compliant with legal obligations and will be monitored by the whole research team at the University of Technology Sydney. At all stages of the research study, you and your partner will be monitored for safety. Any adverse events will immediately be reported to the safety committee which consists of a fertility specialist, a general practitioner, a clinical trial specialist and nutritionist, a psychologist, the lead researcher, and a senior member of the research team to decide if the adverse event is due to the prenatal product or not. Below is the reporting guidelines and flow chart for any adverse events during the trial as stated by the National Health and Medical Research council of Australia.

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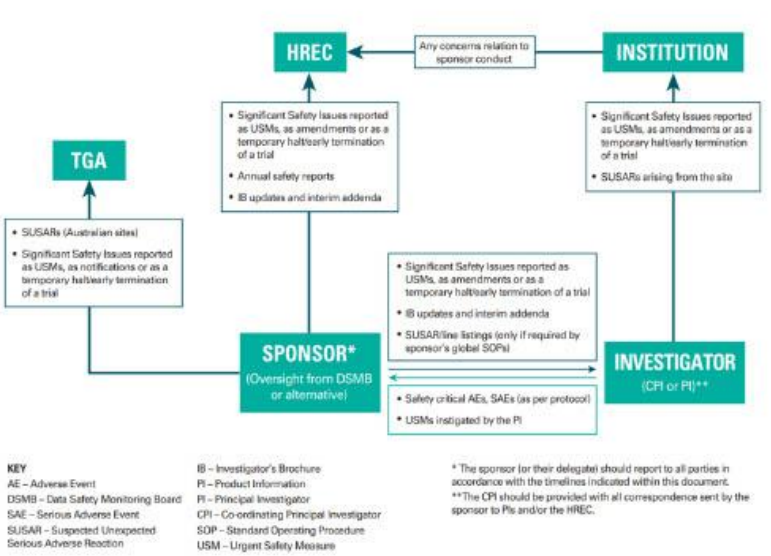

Appendix 1: Report Flowchart for Investigational Medicinal Product Trials

1.7 Who receives the different products?

You will be participating in a pilot study for a further randomised controlled research project. Sometimes we do not know which treatment is best for treating a condition. To find out we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same, each participant is put into a group by chance (random). You will have a 50/50 chance of receiving the investigational product (5-MTHF) or the control (folic acid) product.

You will be participating in a double-blind study. This means that neither you nor your study researchers will know which treatment you are receiving. However, in certain circumstances one of the research team may need to find out which treatment you are receiving.

This research project has been designed to make sure the researchers interpret the results in a fair and appropriate way and avoids researchers or participants jumping to conclusions.

There are no additional costs associated with participating in this research project, nor will you be paid. All prenatal products, tests and medical care required as part of the research project will be provided to you free of charge.

If you have a local doctor, we strongly recommend that you inform them of your participation in this research project as confirmation of your history of recurrent pregnancy loss will be required by your GP.

1.8 What do I have to do?

There will be no physical or lifestyle restrictions placed on you during this study

You will be required to avoid folic acid-fortified foods. A list of foods fortified with folic acid and of foods not fortified with folic acid will be provided to you.

You will be able to continue with your regular medication as long as it does not interfere with folate metabolism.

The list of medications not allowed is included in the exclusion criteria above (see list under 'Section 1.4: Who can participate in this Study?')

You can still donate blood should you wish to during the trial.

It is your responsibility to ensure that you take the prenatal multivitamin each day and that you avoid folic acid fortified foods.

1.9 Other relevant information about the research project

There will be up 60 couples taking part in this research project: 30 couples will be assigned the control product (folic acid-based multivitamin), 30 couples will be assigned the intervention product (5-MTHF based multivitamin). The research project will only be conducted in Australia.

1.10 Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part, you do not have to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

If you do decide to take part, you will be given this Detailed Participant Information Sheet and a Consent Form to sign. You will be given a copy of each to keep.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the University of Technology Sydney.

1.10 What are the alternatives to participation?

You do not have to take part in this research project to receive fertility support. Other options are available; these include consulting with your GP and/or seeing a fertility specialist. Your study researcher will discuss these options with you before you decide whether or not to take part in this research project. You can also discuss the options with your local doctor.

1.11 What are the possible benefits of taking part?

We cannot guarantee or promise that you will receive any benefits from this research; however, possible benefits may include having a successful pregnancy.

1.12 What are the possible risks and disadvantages of taking part?

The disadvantages of you joining this research may be:

- That you do not achieve a successful pregnancy
- That you may have another miscarriage

Supplements often cause side effects. You may have none, some or all of the effects listed below, and they may be mild, moderate, or severe. If you have any side effects, or are worried about them, talk with your study researcher. Your study researcher will also be looking out for side effects.

There may be side effects that the researchers do not expect or do not know about and that may be serious. Tell your study doctor immediately about any new or unusual symptoms that you get.

Many side effects go away shortly after treatment ends. However, sometimes side effects can be serious. If a severe side effect or reaction occurs, your study researcher may need to stop your treatment. Your study researcher will discuss the best way of managing any side effects with you.

Known possible side effects associated with ***folic acid*** supplement use are headaches, migraines, nausea, redness, tiredness, itchiness, rash, slight flushing, vitamin B12 deficiency, short term menstrual cycle changes.

Known possible side effects associated with ***5-MTHF*** supplement use are headaches, migraines, redness, rash, anxiety, depression, irritability, short term menstrual cycle changes. More details about these possible side effects are listed in the table below.

Side Effect	**How often is it likely to occur?**	**How severe might it be?**	**How long might it last?**
Folic acid			
Headaches	unknown	Mild to moderate	While taking folic acid
Nausea	unknown	Mild to moderate	While taking folic acid
Redness	unknown	Mild to moderate	While taking folic acid
tiredness	unknown	Mild to moderate	While taking folic acid
itchiness	unknown	Mild to moderate	While taking folic acid
Rash	unknown	Mild to moderate	While taking folic acid
Slight flushing	unknown	Mild to moderate	While taking folic acid
Vitamin B12 deficiency	After several months of taking folic acid or 5-MHTF	B12 anaemia	Until you stop the folic acid and take Vitamin B12
5-MTHF			
headache	Unknown	Mild to moderate	While taking 5-MTHF
migraine	Unknown	Mild to moderate	While taking 5-MTHF
redness	Unknown	Mild to moderate	While taking 5-MTHF
rash	Unknown	Mild to moderate	While taking 5-MTHF
anxiety	At any time taking 5-MTHF	Mild to moderate	While taking 5-MTHF
depression	At any time taking 5-MTHF	Mild to severe	While taking 5-MTHF
irritability	At any time taking 5-MTHF	Mild to moderate	While taking 5-MTHF

Menstrual cycle changes	Short term (2-3 months)	Mild	Usually only for the first 2-3 months of taking the 5-MTHF

If you become upset or distressed as a result of your participation in the research, the study researcher will be able to arrange for counselling or other appropriate support. Any counselling or support will be provided by qualified staff who are not members of the research project team. This counselling will be provided free of charge.

1.13 What will happen to my test samples?

We are testing the MTHFR gene to evaluate your body's ability to utilise various forms of folate. This gene makes an enzyme which converts folate into its active form.

These tests will be collected by your local pathology centre and no tissue will be banked or stored by the researchers for the purpose of this study.

You can choose whether you receive your MTHFR gene results or not. If you decide to receive your results your lead researcher will speak to you and help you understand the effect of this gene on your folate metabolism. Should you wish to speak to your GP or discuss this with a genetic counsellor you can. For further information about genetic counselling and support services near you, contact Pregnancy, Birth and Baby on 1800 882 436.

Genetic testing involves the study of genetic material (typically DNA) that is shared with your blood relatives. Genetic research in this study is to understand how your body might metabolise folate. This genetic test will not be used to diagnose or treat a disease.

Statutory or contractual duties may require you to disclose results of genetic tests or analysis to third parties (for example, insurance companies, employers, financial and educational institutions), particularly where results provide information about health prospects. If the results of your genetic tests are not available to you or you choose not to have the results given to you, then your future requests for insurance will not be affected by participating in this research.

If you do obtain the results of your genetic tests, you may then be obliged to disclose this on any future application for insurance or employment should it be requested.

1.14 What if new information arises during this research project?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your study researcher will tell you about it and discuss with you whether you want to continue in the research project. If you decide to withdraw, your study researcher will make arrangements for your regular health care to continue. If you decide to continue in the research project, you will be asked to sign an updated consent form.

1.15 Can I have other treatments during this research project?

Whilst you are participating in this research project, you may not be able to take some or all of the medications or treatments you have been taking for your condition or for other reasons. It is important to tell your study researcher and the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture, or other alternative treatments. You should also tell your study doctor about any changes to these during your participation in the research project. Your study researcher should also explain which treatments or medications need to be stopped for the time you are involved in the research project.

1.16 What if I withdraw from this research project?

If you decide to withdraw from the project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to discuss any health risks or special requirements linked to withdrawing.

If you do withdraw your consent during the research project, the study researcher and relevant study staff will not collect additional personal information from you, although personal information already collected will be retained to ensure that the results of the research project can be measured properly and to comply with law. You should be aware that data collected by the researchers up to the time you withdraw will form part of the research project results. If you do not want them to do this, you must tell them before you join the research project.

1.17 Could this research project be stopped unexpectedly?

This research project may be stopped unexpectedly for a variety of reasons. These may include reasons such as:

- Unacceptable side effects
- The form of folate being shown not to be effective

- The form of folate being shown to work and not need further testing

1.18 What happens when the research project ends?

When the research project ends you will have the opportunity to review the results and ask any questions of your lead researcher. Information regarding the form of folate you were taking will be provided if requested and you will be provided with a list of products that you can purchase on an ongoing basis.

A summary of the results will be sent to you at the completion of the project once all data is analysed. We expect this update to be completed by mid-2024.

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PART 2 - HOW IS THE RESEARCH PROJECT BEING CONDUCTED?

2.1 What will happen to information about me?

The data collected in this research study will be re-identifiable (coded). This means that once you consent to be included in this study, only the immediate research team will know your name or personal details. You will be allocated a unique study ID for the duration of the trial so that no one outside the immediate research team has access to any of your personal data or results.

Your personal data will be stored on University of Technology Sydney records keeping systems (Content Manager) that are password protected. No hard copies will be kept for any personal data after the completion of the trial and will be destroyed as soon as these are uploaded to the external hard drive.

The data collected must be stored for at least 15 years from date of final publication in research involving clinical trials. After this time the data may need to be kept for a longer period should the research be re-analysed or re-published. At the end of the 15 years the data will be destroyed further to University of Technology records management program https://www.gsu.uts.edu.au/policies/recordsmgmt.html

Your coded data may be required for further research studies that build on the knowledge gained in this research study. No personal data will be kept on file for future research.

By signing the consent form, you consent to the study researcher and relevant research staff collecting and using personal information about you for the research project. Any information obtained in connection with this research project that can identify you will remain confidential. As discussed above any personal information will only be known by the immediate research team and for the duration of the study you will be given a unique study ID that only the lead researcher will have access to. Your personal information will be kept in the UTS securer recordkeeping software programme and will be password protected so no one outside the research team can access it. Your information will only be used for the purpose of this research project, and it will only be disclosed with your permission, except as required by law.

Information about you may be obtained from your health records held at this and other health services for the purpose of this research. By signing the consent form, you agree to the study team accessing health records if they are relevant to your participation in this research project.

Your health records and any information obtained during the research project are subject to inspection (for the purpose of verifying the procedures and the data) by the relevant authorities, the institution relevant to this Participant Information Sheet, University of Technology Sydney or as required by law. By signing the Consent Form, you authorise release of, or access to, this confidential information to the relevant study personnel and regulatory authorities as noted above.

It is anticipated that the results of this research project will be published and/or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your permission. No personal data will be presented in any form in the results. Only the Study ID may be published.

In accordance with relevant Australian privacy and other relevant laws, you have the right to request access to your information collected and stored by the research team. You also have the right to request that any information with which you disagree be corrected. Please contact the study team member named at the end of this document if you would like to access your information.

Any information obtained for the purpose of this research project and for the future research described in Section 16 that can identify you will be treated as confidential and securely stored. It will be disclosed only with your permission, or as required by law.

2.2 Complaints and compensation

If you have any complaints/issues, please make contact with your lead researcher at any time to notify them or discuss any issues with them at your monthly meeting. The lead researcher will attempt to resolve any issues for you at that meeting and if this cannot be resolved then the wider research team will be notified so that your complaint can be resolved.

Adverse Event

Any adverse event must be reported to the lead researcher immediately by phone. The lead researcher will notify the other investigators within 2-3 hours who will then meet with the Advisory Safety and Monitoring Board within 24 hours.

If the adverse event is not related to the investigational product, it will be recorded, and you will be advised to seek medical advice.

If the adverse event is related to the investigational product and deemed serious you will be advised to cease the product immediately and seek medical advice. This event will be reported to the TGA and relevant bodies within 24 hours.

If the adverse event is deemed non-serious you will be advised to either reduce the dose or cease the product.

If you suffer any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital.

2.3 Who is organising and funding the research?

This research project is being conducted by Carolyn Ledowsky as a contribution to her Doctor of Philosophy at the University of Technology Sydney.

No member of the research team will receive a personal financial benefit from your involvement in this research project (other than their ordinary wages).

Carolyn Ledowsky is the founder of MTHFR support Australia, which is a company aimed at supporting people with fertility issues associated with the MTHFR genetic polymorphisms.

2.4 Who has reviewed the research project?

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the HREC of the University of Technology Sydney.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research (2007)*. This statement has been developed to protect the interests of people who agree to participate in human research studies.

2.5 Further information and who to contact

The person you may need to contact will depend on the nature of your query.

If you want any further information concerning this project or if you have any medical problems which may be related to your involvement in the project (for example, any side effects), you can contact the lead researcher on [redacted] or email: carolyn.j.ledowsky@student.uts.edu.au for any queries.

Name	*Carolyn Ledowsky*
Position	*PhD Candidate*
Telephone	[redacted]
Email	*Carolyn.j.ledowsky@student.uts.edu.au*

Complaints contact person

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Reviewing HREC name	*Research Office, UTS Sydney*
HREC Executive Officer	*Emma Kirk, Senior Research Ethics office*
Telephone	*02 9514 2478*
Email	*Research.ethics@uts.edu.au*

UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Consent Form

Title:	**The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial**
Short Title:	**Folate Trial for recurrent miscarriage**
Protocol Number	**(insert protocol number)**
Project Sponsor	**None**
PhD Candidate	**Carolyn Ledowsky**
Lead Researcher	**Dr Amie Steel**
Co Researcher	**Dr Vanessa Scarf**
Location	**Online**

Declaration by Participant

- I have read the Participant Information Sheet, or someone has read it to me in a language that I understand.
- I understand the purposes, procedures and risks of the research described in the project.
- I give permission for my doctors, other health professionals, hospitals or laboratories to release information to University of Technology Sydney concerning my disease and treatment for the purposes of this project. I understand that such information will remain confidential.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the study without affecting my future health care.

I understand that, if I decide to discontinue the study treatment, I may be asked to attend follow-up visits to allow collection of information regarding my health status. Alternatively, a member

Appendix 15 – Trial Study – REDCap Initial Baseline Collection of Information Form

REDCap Initial participant information sheet

Upon enrolment into the trial and before the baseline visit participants will receive the following form to complete prior to the meeting with the lead researcher.

Name: First/surname

What is your Age? (in years):

What is your gender? Female/male/transgender/other

What is your Ethnicity?

What is your country of birth:

Is English your first language? Yes/no - please state

Address (this is where we will send your prenatal product)

Mobile phone number

Email

What is your relationship status? Married/De facto/other

Name of reproductive partner included in this trial

Mobile phone number

Email address

What is your current weight? (In kg)

How tall are you? (in centimeters)

What is the highest qualification you have completed?

- No formal qualification
- Year 10 or equivalent
- Year 12 or equivalent
- Trade or apprenticeship
- Certificate or diploma
- University degree (eg. Bachelor)
- Higher university degree (eg. Masters, PhD)

What best describes your employment status?

- Full time work (35 hours or more per week)
- Part time work (less than 35 hours per week)
- Casual or temporary work (irregular hours)
- Currently looking for work
- Not currently in the paid workforce, nor looking

What is your current job?

What is your income level?

- <50K
- 51-100K
- 100-150K
- 151+

How would you describe your level of physical activity?

- Sedentary
- Moderate
- Vigorous
- How many hours per week would you exercise?

Female questionnaire:

Fertility History

Did your mother have any pregnancy losses? If so, how many?

Have you ever been pregnant? Yes/no

If yes, how many pregnancies have you had?

How many of those pregnancies were live births?

How old are your children?

Gender

Do your children have any of the following medical conditions?

- Neural tube defect (spina bifida, anencephaly, meningocele, encephalocele)
- Cleft lip or palate
- Hole in the heart

How many of the previous pregnancies had the following complications? Please put the number. If none put 0)

- Miscarriage before 22 weeks
- Pregnancy loss after 22 weeks
- Ectopic pregnancy (or tubal pregnancy)
- Still birth (i.e.: birth of a baby that is not alive, past 24 weeks pregnancy)
- Terminated because of fetal/genetic abnormalities
- Neural tube defects

Were any of these pregnancies due to IVF or any other assisted reproductive technologies?

Please state:

ART methodology;

Pregnancy result in live birth?

Pregnancy miscarried? If so at how many weeks

Were you ever prescribed high dose folic acid (ie: 5mg or more) by your fertility specialist?

If so, what was the resulting outcome of that pregnancy?

How long have you currently been trying to conceive since your last pregnancy loss?

During this time trying to conceive, have you been taking a prenatal multivitamin?

If yes, please list the Manufacturers name, Name of product, number of capsules you take each day

Current menstrual cycle information

Do you have a regular menstrual cycle? If yes how many days is your cycle? Ie: from day 1 of your bleed to next bleed, how many days is it?

If not how long is your cycle? I.e.: from Day 1 of your period to the next period

Do you suffer from PMS symptoms?

- Anxiety
- Pain
- Nausea
- Headaches/migraines
- Heavy periods
- Trouble sleeping

Do you currently see other health professionals for your fertility/reproductive health?

Please provide contact details:

Name

Profession

Email

Phone

Do we have your consent to email information about this trial to your practitioner?

Yes/no

Please provide your GP's contact details:

Name

Clinic practice name

Phone number

Do we have your consent to email information about this trial to your practitioner?

Yes/no

Are you currently seeing a fertility specialist?

If yes, please provide contact details:

Name

Practice Name

Clinic phone number

Do we have your consent to email information about this trial to your practitioner?

Yes/no

Do you take supplements?

If so what brands:

Brand Name, Product Name, How many capsules do you take a day.

Are you currently taking any medications prescribed by your Dr?

If so please list

Does your partner take a prenatal multivitamin?

If so please list manufacturers name, product name, number of capsules taken each day

Are you or have you ever been a smoker? Yes/no

If yes - which of the following have you smoked? Cigarettes/e-cigarettes/other - please state

- If current - how many cigarettes do you smoke per day
- If past - how many cigarettes did you smoke
- When did you start smoking?

Do you drink Alcohol? Yes/no

If yes

- How many alcoholic drinks do you have a day?
- Do you drink alcohol every day
- How many units of alcohol would you drink Monday - Thursday?
- How many units of alcohol would you drink Friday to Sunday?

Dietary information

Please complete the below dietary information table showing your diet over the last week

	Breakfast - please indicate your breakfasts for the last week	Lunch - please indicate your lunch for the last week	Dinner - please indicate your dinner for the last week	Snacks	Drinks and alcoholic beverages
Monday					
Tuesday					
Wednesday					
Thursday					
Friday					
Saturday					
Sunday					

Male Information Questionnaire

1. Have you every had a sperm analysis?
   If yes - please attach a copy of the results
2. Are you or have you ever been a smoker? Yes/no
   If yes - which of the following have you smoked? Cigarettes/e-cigarettes/other - please state
   - If current - how many cigarettes do you smoke per day
   - If past - how many cigarettes did you smoke
   - When did you start smoking?
3. Alcohol use
   - How many alcoholic drinks do you have a day?
   - Do you drink alcohol every day
   - How many units of alcohol would you drink Monday - Thursday?
   - How many units of alcohol would you drink Friday to Sunday?
4. Are you currently taking any supplements?
   If so please list (name/dose)
5. Are you currently taking any medications prescribed by your Dr?
   If so please list (name/dose)
6. Have you ever had children with another reproductive partner?
   If yes, number of children?
7. Have you ever been diagnosed with any reproductive issues?

Dietary information

Please complete the below dietary information table showing your diet over the last week

	Breakfast - please indicate your breakfasts for the last week	Lunch - please indicate your lunch for the last week	Dinner - please indicate your dinner for the last week	Snacks	Drinks and alcoholic beverages
Monday					
Tuesday					
Wednesday					
Thursday					
Friday					
Saturday					
Sunday					

Appendix 16 – Trial Study – Product Tolerability and Adherence Checklist

Product tolerability and adherence checklist

Week 2 of study

Name: First/surname

Question 1: Have you commenced the prenatal multivitamin?

Question 2: Did you introduce the prenatal multivitamin as per the instructions ie: one capsule week 1, two capsules week 2?

Question 3: How many capsules are you currently taking?

Question 3: Did you have any issues with introducing this product:

- Nausea
- Headaches
- Migraines
- Anxiety
- Mood changes
- Changes in sleep patterns
- Changes in menstrual cycle
- Anything else

Question 4: Have you started eliminating folic acid from your foods?

Yes

No

Question 5: Have you had any issues with this? Yes/No

Question 6: Are there any questions you would like to ask about this? Do you need any clarification on what you can or can't eat?

Question 7: Is there anything else you would like to discuss before we set your next meeting time in 6 weeks. Also I'd like to remind you that at any time you wish to meet with me or you have questions, please don't hesitate to contact me in between our meeting times.

Appendix 17 – Trial Study – Six-weekly REDCap Questionnaire for Participants

Ongoing REDCap Questionnaire for participants - Women

Week of study

Name: First/surname

Question 1: Are you taking your prenatal multivitamin as instructed ie: 3 capsules each morning after breakfast?

If not why? Please detail any issues you have had with the prenatal multivitamin since the last appointment Question 2: Have you been avoiding folic acid in your diet? Yes/No

Question 2: Do you require any help with your diet or clarification of any foods that you might be confused about?

Question 3: have you had any side effects from the prenatal multivitamin?

- Nausea
- Headaches
- Migraines
- Anxiety
- Mood changes
- Changes in sleep patterns
- Changes in menstrual cycle
- Anything else

Question 4: Have you started eliminating folic acid from your foods?

Yes

No

Question 5: Have you had any issues with this? Yes/No

Question 6: Are there any questions you would like to ask about this? Do you need any clarification on what you can or can't eat?

Question 7: Are you drinking alcohol? If so how many drinks per week?

Question 8: Are you smoking? Only relevant if they identify as a smoker in the initial intake form

If so how many cigarettes are you smoking each week?

Questions 9: Have you had any menstrual cycle changes since we last met?

Please detail

Question 10: Are you pregnant?

If so how many weeks?

Has this been confirmed through a pregnancy test kit? If so please make sure to send me a photo of the results.

Folate trial for recurrent Miscarriage
6 weekly REDCap questionnaire
Version 2: 24.7.22

Question 11: Are you experiencing any emotional issues or feeling distressed by any aspect of the trial?

Question 11: Are you experiencing any level of anxiety and/or depression?

Question 12: Are you experiencing any difficulty with the online booking procedures or logging in for your meetings?

Question 13: Is there anything else you would like to discuss or do you have any questions before we set your next meeting time in 6 weeks. Please describe.

Question 14: have you completed the necessary blood work if it was due this week?

Question 15: Have you asked your Dr to complete the necessary paperwork should this be required.

I'd like to remind you that at any time you wish to meet with me or you have questions, please don't hesitate to contact me in between our meeting times on or email: carolyn.j.ledowsky@student.uts.edu.au

Appendix 18 – Trial Study- Trial Information Pack for Doctors

UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Doctor Information Pack

Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Short Title	Folate trial for recurrent miscarriage
Protocol Number	*[Protocol Number]*
Project Sponsor	None
PhD Candidate	Carolyn Ledowsky, Tel:
Lead Researcher	Dr Amie Steel
Co-researcher	Dr Vanessa Scarf
Location	Australia (online recruitment)

Dear Dr _____,

Your patient _____ has consented to be involved in a pilot study for a randomised clinical trial comparing the effectiveness of 5-MTHF vs folic acid in couples with recurrent pregnancy loss.
The information below outlines their involvement in the trial and what they are required to do. Should your patient require any medications (including nutrition supplements) for the duration of the trial please advise the lead researcher via email or phone as soon as possible.

Carolyn Ledowsky
Tel:
Email: carolyn.j.ledowsky@student.uts.edu.au
Kind Regards

Carolyn Ledowsky
PhD Candidate

UTS UNIVERSITY OF TECHNOLOGY SYDNEY

What is the purpose of this research?

The aim of this study is to conduct a pilot study for a larger research study to evaluate the feasibility of conducting a study comparing 5-methyl folate with folic acid to achieve successful pregnancies for couples who have experienced recurrent pregnancy loss. Folic acid is the main form of folate given to women who are considering falling pregnant. 5-methyl folate is an active form of folate that is metabolised by the body has not been clinically trialled in women with multiple miscarriage. Our project will help to ensure that a larger study will run smoothly and that patient/practitioner requirements are achievable and support the participants.

This study may help inform new approaches to supporting couples with recurrent pregnancy loss, and the health professionals providing care to these couples.

Many studies have evaluated the importance of folate in the development of the neural tube and to prevent neural tube defects. Folate is essential for gene health which ensures the integrity of both the sperm and egg. In Australia, it is typically recommended that individuals take 500mcg of folate per day.

In Australia, medications, drugs, and devices have to be approved for use by the Australian Federal Government agency, the Therapeutic Goods Administration. *5-MTHF* is approved in Australia as a form of folate to be included in multivitamins and prenatal supplements.

This research has been initiated by the PhD Candidate, Carolyn Ledowsky, and will contribute towards the requirements of her Doctor of Philosophy.

This research has not been funded by a government body and is conducted by the University of Technology Sydney. There is no commercial sponsorship of this research study.

What will your patient be required to do during participation in this research?

1. Take a prenatal multivitamin daily (3 capsules)
2. Avoid foods with folic acid fortification (they will be given a list of what they can and cannot eat)
3. Have blood tests that include Vitamin B12, Folate, homocysteine, unmetabolised folic acid and methylmalonic acid
4. Check for the MTHFR gene polymorphisms that may affect their biologically active folate conversion, 5-MTHF
5. Meet with the PhD candidate once every 6 weeks of the trial for the duration of the time they are in the trial

6. Their reproductive partner must consent to be included in the trial as well and agree to the above requirements, however, will not be required to continue with these protocols once his partner conceives.

How long will the trial run?

There will be a recruitment stage that will run from January 2023 until April 2023 and the trial will finish December 30, 2023.

Eligibility Criteria for the trial

a) To qualify for the definition of multiple miscarriages the patient must have had 2 or more pregnancy losses before 22 weeks
b) Be able to speak and understand English sufficiently to comprehend the purpose and risks of the study and to provide consent
c) Ability to visit a local pathology collection centre for collection of bloods
d) Be willing to not conceive for 2 reproductive cycles from commencement of the folate
e) Be willing to avoid folic acid-based foods and swap to similar products with no folic acid fortification (a detailed list will be provided)

They are **ineligible** for this study if they:

a) Are currently pregnant or breastfeeding
b) Are currently undergoing any assisted reproductive technologies such as IVF, ICSI.
c) Are currently taking medication that might interfere with folate absorption such as:
   - Phenytoin (i.e., Dilantin, phenytek)
   - Valproic acid (i.e., Depakene, Stavzor)
   - Valproate sodium
   - Carbamazepine (i.e., Tegretol, Equetro, Epitol, Tegretol XR, Carbamazeipine chewtabs, carbamazepine CR, Carbatrol, Teril, Carnexiv
   - Methotrexate
   - Trimethoprim hydrochloride (i.e., antibiotic under the name of Primsol, proloprim, TMP)
   - Trimethoprim sulphate
   - Trimethoprim
   - Aminopterin sodium

- Phenobarbital
- Phenobarbital sodium
- Primidone (i.e., Mysoline)
- Divalproex (i.e., Depakote, Depakote ER, Depakote Sprinkles)

d) Have a known sensitivity to folic acid or methyl folate (5-methyltetrahydrofolate)
e) Have a history of a psychiatric disorder, autoimmune disease, cancer, antiphospholipid syndrome, endometriosis, Factor V Leiden, fallopian tube blockage, sexually transmitted infections, polycystic ovarian syndrome, or uterine fibroids.

What do you need to know as prescribing Doctor?

1. You will need to confirm that the participant has a history of recurrent pregnancy loss (definition: pregnancy losses prior to 22 weeks gestation) (see attached form)
2. You will need to confirm a viable pregnancy via a dating scan between 18- 22 weeks gestation (see attached form)
3. Other medications may be allowed during the trial, however the PhD candidate will need to be notified before commencement of medication
4. Other supplements may be allowed during the trial, however the PhD candidate will need to be notified before commencement of the supplement
5. Any concerns regarding health conditions throughout the trial will be referred back to you.
6. All blood test referrals will be provided by the researchers. You are not required to refer for any tests to do with the trial.
7. There are no physical or lifestyle restrictions placed on the participant during this study.

Human Ethics

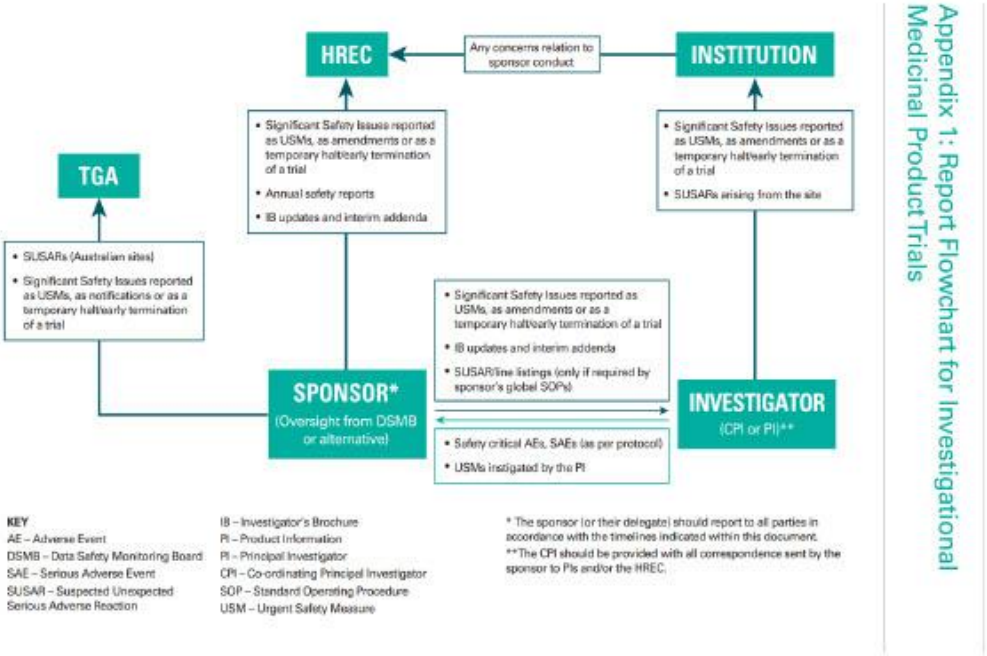
This trial has been approved by the UTS human ethics team.
Ethics approval number:

Human research is governed by Australian law that establishes rights for participants and imposes general and specific responsibilities on researchers and institutions. This research will be compliant with legal obligations and will be monitored by the whole research team at the University of Technology Sydney. At all stages of the research study, the participants will be monitored for safety. Any adverse events will immediately be reported to the safety committee which consists of a fertility specialist, a general practitioner, a clinical trial specialist, nutritionist,

and a psychologist. Below is the reporting guidelines and flow chart for any adverse events during the trial as stated by the National Health and Medical Research council of Australia.

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the HREC of the University of Technology Sydney.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research (2007)*. This statement has been developed to protect the interests of people who agree to participate in human research studies.



Appendix 1: Report Flowchart for Investigational Medicinal Product Trials

Patient randomisation of the products

This is a randomised controlled research project. Each participant and their partner will be randomly assigned the folic acid group or the 5-MTHF group. They will have a 50/50 chance of receiving the investigational product (5-MTHF) or the folic acid product.

Neither the participants nor the researchers will know which treatment they are receiving.

Other relevant information about the research project

There will be 60 couples taking part in this research project: 30 couples will be assigned the control product (folic acid-based multivitamin), 30 couples will be assigned the intervention product (5-MTHF based multivitamin). The research project will only be conducted in Australia.

Known possible side effects associated with ***folic acid*** supplement use are headaches, migraines, nausea, redness, tiredness, itchiness, rash, slight flushing, vitamin B12 deficiency

Known possible side effects associated with ***5-MTHF*** supplement use are headaches, migraines, redness, rash, anxiety, depression, irritability, short term reproductive cycle changes. More details about these possible side effects are listed in the table below.

Side Effect	How often is it likely to occur?	How severe might it be?	How long might it last?
Folic acid			
- Headaches	unknown	Mild to moderate	While you are taking the folic acid
- Nausea	unknown	Mild to moderate	While you take the folic acid
- Redness	unknown	Mild to moderate	While you take the folic acid
- tiredness	unknown	Mild to moderate	While you take the folic acid
- itchiness	unknown	Mild to moderate	While you take the folic acid
- Rash	unknown	Mild to moderate	While you take the folic acid
- Slight flushing	unknown	Mild to moderate	While you take the folic acid
- Vitamin B12 deficiency	After several months taking the prenatal	B12 anaemia	Until you stop the folic acid and take Vitamin B12

5-MTHF			
- *headache*	Unknown	Mild to moderate	While you are taking the 5-MTHF
- *migraine*	Unknown	Mild to moderate	While you are taking the 5-MTHF
- *redness*	Unknown	Mild to moderate	While you are taking the 5-MTHF
- *rash*	Unknown	Mild to moderate	While you are taking the 5-MTHF
- *anxiety*	At any time taking the 5-MTHF	Mild to moderate	While you are taking the 5-MTHF
- *depression*	At any time taking the 5-MTHF	Mild to severe	While you are taking the 5-MTHF
- *irritability*	At any time taking the 5-MTHF	Mild to moderate	While you are taking the 5-MTHF

Further information and who to contact

If you want any further information concerning this project or if you have any medical questions, please contact the lead researcher on [redacted] for any queries.

Researcher Contact Information

Name	*Carolyn Ledowsky*
Position	*PhD Candidate*
Telephone	[redacted]
Email	*Carolyn.j.ledowsky@student.uts.edu.au*

Appendix 19 – Trial Study – Doctor's Confirmation of Pregnancy Form – 8 Weeks

Participant confirmation of pregnancy – 8-week scan

Protocol Number	**TBA**
Study Title	Folate for Pregnancy Success: A phase 2 *randomised, double-blind,* placebo-controlled, double-blind *trial assessing the comparative effectiveness of 5-methlytetrahydrofolate and folic acid intake in couples with a history of recurrent pregnancy loss*
Study Site	**University of Technology Sydney, Ultimo**
HREC Approval Number	**TBA**
Principal Investigator	**Carolyn Ledowsky**
Co-Investigators	**Dr Amie Steel, Dr Vanessa Scarf, Dr Abela Mahimbo**
Participant's Name	
Medical Practitioner Name	
Date	

I confirm that _____ (Name) has a confirmed pregnancy.
The scan was conducted on_____(Date) at
_____(place) by a scan when the participant was approximately _____ weeks gestation.
The participants last menstrual cycle commenced on
_____(Date).

Signed: _____

Name of Medical Practitioner: _____

Date: _____

Appendix 20 – Trial Study - Doctor's Confirmation of Pregnancy Form - 22 Weeks

UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Participant confirmation of pregnancy sheet – 18-22 week scan

Protocol Number	TBA
Study Title	The effectiveness of 5-methyltetrahydrofolate versus folic acid prenatal multivitamins in couples with a history of recurrent pregnancy loss: A feasibility randomised controlled trial
Study Site	University of Technology Sydney Campus
HREC Approval Number	TBA
PhD Candidate	Carolyn Ledowsky
Lead Researcher	Dr Amie Steel
Co Researcher	Dr Vanessa Scarf
Participants Name	
GP Name	
GP Signature	
Date	

I confirm that _____ (Name)
has had a scan between 18-22 weeks of gestation and has a viable pregnancy.
The number of foetuses: _____
Estimated gestation: _____
Confirmation of the delivery due date _____

The scan was conducted on _____(Date)
The scan was conducted at _____ (place)

Folate Trial for recurrent miscarriage
Confirmation of pregnancy 22 weeks form
Version 2: 24.7.22
PRIVATE AND CONFIDENTIAL

UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Signed: _____
Name of Medical Practitioner: _____
Date: _____

Appendix 21 – Trial Study – Advertising Flyer

Appendix 22 – Trial Study – Adverse Events Log

Adverse Event Log

This log facilitates tracking and timely reporting of all applicable adverse events occurring during the project. Adverse events are any unfavourable medical occurrence, including any abnormal signs, symptoms, or disease, whether or not they are associated with the participant's participation in the research project. All events must be reported to Human Ethics Research Committee (HREC) within the time frame indicated by the approving HREC. Please ensure to sign your initials with every entry below.

Protocol Number	
Study Title	Folate for Pregnancy Success: A phase 2 *randomised, double-blind,* placebo-controlled, double-blind *trial assessing the comparative effectiveness of 5-methlytetrahydrofolate and folic acid intake in couples with a history of recurrent pregnancy loss*
Study Site	**UTS Sydney**
HREC Approval Number	
Lead Researcher	**Carolyn Ledowsky**
Co-Investigator	**Dr Amie Steel, Dr Vanessa Scarf**

Severity	Study Intervention Relationship	Action Taken Regarding Study Intervention	Outcome of Adverse Event
1 = Mild 2 = Moderate 3 = Severe	1 = Definitely related 2 = Possibly related 3 = Not related	1 = None 2 = Discontinued permanently 3 = Discontinued temporarily 4 = Reduced Dose 5 = Increased Dose 6 = Delayed Dose	1 = Resolved, No Sequel 2 = AE still present- no treatment 3 = AE still present-being treated 4 = Residual effects present-not treated 5 = Residual effects present- treated 6 = Death 7 = Unknown

Participant Code	Participant Initials	Date of Onset Date Ended	Description	Severity See p.1 for CAT	Relationship See p.1 for CAT	Action Taken See p.1 for CAT	Outcome of AE See p.1 for CAT	Initials

Appendix 23 – Trial Study – Adverse Events Report Form

Participants ID: _____

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Adverse Event Report Form

This report facilitates tracking and timely reporting of all significant adverse events occurring during a research project. Adverse events are any unfavourable medical occurrence, including any abnormal signs, symptoms, or disease, whether or not they are associated with the participant's participation in the research project.

ANZCTR Protocol Number	
Study Title	Folate for Pregnancy Success: A phase 2 *randomised, double-blind,* placebo-controlled, double-blind *trial assessing the comparative effectiveness of 5-methlytetrahydrofolate and folic acid intake in couples with a history of recurrent pregnancy loss*
Study Site	**UTS Sydney**
HREC Approval Number	
Lead Researcher	**Carolyn Ledowsky**
Co-Investigator(s)	**Dr Amie Steel, Dr Vanessa Scarf**

Please complete as much information as is known.

Date:

Initial report ☐ Follow Up report ☐ Final report ☐

Details of AE

Please complete as much information as possible and submit and **Initial Report** to the Lead Researcher within 24 hours of becoming aware of the AE.

Description of AE

Diagnosis (if known):

Relevant tests, laboratory results, PMHx:

Participants ID: _____

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Onset date:

Stop date (OR Ongoing):

☐ Ongoing

Relationship to study drug/IMP?

☐ Definitely related
☐ Probably related
☐ Possibly related
☐ Unlikely related
☐ Not related

If the AE is related to IMP, does the AE meet the definition of an SAE?

☐ Yes – participant to cease IMP immediately. Notify investigators immediately.

☐ No

If No Implement Non-serious AE protocol

☐ No – participant to cease Prenatal and follow Non-serious protocol

Action regarding IMP as a result of the AE?

☐ None
☐ Reduced dose
☐ Discontinued permanently
☐ Increased dose
☐ Discontinued temporarily
☐ Delayed dose

Severity

☐ Mild ☐ Moderate ☐ Severe

Outcome of AE

☐ Resolved
☐ Resolved with sequelae
☐ Ongoing
☐ Death
☐ Unknown

Participants ID: _____

UNIVERSITY OF TECHNOLOGY SYDNEY

Concomitant Medications

Please record any changes or additions to Concomitant Medications as a result of the AE.

Drug	Dose	Route	Indication	Start date	Stop date

Lead Researcher Name **Lead Researcher signature** **Date**

Appendix 24 – Trial Study – Non-serious Adverse Events Protocol

Participants ID: _____

UNIVERSITY OF TECHNOLOGY SYDNEY

Non-serious Adverse Events protocol

ANZCTR Protocol Number	
Study Title	**Folate for Pregnancy Success Trial: A Phase 2 controlled, double-blind randomised clinical trial comparing the effectiveness of 5-methyltetrahydrofolate and folic acid intake in couples with recurrent pregnancy loss.**
Study Site	**UTS Sydney**
HREC Approval Number	
Lead Researcher	**Carolyn Ledowsky**
Co-Investigator(s)	**Dr Amie Steel, Dr Vanessa Scarf**

The following are considered to be a non-serious adverse event and will mean that the research team will follow the protocol below. If the adverse event is not resolved and the participant cannot resume taking the prenatal multivitamin they will be withdrawn from the study.

- Headaches
- Nausea
- Dizziness
- Sleep disturbances – sleep onset, sleep latency
- Cycle changes – change in cycle length, reduced menstrual flow, increased menstrual flow, menorrhagia,
- Anxiety
- Skin flare – flare in eczema, rash, pruritis, erythema,
- Stomach and/or bowel conditions – constipation and/or diarrhoea, cramping
- Muscle pain
- Nausea and vomiting due to pregnancy (first trimester), hyperemesis Gravidarum
- Food poisoning
- Cold or flu symptoms
- COVID-19

Protocol for those not pregnant

Step 1: advise participant that these reactions may be a normal side effect from an increase in folate dose.

Step 2: Advice to participant

a. Discontinue until symptoms subside (maximum 1 week)

b. Week 3: re-introduce the prenatal multivitamin at one capsule per day. Wait one week

c. Week 4: if tolerated, re-introduce the prenatal multivitamin at two capsules per day. Wait one week

UNIVERSITY OF TECHNOLOGY SYDNEY

Participants ID: _____

d. Week 5: if tolerated, re-introduce dose at 3 capsules per day.
e. If participant can tolerate re-introduced dose they continue in the trial. If participant cannot tolerate, they will be withdrawn from the trial.

Protocol for those pregnant where early pregnancy is contributing to nausea and/or vomiting

Step 1: Advise participant to cease the prenatal multivitamin for 3 days

Step 2: After three days, try to include one prenatal multivitamin after lunch. Continue for one week

Step 3: If after one week, they have tolerated the one capsule per day, ask them to increase to two caps per day. If they can tolerate continue to step 4.

If they cannot tolerate continue with one capsule daily. Reduced dose can be continued until vomiting and/or nausea ceases but no longer than week 13 of pregnancy. Following week 13 of pregnancy they must titrate to the three capsules per day or withdraw from the study.

Step 4: If after one week, they have tolerated two capsules per day, ask them to increase to three capsules per day. If they can tolerate continue to Step 5.

If they cannot tolerate continue with two capsules daily. Reduced dose can be continued until vomiting and/or nausea ceases but no longer than week 13 of pregnancy. Following week 13 of pregnancy they must titrate to the three capsules per day or withdraw from the study.

Step 5: Continue with study involvement. Record all modification to dose, including duration and number of capsules tolerated at each step.

Appendix 25 – Safety protocols for the Pilot Study

Distress and Safety Protocols

Distress and Safety Protocol: Women and Men

The following protocol was developed should a participant become distressed and require additional or ongoing assistance.

Distress Protocol

The protocol below was employed for the following:

- Should the participant have a pregnancy loss
- Should the participant experience psychological harms – feelings of worthlessness, stress, guilt, anger or fear, sadness or despondency related to her inability to conceive
- Anxiety and depression rating – should the participant indicate in their follow up meetings that they are experiencing any anxiety and/or depression, the DASS-21 scale was utilised to measure the emotional states of depression, anxiety and stress. The following protocol was used.

Step 1 – participant becomes distressed during an interview or emails the researcher to advise that they are in distress. The researcher determined the level of participant distress by using the Subjective Units of Discomfort scale (SUDs) where a participant rates themselves on a scale of 1-10, where 0= no distress and 10= extreme distress. Should the distress be transient, it was expected that the SUDs would reduce by 2-3 points within 15 minutes. The researcher was to remain online with the participant during this time to ensure that a rating of 3/10 or less was achieved, indicating mild distress and that the participant was not experiencing any lingering harm and that they could 'self-regulate' the distress. The researcher encouraged the participant to share their feelings with their regular support networks and/or someone they trust (e.g. partner, friend, family, colleagues) and to monitor their feelings over the next two days to see whether they resolve, continue, or become worse. Should their distress persist, they were to contact the UTS support services contacts provided in the participant information sheet, or contact their local GP. A follow-up phone call was made to the participant every day following the event to ask the participant about their distress and discuss whether participation should continue or discontinue. Should the participant not achieve the 3/10 or below within 15 minutes, the following protocol was followed:

Step 2 – Researcher offered support and extended the opportunity to:

a) Stop the interview – A member of the research team would provide assistance (the researcher had mental first aid course certification) to the participant to discuss their concerns and support them.

b) The researcher was then to ask the participant how they would like to proceed:

- Cease for a short break
- Reschedule for another day/time
- Cease participation altogether

c) Continue – if the participant wished to continue, the researcher would remind him/her of the support services open to him/her, i.e. UTS counselling services via 9514 1177. He/she was reminded that at any time throughout the trial he/she could withdraw from the study.

Step 3 – If the participant's distress was acute or a safety concern beyond what would be expected in an interview the following action was followed:

- Cease the interview
- Appropriate person to provide immediate support to the participant
- Seek the above SUDs rating as above to measure the severity of distress
- Recommend the participant speak to a professional as above
- Notify the chief investigator (supervisors) and ASMB immediately

Step 4 – if the participant's distress reflects imminent danger:

- Cease the interview
- Contact emergency services (000)
- Notify the chief investigator (supervisors) and ASMB immediately

A follow up phone call was made to the participant within 1-3 working days (or each day if required) to ask the participant about their distress and discuss whether participation should continue or discontinue.

Table 1 - Research Interview and Distress Protocol

Indications of Distress During Interview	Follow-Up Questions	SUD Rating/DASS-21 Rating	Participants Response/Behaviours	Acute Emotional Distress or Safety Concern? (Y or N)
Indicate they are experiencing a high level of stress or emotional distress, OR exhibit behaviours suggesting that the interview is too stressful such as uncontrolled crying, incoherent speech etc.	1. Stop the interview 2. Offer support and allow the participant time to regroup 3. Assess level of distress - SUD rating 4. Assess mental status (DASS-21): a. Tell me what thoughts you are having b. Tell me what you are feeling right now c. Do you feel able to go on about your day? 5. Determine if the person is experiencing acute emotional distress beyond what would normally be expected in an interview about a sensitive topic.			
Indicating they are thinking of hurting themselves or have rated above 3/10 in the SUD	1. Stop the interview 2. Offer support - express concern and conduct a safety assessment 3. Assess mental status: a. Tell me what thoughts you are having b. Tell me what you are feeling right now c. Do you intend to harm yourself? 4. Provide resources for support 5. Immediately report to supervisors and ASRB 6. Continue to follow up 1-3 days			

Removal of participant from the trial

- Should the participant wish to withdraw from the study, they would be advised that their data to-date will be included in the trial, but no further data will be recorded.
- The participant was advised that the researcher was to still contact him/her in the next 1-3 days to follow up and enquire about the participant's wellbeing but make it clear that the purpose is not to revisit his/her decision to withdraw.

Strategies to Assist Those Distressed During an Interview

If a participant became uncomfortable or distressed while discussing any topic during the interview, the following actions were taken by the interviewer:

1. The researcher suggested that the interview be terminated.

2. If the participant wished this to happen, the interview was ended.

3. In the case where a counsellor or GP was not readily accessible, a member of the research team who is a health professional was to spend time with the participant and provide assistance, within the scope of their abilities, to discuss their concerns and support them.

4. After seeking advice from the lead researcher, a recommendation was made that the participant speak to a counselling professional to discuss their concerns and, if they agreed, a referral was made.

5. UTS Faculty of Health Clinical Fellow, Dr Athena Hammond (PhD Midwifery, Graduate Certificate in Counselling) was available.

6. If the participant had a general practitioner (GP) involved in their care and it was more appropriate to refer them to their GP who was already familiar with their history and could provide continuity of care, then this was done.

7. The intended outcome of the activation of this protocol was a comprehensive assessment and the presentation of options regarding ongoing counselling or other management as appropriate.

8. A follow-up phone call was made by the interviewer the following day to ensure that the participant was well and determined the feasibility of a follow-up interview if required.

Distress and Safety Protocol: Researcher

The following protocol was developed should one of the researchers become distressed (Figure 12), or be at risk and require emergency, additional, or ongoing assistance. A range of services were to be offered depending on the circumstances.

Strategies to Assist Those Distressed During an Interview

1. Regular meetings with her supervisors.

2. The researcher was to be referred to a counselling professional to discuss their concerns or a referral made to their Employee Assistance Program.

It was the researcher's duty of care to ensure that there was a balanced consideration of the benefits against the risks. These strategies were put in place prior to commencing the interviews or discussions. Below is the step by step guided protocol adapted from Draucker (2009) [422].

Notifying the Lead Researcher

Any trial-related unanticipated problem that posed a risk of harm to participants or others, and any type of serious adverse event, was reported to the lead researcher within 24 hours of the event (Associate Professor Amie Steel). An Adverse Event Form *(Appendix 23, Adverse Event report form)* was completed by the researcher, and the researcher called the Principal Investigator to notify them of the Serious Adverse Event, and a form was also sent via email. The trial lead researcher then informed the Advisory Safety and Monitoring Board (ASMB) to discuss the event with regards to causality, and any action that was required.

Notifying the Oversight Authorities: HREC

In line with the NHMRC National Statement on Ethical Conduct in Research Involving Humans, the investigators:

- Informed the HREC of all serious adverse events that occurred during the pilot study and could possibly affect the conduct of the pilot study or the safety of the participants or their willingness to continue participation in the pilot study (Significant safety issues), using the *UTS HREC Adverse Incidents and Events Report form*.
- Informed the HREC as soon as possible of any new information from other published or unpublished studies which may have an impact on the continued ethical acceptability of the pilot study, or which may indicate the need for amendments to the pilot study protocol.
- Within the following 48 hours, the investigators were to provide further information on the serious adverse event or the unanticipated problem in the form of a written narrative. This included a copy of the completed Serious Adverse Event form, and any other diagnostic information that assisted the understanding of the event. Significant new information on ongoing serious adverse events was provided promptly.
- All other adverse and serious adverse events were documented in the source files, and a table was created to provide an overview of all events for the HREC (annual report).

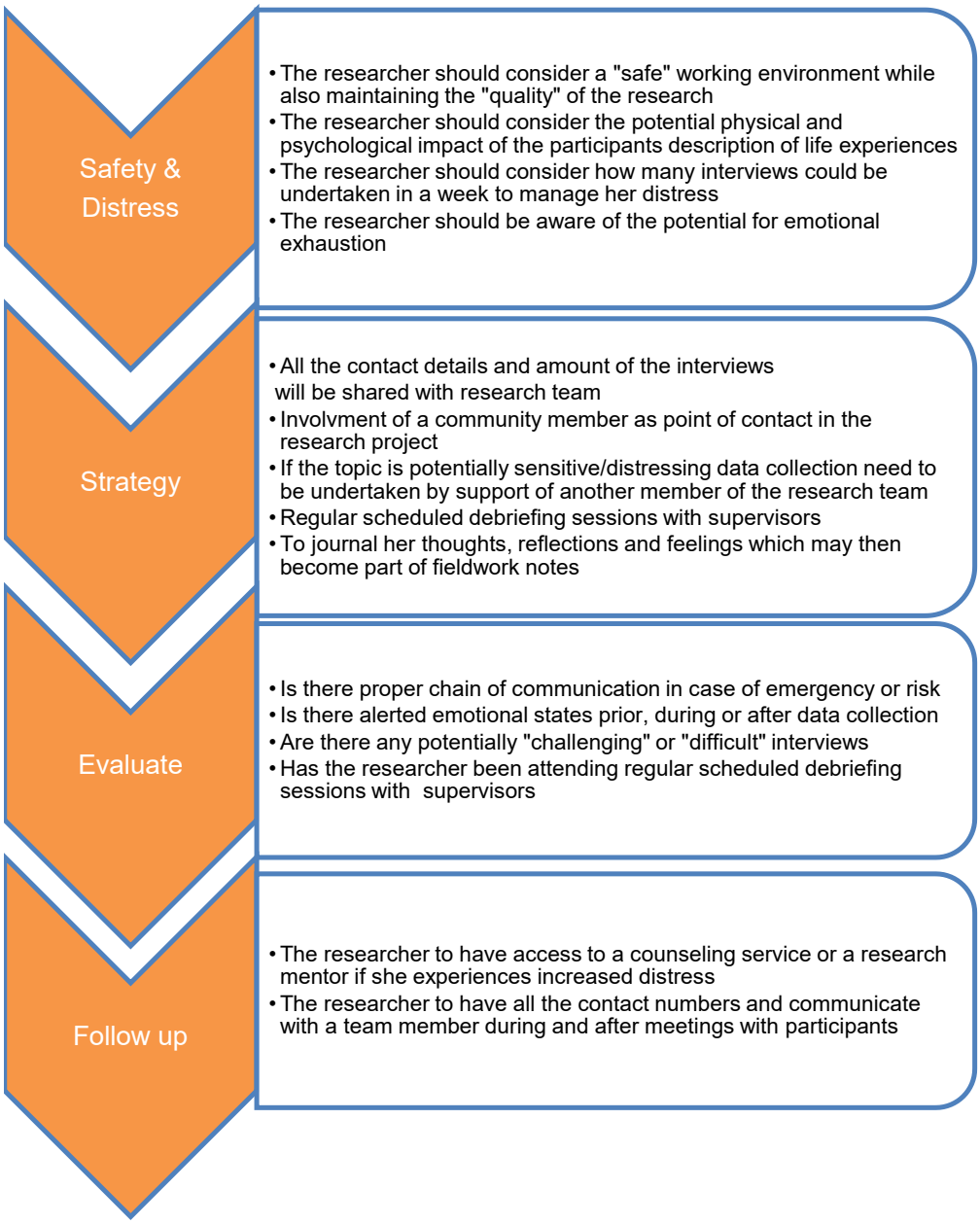

Figure 1- Researcher safety protocol

Notifying the Oversight Authorities: Therapeutic Goods Association (TGA)

There were no serious adverse reactions related to either trial product that were required to be reported to the Adverse Drug Reaction Unit of the TGA.

In line with the NHMRC National Statement on Ethical Conduct in Research Involving Humans, the investigators must:

- Inform the TGA of all serious adverse events that occurred during the pilot study and may affect the conduct of the pilot study or the safety of the participants or their willingness to continue participation in the pilot study (Significant safety issues).

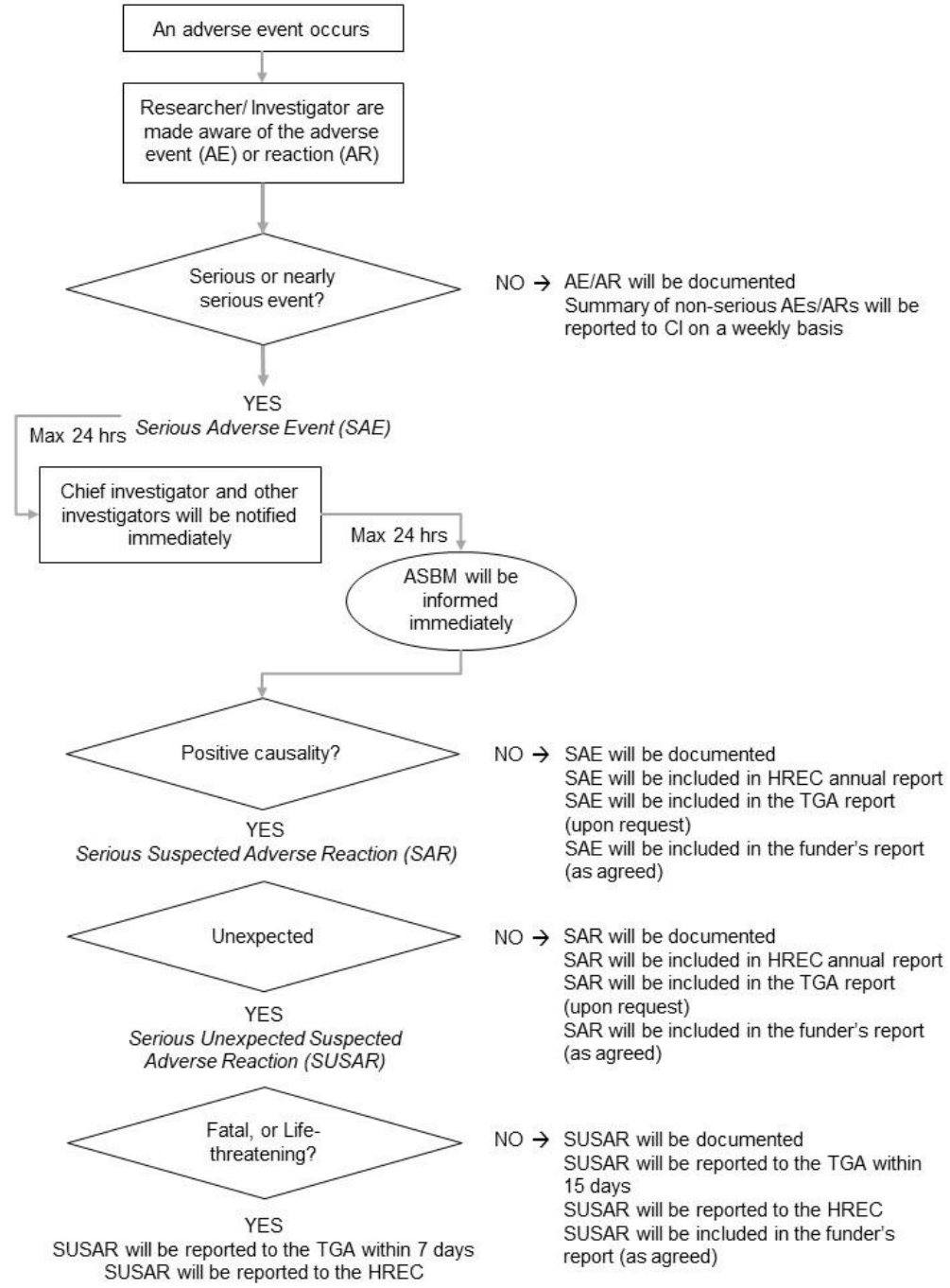

Figure 1- Adverse Events management flow chart

Other Reportable Events

The following events were also to be reported to the HREC and the TGA immediately, within 48 hours of obtaining knowledge of the event.

- Any adverse experience that, even without detailed analysis, represented a serious unexpected adverse event that is rare in the absence of drug exposure.
- Any adverse event that would cause the researchers to modify the protocol, or informed consent form, or would prompt other action by HREC for the protection of human participants.
- Information that indicated a change to the risks or potential benefits of the research, in terms of severity or frequency. For example:
    - An interim analysis indicates that participants have a lower rate of response to treatment than initially expected.
    - Safety monitoring indicates that a particular side effect is more severe, or more frequent than initially expected.
    - A paper is published from another study that shows that an arm of the research study is of no therapeutic value.
    - Breach of confidentiality.
    - Complaint of a participant when the complaint indicates unexpected risks, or the complaint could not be resolved by the research team.
    - Protocol violation (meaning an accidental or unintentional deviation from the approved protocol) that in the opinion of the investigator placed one or more participants at increased risk or affects the rights or welfare of subjects.
    - Abnormal laboratory values

Hospitalisation, Prolonged Hospitalisation or Surgery

No adverse event resulted in hospitalisation or prolonged hospitalisation.

Auditing

The auditing process involved inspection of the following to ensure protocol compliance:

- Informed consent forms
- Documentation of the consent process
- Reported data

- Regulatory records
- Source document

Self-audits were carried out by the researchers to ensure a systematic, disciplined approach to evaluate and improve the effectiveness of risk management, control, and governance processes. (*see Office for Research Audit Checklist, Appendix 26*).

Appendix 26 - Trial Study – Office for Research Audit Checklist

UNIVERSITY OF TECHNOLOGY SYDNEY	**Audit Checklist – Research Project**

Section 1 – Team Details *Manager to complete all sections*

Research Team Name			
Primary Site :		Position / Title :	
Responsible Manager's Name :		Date of audit	
Auditor's name:			

Section 2 – Adminsitrative information

No.	Description	Required	Completed
2.1	Is there a membership list of research team?	Yes / No / NA	❑
2.2	Has the research team completed a self-acceditation form?	Yes / No / NA	❑
2.3	Have all relevant departmental approvals been obtained?	Yes / No / NA	❑
2.4	Does the team have a separate research account?	Yes / No / NA	❑
2.5	Does the team have a team charter or contract ?	Yes / No / NA	❑
2.6	Does the team have adequate working space?	Yes / No / NA	❑

Section 3 – Site qualification, adequacy of resources and training

No.	Description	Required	Completed
3.1	Do team members have skills and experience to conduct the research being undertaken?	Yes / No / NA	❑
3.2	Do the staff meet all the qualifications specified by the applicable regulatory requirement(s) eg. Current medical practitioner registration?	Yes / No / NA	❑
3.3	Are the team able to demonstrate a potential for recruiting the required number of suitable subjects within the agreed recruitment period?	Yes / No / NA	❑
3.4	Are all persons assisting with the trial adequately informed about the protocol, the investigational product(s), and their trial-related duties and functions?	Yes / No / NA	❑
3.4	Are records kept relating to delegation of tasks and are the tasks delegated to appropriately skilled and experienced staff?	Yes / No / NA	❑
3.5	Is all documentation of trial related training kept current and available for review?	Yes / No / NA	❑
3.6	Are all essential documents maintained according to ICH-GCP?	Yes / No / NA	❑

Section 4 – Access to Key information

No.	Description	Required	Completed
4.1	Does the team have access to National Statement on Ethical Conduct in Human Research 2007?	Yes / No / NA	❑
4.2	Does the team have access to Research Governance Office & HREC SOPs?	Yes / No / NA	❑
4.3	Does the team have access to [insititute] Policies & SOPs?	Yes / No / NA	❑
4.4	Does the team have access to Australian Clinical Trial Handbook TGA 2006?	Yes / No / NA	❑

Research Governance Office Audit Checklist – Researcher *continued*

4.5	Does the team have access to VMIA Guidelines for Clinical Trials for Victorian Public Hospitals 2009 or as updated from time to time?	Yes / No / NA	❑
4.6	Does the team have access to Access to Unapproved Therapeutic Goods – Clinical trials in Australia TGA 2004?	Yes / No / NA	❑
4.7	Does the team have access to Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) annotated with TGA comments TGA, 2000?	Yes / No / NA	❑
4.8	Does the team have access to Australian Code for the Responsible Conduct of Research NHMRC 2007?	Yes / No / NA	❑
4.9	Does the team have access to Privacy Act 1988?	Yes / No / NA	❑

Comments:

Name Signature of Manager: _____

Signature of Manager: _____ Date: ____/____/____

Appendix 27 – Trial Study – Stash Management plan

Folate for pregnancy success trial

Description

We will be conducting a randomised clinical trial comparing 5-MTHF with folic acid in couples with recurrent pregnancy loss. There will be two arms: control arm will be the folic acid arm, intervention is the 5-MTHF arm.
The trial will recruit 130 women of childbearing age (20-49 years of age) and their male reproductive partners. They will be taking the pre-natal multivitamin for 3 months of preconception care and 10 months of trying to conceive/pregnancy.

Expand/Collapse all

- Project

Project overview

Project name
Folate for pregnancy success trial

Research Master Project Code / Student ID

HDR student project

Project description
We will be conducting a randomised clinical trial comparing 5-MTHF with folic acid in couples with recurrent pregnancy loss. There will be two arms: control arm will be the folic acid arm, intervention is the 5-MTHF arm.
The trial will recruit 130 women of childbearing age (20-49 years of age) and their male reproductive partners. They will be taking the pre-natal multivitamin for 3 months of preconception care and 10 months of trying to conceive/pregnancy.

Keywords

folate

folic acid

pregnancy loss

miscarriage

preconception

pregnancy

Start date
01/09/2022

End date
01/09/2022

Funders

no funding

Grant ID

None

- People

People

First-named chief investigator / UTS supervisor

Name	Email	Project Role	ORCID
Amie Steel	Amie.Steel@uts.edu.au	Chief Investigator	

Data manager

Name	Email	ORCID
Carolyn Ledowsky	carolyn.j.ledowsky@student.uts.edu.au	

Contributors

Name	Email	ORCID

Additional supervisors

Name	Email	ORCID
Abela Mahimbo	Abela.Mahimbo@uts.edu.au	
Vanessa Scarf	Vanessa.L.Scarf@student.uts.edu.au	

- Ethics and security

Ethics and Security

Initially your research data is classifed as UTS Internal. To improve the accuracy of this classification, please answer the following

Information Security Classification
UTS: Confidential

Does the research involve:
Human participant data
Clinical trials
Other sensitive data

Other, not listed above
blood test results, genetic testing results

Will the data you collect from individuals include personal information?
Yes

Will the data you collect from individuals include sensitive personal information other than health information?
Yes

Will the data you collect from individuals include health information?
Yes

Will any data or information be individually identifiable or potentially re-identifiable (i.e. include codes) at any stage of the research?
Yes

Outline any potential risk to participants from accidental disclosure of the data and any strategies for minimising those risks
Blood test results will be individually identifiable. All results will be de-identified by the lead reseacher and no other member of the team will have access to the personal details of participants. The information will be held in secure folders by UTS data management system. No data will be held outside this system.

If you are collecting data from residents of countries other than Australia, which countries?
NA

Is Ethics approval required for your project?
Yes

– Data collection and storage

Data collection and storage

Please provide a brief description of your data collection methodology

3.1 DATA COLLECTION METHODS

Data will be collected online (REDCap) for the primary and secondary outcomes. All data will export to Stata statistical analysis software for analysis. At baseline, basic sociodemographic and clinical data will be collected, they will be used to describe the sample, as well as serve as confounding variables in the statistical analyses. The following participant reported outcomes will be used for outcome assessment:

The primary outcome will be assessed as a viable pregnancy > 22 weeks gestation. This will be confirmed by the participants' GP and/or fertility specialist.

The secondary outcome measures include:

Secondary Outcomes;

● Modification of pregnancy success based on MTHFR polymorphisms (C677T and A1298C). Blood tests will determine if any polymorphisms exist for the group.

● Any adverse events associated with using 2,500mcg of 5-MTHF

● Time to pregnancy

● Folate absorption

- Measurement of methyl donors via blood collection methods:

- Fasting homocysteine (baseline collection)

- Red cell folate (Baseline), end of preconception period (3 months), 6months, 12 months

- Serum folate (Baseline), end of preconception period, 6 months, 12 months

- Vitamin B12 – serum B12 (Baseline), end of preconception period (3 months)

Participants will receive a referral for the above blood work to be collected as a commercial pathology lab near where the participant is located. Red blood cell folate will be assessed using a microbiologic assay as indicated by Marchetta et al, as this methodology is not affected by MTHFR genotype (Marchetta & Hamner, 2016).

● Live birth

● Birth abnormalities

Safety: Adverse events (AE) will be collected throughout the trial. They will be defined as any symptom, sign, illness, or experience that develops or worsens in severity during the course of the study, including intercurrent illnesses or injuries and abnormal diagnostic results. Participants will be asked at each follow-up to provide information on any adverse event since the last visit.

An overview of the outcome measurements, and time points can be found in Table 1.

A detailed description of the visits can be found below.

Table 1: Overview of time points and data collection methods

Pre-screening

Confirmation interview Baseline

(Day 0) F/U up

Week 2 F/U

Wk. 8,14,20,26,32 F/U

Wk. 44

Screening x x

Written informed consent x

Randomisation x

Sociodemographic information, medical history (CRF) x

MTHFR Polymorphisms x

Homocysteine blood test x

Serum B12 blood test x
Serum folate blood test x x x x
Red cell folate blood test x x x x
Safety (AE) x x x
Compliance, medications, adverse events (Diary) I---------- ---------- ---------- ---------
--
Accounting for investigational products x x x
End-of-trial evaluation x

Predominant file type(s), e.g. textual, tabular, image or recording. Give file format if known
textual, tabular

Data storage location
UTS provided survey platform (e.g Qualtrics, REDCap, etc)
eResearch storage platforms (e.g Omeka, Geoserver etc)

If other, provide further details: (Including access arrangements for the minimum retention period)

Describe the form(s) of the identifiable or re-identifiable data
Name, address, blood test results, health history, MTHFR gene test

What platforms or tools will you use to collect or import identifiable or re-identifiable data?
RedCap
Qualtrics

Where will the identifiable or re-identifiable data be stored?
eResearch Store

Will you be seeking prior informed consent to publish identifiable participant data?
Yes

Will identifiable or re-identifiable data be transferred in or out of secure UTS storage (e.g. in from linkage agency, out for transcription)?
No

Will you de-identify the data?
Yes

Outline how and when (e.g. after transcription, before analysis) you will de-identify the data:
Data will be de-identified after transcription. As participants enter the study they will be de-identifed.Data that is collected on hard copies (e.g., forms completed by doctors) will be manually entered or scanned and uploaded into the REDCap database by the researcher. The REDCap database may contain identifying information, which will be needed to distribute the questionnaire online, it will be flagged as identifying information and not be exported into the dataset for data analysis. REDCap has been specifically developed for research and provides a secure environment for data management. All hard copy files will be kept in a locked filing cabinet in a locked room at the UTS Sydney campus, accessible to UTS clinical trials researchers. Files for statistical analysis will be de-identified using the participant code and will be kept on password protected drives on UTS computers, accessible to those involved in the clinical trial only. Once the database is locked, only the CI/PI and Co-I's will have access to the data for analysis. Before analysis, all data will be checked for plausibility (via descriptive statistics such as mean, median and range), and data recoded to create summary scores of the questionnaires. The syntax and a log of any

data processing will be stored in the same folder as the original data. Upon study completion all electronic files will be saved in STASH.

Where will any link files (files that match pseudonyms/codes to identifiable information) be stored?
on eResearch Strore

- Data retention and disposal

Data retention and disposal

Minimum retention period
15 years (clinical trials or research with potential long term effects on human subjects)

The data steward is:
Amie Steel

Have you made commitments to destroy part of the data prior to end of retention period (e.g original recordings, linking/code files)?
Yes

Which data?
In accordance with ethics approval and participant information and consent forms, data will be stored in a re-identifiable (coded) format for the duration of the study as well as the following 15 years as required by the UTS HREC. All data will then be destroyed.

When should it be destroyed?
30/12/2039

- Access and rights

Access and rights

Copyright and intellectual property owners of data created in project
Higher Degree Research Student

Please list any other owners:
University of Technology Sydney

Access after the project will be
Mediated, by permission from the data manager

Are you using any secondary or third-party data?
No
Licences or Agreements:

Appendix 28 – Publication Trial Paper

Nutrition Research 146 (2026) 68–81

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/NTR

Original Research

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]

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5-MTHF
Folic acid
Infertility
Unmetabolized folic acid

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.

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Health professionals require more defined protocols, better funding and patient resources to support couples with recurrent pregnancy loss

Carolyn Ledowsky[1], Vanessa Scarf[2] & Amie Steel[1]

The risk of pregnancy loss prior to 24 weeks gestation has been found to be 15.3% of all pregnancies, around 23 million losses each year. Recurrent pregnancy loss (RPL) - two or more pregnancy losses - are now presumed to affect 5% of couples. Clinical guidelines for health professionals for RPL are conflicting, lacking in good quality evidence and fail to involve those who experience RPL, especially the male partner. Despite folate being recommended for preconception support, no mention of folate exists in RPL guidelines. A national cross-sectional survey which assessed health professionals' recommendations, testing, prescribing and referral practices when providing care for couples in preconception and RPL was conducted. Clinical guidelines for RPL were found to be critically important for health professionals to provide care to couples experiencing RPL, however health professionals (n=175) require stronger clinical guidance and more specialised training (47.3%), education (62.8%) and funding to better assist couples with RPL. Only 34% of health professionals reported having the necessary resources to support couples with RPL. Health professionals are aware of how important folate is to support preconception in females but are unaware of the critical role it plays in male fertility and as a result males are largely ignored in preconception care. Given the importance of folate supplementation in preconception and pregnancy, the guidelines for RPL fail to include dosing recommendations or variations in the type of folate that should be prescribed. Improved clinical guidelines, better resourcing and funding are required for health professionals to better support couples with RPL. Male partners need to be included in the preconception care and folate form and dosing must be considered.

Keywords Recurrent pregnancy loss, Miscarriage, Clinical guidelines, Folate, Male

Abbreviations

BMI	Body mass index
FA	Folic acid
FVL	Factor V Leiden
GPs	General practitioners
HPs	Health practitioners
MTHFR	Methylenetetrahydrofolate reductase
STIs	Sexually transmitted infections
RPL	Recurrent pregnancy loss
UPL	Unexplained pregnancy loss

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