The Pharmacology of Periconceptional Folic Acid Supplementation

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Pharmaceutical Sciences
University of Toronto

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University of Toronto
2014

Abstract
Periconceptional folic acid supplementation is associated with a reduction in the risk of neural tube defects, other birth defects, as well as positive maternal outcomes during pregnancy. Current healthcare recommendations regarding the timing and dosage of prenatal supplementation are based on studies in non-pregnant women of childbearing age. The overall objective of this thesis is to characterize risk factors associated with the periconceptional period, validate the effectiveness of folic acid supplementation based on pregnancy-related pharmacokinetics, analyze the current folate status of women in Toronto, as well as explore recruitment strategies for clinical trials in pregnant women.

The first study was a two-arm, randomized, clinical trial assessing the long-term folate kinetics in pregnant women supplementing with either 1.1mg (regular dose) or 5mg (high dose) of folic acid. This study evaluated dose-dependent and gestational age-dependent changes in the pharmacokinetics among pregnant women, which have not been previously studied at these doses of supplementation. We found non-linear folate pharmacokinetics in the 5mg group, as well as altered pharmacokinetics in both dose-groups due to pregnancy-related changes. The second study was based on secondary analysis emerging from the randomized clinical trial,
where we tackled the challenges associated with recruitment of pregnant women (a vulnerable population) into a study on folic acid supplementation at a time when a high degree of supplementation awareness already exists among women in Toronto. We used social media as a novel tool for recruitment, and found a 12-fold increase in recruitment rate. The third study was a systematic review and meta-analysis investigating the effect of oral contraceptive use on blood folate concentrations among women. We found that oral contraceptives had a folate-lowering effect and recommended continued folate supplementation among oral contraceptive users. Finally, in our fourth population-based cohort study, we evaluated blood folate concentrations among women in Toronto and found that 93% of women of childbearing age had optimally protective blood folate concentrations against neural tube defects.

Recommendations for periconceptional folic acid supplementation should be re-evaluated based on population-level data, and be tailored to individual risk factors such oral contraceptive use or altered folate demands during pregnancy.
Acknowledgements

“What you seek is also seeking you”    -Rumi

Firstly, thank you to my supervisor and mentor, Dr. Gideon Koren, for giving me the opportunity to be a part of your lab and work on this project. Thank you, for your endless support and guidance at every step. For your insights, creativity and truly inspiring leadership, which have helped me nurture true scientific curiosity as part of, and well beyond, my graduate pursuits.

I would also like to extend my sincere gratitude to all of my advisors: To Dr. Bhushan Kapur for your invaluable advice and guidance through every hurdle. I am also indebted to Dr. Shinya Ito and Dr. Ahmed El-Sohemy, you have provided a wealth of knowledge and guidance in shaping the scope of my projects, and have challenged me to carefully explore each aspect of my project with its multi-faceted approaches.

I would like to thank Dr. Deborah O’Connor and her lab, for sharing your expertise with me as I trained within the area of folate research. As well, I am grateful to all of my colleagues at Motherisk for truly making Motherisk my home away from home.

Finally, thank you to my family and friends for enduring me during stressful times, and for your continued love and support, which have not only made this pursuit possible, but also worthwhile. Thank you for all your genuinely curious and incisive questions about my work that have pushed me to truly explore its broader implications, and have helped me fall in love with my research along the way.

Thank you to everyone who has inspired every little eureka moment along the way – if I have seen [anything], it is only by standing on the shoulders of giants.
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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>5-methyl-THF</td>
<td>5-methyl tetrahydrofolate</td>
</tr>
<tr>
<td>5,10-methylene-THF</td>
<td>5,10-methylene tetrahydrofolate</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate Intake</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CHDs</td>
<td>Congenital heart defects</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DFEs</td>
<td>Dietary Folate Equivalents</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intake</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated Average Requirement</td>
</tr>
<tr>
<td>FBP</td>
<td>Folate binding protein</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal-growth restriction</td>
</tr>
<tr>
<td>FPGS</td>
<td>Folypolyglutamate synthetase</td>
</tr>
<tr>
<td>FR-α</td>
<td>Folate receptor (alpha)</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GCPII</td>
<td>Glutamate carboxypeptidase II; (\gamma)-glutamyl hydrolase</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
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<tr>
<td>MRP3</td>
<td>Multidrug resistance protein 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NTDs</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>OC</td>
<td>Oral contraceptive</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton-coupled folate receptor</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFC1</td>
<td>Reduced folate carrier 1</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SOGC</td>
<td>Society of Obstetricians and Gynaecologists of Canada</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable Upper Intake Limit</td>
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1 CHAPTER 1.

General Introduction

1.1 REVIEW OF THE LITERATURE

1.1.1 Folate

1.1.1.1 Chemistry and function

Folic acid, also known as pteroylmonoglutamate, consists of a pterin (2-amino-4-hydroxy-pteridine) moiety linked via a methylene group at the C-6 position to a glutamate (p-aminobenzoyl-glutamate) moiety [1]. Folate metabolism consists of three major reactions: the reduction of the pyrazine ring of the pterin moiety to the coenzymatically active tetrahydro form; the elongation of the glutamate chain through the addition of glutamate residues in the γ-peptide linkage; and the oxidation or reduction of one-carbon units at the N-5 and N-10 positions [1]. Subsequent folate derivatives are obtained from the above reactions. Generally, the reduced form of folate is more chemically unstable than the oxidized form (Figure 1).
Within the body, folate coenzymes function as acceptors or donors of one-carbon units in reactions involving nucleotide and amino acid synthesis, through one-carbon metabolism [1]. Mammals are incapable of de novo folate synthesis, which is why it is essential that folate is obtained through the diet. Naturally occurring folates are reduced derivatives, whereas folic acid is the completely oxidized form of folate, without any carbon substitutions [2]. Because of the high stability of oxidized folate, folic acid is the preferred form used in supplements and fortification [2]. The loss-of-stability of reduced folates depends on the type of the one-carbon substitution.

1.1.1.2 Sources

Folate-rich foods include dark, leafy green vegetables, animal liver, legumes, oranges, and strawberries, as well as folate-fortified foods such as flour, cornmeal, enriched grain and cereal products. Folate can also be taken through folate supplements, or through multivitamin/mineral supplements containing folic acid [3, 4].
1.1.2 **Folate Pharmacokinetics**

1.1.2.1 **Absorption**

Folates are absorbed differentially through the intestinal mucosa depending on their form. Dietary folates are polyglutamate derivatives, and are hydrolyzed to monoglutamate forms prior to intestinal absorption, because only the monoglutamyl forms are able to cross cell membranes [5, 6]. This hydrolysis of folypolyglutamates is catalyzed by glutamate carboxypeptidase II (GCPII) at the intestinal brush border in the proximal jejunum, which functions at an optimal pH of 6.5 [5, 7, 8]. In contrast to dietary folates, supplemental or fortified folic acid is a monoglutamatym form, and does not require conversion prior to absorption.

The exact mechanism of folate transport through the mucosal cell is not fully understood, however, it is known that it consists of both diffusion and transporter-mediated absorption. About 20-30% monoglutamyl folate is passively absorbed through diffusion, regardless of concentration [5, 8]. The major transporters responsible for folate transport across the intestine include the proton-coupled folate transporter (PCFT) and to a lesser extent, the reduced folate carrier I (RFC1). During the process of absorption, metabolism of reduced folates and folic acid to 5-methyl-tetrahydrofolate (5-methyl-THF) can occur within the mucosal cell, but the degree of metabolism is dependent on the folate dose, and it is a saturable process [9]. Folate efflux from the intestinal mucosa to the portal circulation is mediated by the multidrug resistance protein 3 (MRP3) [7, 10] (Figure 2).
1.1.2.2 Distribution

The circulating form of folate is 5-methyl-THF, a pteroylmonoglutamate. After folate absorption into the portal circulation, it can either be taken up by the liver to be stored after metabolism to polyglutamate derivatives, or it can be released into blood or bile. Some circulating folate is bound to low-affinity protein binders, including albumin, but the proportion of bound-folate is variable depending on certain physiological states, such as folate deficiency or pregnancy [10]. Erthrocytes primarily contain 5-methyl-THF polyglutamates, which are incorporated during erythropoiesis. Transport into peripheral tissues is mediated mainly by RFC1, and PCFT to a lesser extent [12]. Folate transport into the cerebrospinal fluid (CSF) through the choroid plexus depends on PCFT, RFC1 and folate receptor alpha (FR-α), yet the exact mechanism of transport is not well understood [7, 13]. Finally, folate is reabsorbed by the renal proximal tubule through FR-α [7, 10, 12].
1.1.2.3 Metabolism

Metabolism to polyglutamate derivatives is not merely a method of folate storage, but polyglutamyl folates are actually the active coenzyme species involved in one-carbon metabolism [10, 14] (Figure 3). Folic acid is reduced to tetrahydrofolate (THF) through the two-step reduction mediated by dihydrofolate reductase (DHFR), with the first step being rate-limiting [7]. Folates in tissues function as one-carbon donors and acceptors, and are involved in one-carbon metabolism. Tissue folate accumulation requires the metabolism to polyglutamate derivates in the cytosol or mitochondria of cells, and is mediated by different isozymes of folypolyglutamate synthetase (FPGS) [15, 16].

In humans, whole-body folate turnover entails the catabolism of folates to cleavage products (N-acetamidobenzoyl-glutamate and p-aminobenzoyl-glutamate), occurs at a much slower rate than tissue turnover, and is also dependent on folate dose [10]. Thus, tissue turnover involves folate hydrolysis to monoglutamates with the release of intact folates into circulation, followed by reuptake into tissues [15, 17].
Figure 3. Role of folate in one-carbon metabolism. Adapted from Wani et al. (2008) [18]

Folate provides methyl groups for nucleotide synthesis via 5,10-methylenetetrahydrofolate (5,10-methylene-THF), or to methylation reactions via 5-methyltetrahydrofolate (5-methyl-THF) and S-adenosylmethionine (SAM). These two pathways of folate metabolism are separated by an irreversible reaction mediated by the methylenetetrahydrofolate reductase (MTHFR) enzyme.

1.1.2.4 Elimination

After folate filtration through the glomerulus, it is primarily reabsorbed through the proximal tubule and its cleavage products are eliminated through urine. Though urine contains some folate derivatives, the majority of excretion products in humans are folate cleavage products [5, 17, 19].

Some intestinal reabsorption does occur, after which, up to 100µg/day of folate is eliminated via biliary excretion [2, 10].

1.1.2.5 Altered folate demands during pregnancy

Changes in folate status including a decrease in circulating maternal blood folate concentrations, and increased folate requirements during pregnancy have been well-documented
within the literature. Several pregnancy-related changes have been implicated based on their effect on folate status. Some of these are presented below.

1.1.2.5.1 Expansion of tissues and plasma volume

Pregnancy is a time associated with embryonic and fetal growth, as well as growth of uteroplacental and breast tissues. Thus, expanding maternal and fetal tissues increase the demand for folate during pregnancy [20]. Bruinse et al. also demonstrated a decrease in plasma folate concentrations due to the expansion of blood volume during pregnancy. However, results from this study showed that the decline in serum folate (42%) was much greater than the decline in total circulating folate (28%), suggesting that hemodilution, or the expansion of plasma volume, cannot solely explain the decrease in serum folate concentrations observed in pregnancy [21].

1.1.2.5.2 Intestinal absorption (bioavailability)

Studies investigating the impact of pregnancy-related changes in the absorption of folate have provided mixed results. A study by Chanarin et al. reported lower peak serum folate levels after the consumption of a single-dose folic acid supplement in pregnant women vs. non-pregnant women [22]. In contrast, a study by Landon and Hytten found no significant differences in plasma folate concentrations after a single oral dose of folic acid between pregnant women and their post-partum levels, as well as compared to levels in adult males [23].

1.1.2.5.3 Folate catabolism

An increase in the excretion of folate catabolites has been reported in late-pregnancy [24], but not during mid-pregnancy [25] or in the second trimester [26], in comparison to non-pregnant women. This suggests that late pregnancy is associated with an increase in folate catabolism.
1.1.2.5.4 Folate clearance

A study investigating folate clearance after an intravenous injection of folic acid reported higher clearance in pregnant women compared to non-pregnant women, and that clearance continued to increase as the pregnancy progressed [22]. Similar results were found by Landon and Hytten who compared 24-h urinary excretion in women during pregnancy and post-partum [27]. Thus, the evidence points towards increased folate clearance in pregnancy.

1.1.2.5.5 Hormonal effect

Several researchers implicate hormonal as a physiological response to pregnancy, in affecting folate metabolism and hence, folate status during pregnancy [20, 28]. However, the exact mechanism and the nature of hormones involved is not well-defined.

1.1.3 Folic acid in the prevention of birth defects and pregnancy complications

Due to its involvement in one-carbon transfer reactions, folate plays a critical role in DNA replication, DNA repair, and cell division, and therefore, in embryonic development. Most notably, research has shown that supplementation with folic acid in the periconceptional period is associated with a decrease in the risk of neural tube defects.

1.1.3.1 Neural tube defects

Neural tube defects are severe and debilitating malformations of the central nervous system that occur due to an improper closure of the neural tube during organogenesis. The neural tube typically completes closure 21-28 days post-conception [29-31]. There are two main forms of neural tube defects depending on whether the cranial or caudal end is involved: anencephaly involves the incomplete closure of the cranial end, and is lethal, such that death occurs either
before or shortly after birth, whereas spina bifida is characterized by incomplete closure at the caudal end, and can cause severe disabilities including paraplegia and paralysis of the lower extremities [30, 31] (Figure 4).

**Figure 4. Neural tube defects caused due to an improper closure of the cranial or caudal neurpores.** Adapted from Botto et al., 1999 [31].

The prevalence of neural tube defects is approximately 1 in 1000 births in the Western world, however, the incidence of NTDs varies across the world in relation to ethnicity, geography, and folate intake. Worldwide estimates range from 0.5 to 8 in 1000 births [32].

While the exact etiology of neural tube defects is not well understood, epidemiological studies have shown that both environmental, genetic, or a combination of these factors influence the risk
of these major malformations [31]. Mechanisms involved are thought to impair processes related to cellular proliferation, vascularization, DNA methylation, and proper shape formation of the neural tube during neurulation [33].

Despite the complex mechanisms, about 50-75% of neural tube defects are responsive to and preventable by folic acid supplementation [34]. With respect to the role of folate in the prevention of neural tube defects, several hypotheses have been proposed but the exact mechanism still remains unknown. Rothenberg et al. showed that 75% of mothers who had given birth to an NTD-affected infant had auto-antibodies to the folate receptor (FR) [35]. In contrast, only 10% of mothers had these auto-antibodies in non-NTD-affected infants, suggesting that maternal auto-antibodies targeting the FR may impair the intracellular uptake of folate by epithelial cells, and may lead to NTDs [35]. Studies analyzing postpartum blood concentrations of women with NTD-affected pregnancies vs. controls have also shown that altered folate metabolism may contribute to abnormal neural tube development [34, 36-38]. Genetic variation in folate metabolism due to a single nucleotide polymorphism (SNP) in the methylene tetrahydrofolate reductase (MTHFR) gene (MTHFR C667T) has strongly implicated the methylation hypothesis, which suggests that impaired MTHFR function leads to decreased methylation (and elevated plasma homocysteine), which affect the actin function and microfilament contraction necessary for the inward folding of neural folds [34, 39, 40].

The data surrounding the association of folic acid supplementation and the risk-reduction of neural tube defects is vast and varied in its scientific rigour. In 1976, Smithells and colleagues were the first to compare maternal blood folate concentrations among mothers who gave birth to NTD-affected infants in comparison to controls through an observational study [41], and found significantly lower erythrocyte folate concentrations among mothers of infants with NTDs.
Smithells et al. (1980) then conducted a non-randomized prospective trial to evaluate the efficacy of prenatal multivitamin supplementation in reducing the recurrence of NTDs among women who had a previously occurring NTD-affected pregnancy. Overall, authors found that 4.2% of women who had not been supplementing with multivitamins (controls) gave birth to an NTD-affected infant, whereas only 0.5% of women who were supplementing with multivitamins gave birth of infants with NTDs [42]. The risk-reduction was even more significant among mothers who had two or more affected pregnancies with NTDs [42, 43]. While this study was the first to show a clear association in the reduction of recurrence of NTD-affected pregnancies through multivitamin supplementation, the non-randomized nature of the investigation was criticized [20, 42].

The Medical Research Council (MRC) Vitamin Study became the first multi-center, double-blind, randomized clinical trial to evaluate folate-specific supplementation and overall multivitamin supplementation effects in 1991 [44]. Among 33 centers in 7 countries, 1817 high-risk women who had a previous affected pregnancy with a NTD were randomized. Upon enrollment, women were randomized to one of four intervention groups: 1) 4mg folic acid (no other vitamins), 2) 4mg folic acid + other vitamins (vitamin A, D, B1, B2, B6, C, and nicotinamide), 3) no vitamin supplementation, 4) other vitamins (no folic acid). Birth outcomes and the presence of a neural tube defect among the infants were evaluated at birth. The authors found a 72% reduction in the recurrence of NTDs among the folic acid-supplemented groups (RR 0.28, 95% CI 0.12-0.71), yet found no significant risk reduction among the other vitamins or non-supplemented groups [44].

Though the important role of folic acid in preventing the recurrence of NTDs had been proven, its role in preventing the first-time occurrence of NTDs still required validation. Hence,
Czeizel and Dudas (1992) conducted a randomized controlled trial to evaluate the effect of multivitamin supplementation on malformations among mothers without a previous history of an NTD-affected pregnancy [45]. Women were either randomized to take a multivitamin supplement (including 0.8mg folic acid) or a trace-element supplement (containing no folic acid) prior to conception. Among 2391 women in the trace-element supplement group, there were 6 cases of NTDs observed, whereas, among the 2471 multivitamin supplement group, there were no observed NTDs (p = 0.02). The authors also found a significantly higher incidence of congenital malformations in the trace-element group (22.9 in 1000) in comparison to the multivitamin supplement group (13.3 in 1000) [45].

1.1.3.2 Orofacial clefts

Orofacial clefts are the most common congenital malformation with a worldwide incidence of 15.21 per 10,000 births [46]. Orofacial clefts can either include a malformation of the cleft lip with or without the cleft palate (prevalence: 1 in 1000 births), or just a posterior cleft palate (prevalence: 0.4 in 1000 births) [46, 47]. The neural tube cells that are involved in the formation of the lip and palate are highly responsive to folate supplementation, and share an embryonic origin with the cranial neural crest cells that mediate lip and palate closure, which completes formation around 12 weeks gestation [44].

The scientific literature on maternal folate supplementation and the reduced risk of orofacial clefting in the offspring is controversial. Much of the early literature suggested an association between maternal folic acid use and a risk-reduction in the recurrence of orofacial clefts, as was seen with neural tube defects [48-52]. A population-based case-control study in California reported a 50% reduction in the occurrence of cleft lip among infants of women who used folic acid-containing supplements early in pregnancy [53]. Similar results were found by an
interventional trial in China, as they reported a reduction in cleft lip with or without cleft palate, but not in cleft palate alone [54]. In contrast, the National Birth Defects Prevention Study showed no significant risk-reduction in orofacial clefts, as an effect of folic acid supplementation or dietary folate [55]. A recent meta-analysis summarizes the data available through interventional and observational studies, and has shown that the use of folic acid supplements during pregnancy is associated with a reduction in the risk of cleft lip with or without cleft palate, but not in the risk of cleft palate alone [56].

### 1.1.3.3 Congenital heart defects

Throughout the world, congenital heart defects (CHDs) account for the most infant deaths compared to any other congenital anomaly [57, 58], and only 15% of heart defects are attributed to a known cause [58]. Several studies have demonstrated an association between folic acid supplementation and reduced risk for ventricular septal defects and conotruncal defects in infants [10], yet the literature lacks consensus. A large population-based cohort study from California showed a 30% reduction in the occurrence risk of conotruncal defects among the children of women supplementing with folic acid in early pregnancy [59]. In a randomized clinical trial in which pregnant women supplemented with a multivitamin containing 0.8mg folic acid vs. women supplementing with trace elements (controls), the risk of congenital heart defects was significantly reduced by 50% [60]. In contrast, two case-control studies did not observe a decrease in the risk of congenital cardiovascular anomalies post maternal folic acid supplementation [61, 62]. Hobbs et al. implicated homocysteine (Hcy), as well as S-adenosylhomocysteine (SAM) and methionine, as important biomarkers in predicting the risk of congenital heart defects [63].
1.1.3.4 Other birth defects

In contrast to the evidence surrounding neural tube defects, orofacial clefts, and congenital heart defects, the reports of folate reducing the risk of other birth defects have been scarce or inconsistent, thus limiting the strength of association. Some studies have shown that the use of multivitamins including folate in early pregnancy is associated with decreased risk of urinary tract defects [62, 64]. Similarly, some studies show a protective effect of folate on limb deficiencies [59, 62, 65]. A Hungarian study found that high-dose of folic acid supplementation (6mg) was associated with a decrease in the risk of trisomy 21, the chromosomal abnormality implicated in Down’s syndrome [66]. Decreased incidence of omphalocele, an abdominal wall defect, was observed in incidence whose mothers used multivitamins in early pregnancy [67], and in general after the initiation of folic acid fortification [68].

1.1.3.5 Pediatric cancers

Prenatal supplementation with a multivitamin containing folic acid has also been associated with a reduction in the development of certain pediatric cancers, including childhood brain tumours, neuroblastoma and leukemia, based on published studies as well as a recent meta-analysis [69-71].

1.1.3.6 Pregnancy complications

Apart from the data surrounding periconceptional folic acid supplementation and the reduction in the risk of birth defects, it has also been correlated with a reduction in the risk of adverse pregnancy outcomes and pregnancy complications. Yet again, findings in the literature lack consensus and have not been able to establish causation because of the multifactorial nature of most pregnancy-related complications.
Researchers have shown associations between folate deficiency and an elevated risk for placental abruption [72]. Implicated mechanisms include the vascular toxicity caused by hyperhomocysteinemia (as a byproduct of folate deficiency), or polymorphisms of genes involved in folate and homocysteine metabolism [73-75].

In exploring the mechanisms of preeclampsia, recent research has explored the relationship between folate status and preeclampsia. Studies have found higher homocysteine concentrations, but not significantly different plasma folate concentrations among pregnant women with preeclampsia [75-77]. Thus, in light of the evidence, elevated levels of homocysteine are involved in the metabolic pathway leading to preeclampsia, but the exact mechanism is not well understood [75]. A large prospective cohort study conducted by Wen et al. showed that women taking folic acid-containing multivitamin supplements were 63% less likely to experience preeclampsia than women not supplementing with folic acid [78]. However, an ecological study conducted in Canada demonstrated no significant differences in the risk of preeclampsia pre- and post- folic acid supplementation [79].

Despite overall inconclusive results, some studies have associated poor folate status as a risk factor for spontaneous abortion (pregnancy loss before 20 weeks gestation) and stillbirth (pregnancy loss after 20 weeks gestation). A population-based case-control study showed that women with low plasma folate concentrations were at a greater risk of miscarriage than those with higher plasma folate, especially if fetal chromosomal aberrations existed [80]. Some researchers implicated elevated plasma homocysteine concentrations in increasing the risk of early pregnancy loss [81, 82], yet folic acid supplementation studies were not able to demonstrate differences in the risk of early pregnancy loss after supplementation [83, 84]. Similarly, Vollset et al.’s large-scale Norwegian cohort study assessed homocysteine
concentrations among women with a high risk for stillbirth, and found that homocysteine concentrations were highest in the highest quartile of women for stillbirth risk [85]. However, studies evaluating this association have been criticized for their limitations, and further research is necessary to understand the role of the potential impairment of folate metabolism in influencing the risk of stillbirth.

1.1.3.7 Fetal growth

Because of the role of folate in embryonic and fetal growth and development, several researchers have evaluated the effect of maternal folate status on infant birth weight.

While the exact mechanism of this effect has not been characterized, homocysteine has been identified as being inversely associated with fetal growth. Linblad and colleagues reported that in full-term, but not preterm pregnancies with fetal-growth restriction (FGR), maternal and cord blood folate concentrations were 50% less than those in the deliveries of healthy birth weight infants [86]. Similar data showed an inverse effect of homocysteine concentrations on fetal growth. Burke et al. were the first to report an elevated risk of FGR with high plasma homocysteine concentrations in mothers [87]. This has been confirmed by various other studies [75, 85, 88], and a recent Japanese study has reported that a 1.0µmol/L increase in plasma homocysteine concentrations is associated with a 151g decrease in birth weight [89].

Several studies have confirmed the use of folic acid supplementation during pregnancy and its association with higher birth weight [90-92], however, many of these studies are confounded by factors such as socioeconomic class, maternal size, nutritional habits and maternal lifestyle that also influence birth weight.
The association between maternal folate intake and preterm delivery has also been investigated based on the hypothesis that poor folate status leads to elevated homocysteine concentrations, and impaired folate-homocysteine metabolism leads to decidual vasculopathy [10, 93]. The role of periconceptional folic acid supplementation in the risk-reduction of preterm birth was established by a recent cohort study that reported a 70% reduced risk of preterm delivery between 20-28 weeks gestation, and a 50% reduced risk of preterm delivery between 28-32 weeks gestation among mothers supplementing with folic acid [94].

1.1.4 Prenatal and periconceptional recommendations

1.1.4.1 The periconceptional period

The periconceptional period is associated with the period around conception leading up to early pregnancy. The Development Origins of Health and Disease hypothesis suggests that in utero development and the maternal environment experienced during the periconceptional period are part of a critical window whose effects mediate the health and disease of the fetus (and its later life) [95].

Given this understanding, as well as the fact that optimal blood folate concentrations are necessary for the proper closure of the neural tube, which closes on day 28 post-conception [31], intensive efforts are employed by regulatory agencies and healthcare professionals to identify risk factors associated with the periconceptional period for a woman, and adjust the prenatal and periconceptional recommendations for folic acid supplementation based on these risk factors.

Since about 50% of pregnancies are unplanned, many women who may be at high risk of suboptimal folate status may not even realize that they are pregnant and incur a preventable
NTD. It is hence essential that periconceptional supplementation guidelines target high-risk women as well as counsel regarding supplementation routinely.

### 1.1.4.2 Women at high risk of suboptimal folate status

Several risk factors associated with low folate status leading to inadequate protection against NTDs among women of childbearing age are reported below (Table 1).

**Table 1. Risk factors associated with low folate status and/or high risk of neural tube defects.** Adapted from Tam et al. [96]

<table>
<thead>
<tr>
<th>Factor</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lifestyle and demographic factors</strong></td>
<td>Poor supplement compliance</td>
</tr>
<tr>
<td></td>
<td>Restricted diets</td>
</tr>
<tr>
<td></td>
<td>Food insecurity</td>
</tr>
<tr>
<td></td>
<td>Low socioeconomic status</td>
</tr>
<tr>
<td></td>
<td>Adolescence</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
</tr>
<tr>
<td></td>
<td>Substance use/abuse</td>
</tr>
<tr>
<td></td>
<td>Alcohol</td>
</tr>
<tr>
<td><strong>Maternal obesity</strong></td>
<td>Prepregnancy BMI is inversely associated with folate intake</td>
</tr>
<tr>
<td></td>
<td>Obesity itself may also be a risk factor for NTDs</td>
</tr>
<tr>
<td><strong>Malabsorption diseases</strong></td>
<td>Celiac disease</td>
</tr>
<tr>
<td></td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td><strong>Epilepsy or use of antiseizure medications</strong></td>
<td>NTD risk increased in women taking antiseizure medications: valproic acid (1-2%), carbamazepine (1%)</td>
</tr>
<tr>
<td></td>
<td>Blood folate levels are lower in pregnant and nonpregnant women taking antiseizure medications</td>
</tr>
<tr>
<td><strong>Use of folate antagonists</strong></td>
<td>Carbamazepine</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim +/- sulfonamide</td>
</tr>
<tr>
<td></td>
<td>Aminopterin</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
</tr>
<tr>
<td><strong>MTHFR genotype</strong></td>
<td>MTHFR 677TT polymorphism produces a thermolabile enzyme, resulting in decreased RBC folate and increased risk for NTDs</td>
</tr>
<tr>
<td><strong>NTD history</strong></td>
<td>Individuals with an NTD risk (4% risk)</td>
</tr>
<tr>
<td></td>
<td>Previous child with an NTD (2-5% risk)</td>
</tr>
<tr>
<td><strong>Family history of NTD</strong></td>
<td>Siblings and second-degree relatives (1-2% risk)</td>
</tr>
<tr>
<td></td>
<td>Third-degree relatives (0.5-1% risk)</td>
</tr>
<tr>
<td><strong>Pregestational diabetes</strong></td>
<td>Poorly controlled type I or II diabetes mellitus (1% risk)</td>
</tr>
<tr>
<td><strong>Maternal ethnicity</strong></td>
<td>Sikh</td>
</tr>
<tr>
<td></td>
<td>Northern Chinese</td>
</tr>
<tr>
<td></td>
<td>Native American</td>
</tr>
</tbody>
</table>
1.1.4.2.1 Obesity

Several studies have found a relationship between high BMI and inadequate folate intake due to poor diet quality [97]. Studies have also found an effect of high pre-pregnancy BMI and an increased risk of NTDs, independent of folate intake [98, 99]. Currently given the high prevalence of obesity among women prior to pregnancy, the adjustment of folic acid supplementation recommendations based on high maternal BMI represents an important issue.

1.1.4.2.2 Substance use

Several studies have confirmed the negative effects of smoking, alcohol and substance abuse on the risk of NTDs. Significantly lower blood folate concentrations are found in pregnant women who smoke [100] compared to non-smokers. Another study reported impaired folate transport to the fetus through significantly decreased cord blood folate concentrations among smokers [101]. Finally, substance use including smoking and alcohol risk are associated with an elevated risk of NTDs [102].

1.1.4.2.3 MTHFR polymorphism

Women who are homozygous for the MTHFR C677T polymorphism have an increased risk for lower folate concentrations and an affected-pregnancy with NTDs [103-105]. The enzyme MTHFR normally catalyzes the reduction of 5,10-methylene-THF to 5-methyl-THF during folate-dependent one-carbon metabolism. The homozygous mutation of MTHFR C677T affects 5-10% of the population, whereas the heterozygous form is present in 30-40% of the population [105]. Among individuals with the homozygous mutation, MTHFR is thermolabile such that enzyme activity is only 50% of normal mean activity, and after heat inactivation at 46°C, the residual activity may be <36% of the initial activity [103].
1.1.4.2.4 Ethnicity

Several ethnicities are at high risk of having children with neural tube defects. It is unclear whether this increased risk is based on some genetic predisposition, cultural dietary preferences or a combination of these factors but it is essential that these ethnic groups are appropriately targeted for periconceptional supplementation [106]. Some of these ethnicities include Sikh, Celtic, Native American and Northern Chinese women [107-109].

1.1.4.2.5 Co-morbidities

Pre-existing health conditions often increase NTD-risk or are associated with defects in folate metabolism leading to lower blood folate levels among expecting mothers. For instance, diabetes mellitus during pregnancy is associated with increased risk of CNS-related birth defects [110]. Women with celiac disease [111, 112], Crohn’s disease [113] or irritable bowel syndrome [114] may also be at a higher risk of folate malabsorption, and consequently at higher risk of a pregnancy with an NTD. Finally, women who are epileptic and use anticonvulsant medications also have an increased risk of NTDs [115-117].

1.1.4.2.6 Anti-folate medications

Women on pharmacotherapy consisting of folate antagonists such as valproic acid, carbamazepine, trimethroprim, aminopterin, methotrexate, etc. are also at high risk of NTDs because of their negative effects on normal folate metabolism [118].

1.1.4.2.6.1 Oral contraceptives

While oral contraceptives are not generally perceived as clinically significant folate antagonists, there is much controversy in the literature surrounding their blood folate-lowering effect. Shojania et al. (1968) were the first to report lower serum folate concentrations in women who use oral contraceptives, in a manner similar to pregnancy [119]. Later studies suggest that
oral contraceptive use may impair folate metabolism in a mild way that may have limited clinical significance [120]. However, given that 50% of pregnancies are unplanned, women using oral contraceptives may represent a special population in need of higher folate intake if they conceive shortly after the cessation of oral contraceptive therapy, to counter the folate-lowering effect of oral contraceptives.

1.1.4.3 Supplementation guidelines

Based on guidelines by the Society of Obstetricians and Gynaecologists of Canada (SOGC) [106], healthy women are recommended to begin supplementation with 0.4-1mg folic acid per day, at least 2-3 months prior to conception, and continue supplementation throughout pregnancy and during the post-partum period. Women with risk factors associated with impaired folate status or an increased risk of NTDs are recommended evaluation on a case-by-case basis, and are recommended supplementation with 5mg folic acid in the periconceptional period until early pregnancy or throughout the course of pregnancy and postpartum depending on the severity of risk, or the presence of multiple risk factors. In addition to some of the risk factors presented in the Table and sections above, the SOGC also includes poor compliance to multivitamin supplements as a risk factor associated with inadequate folate intake [106].

1.1.5 Blood folate concentrations associated with optimal risk-reduction of NTDs

While the majority of evidence pointed towards the efficacy of periconceptional folic acid supplementation, Daly and colleagues in 1995 were the first to examine the correlation of specific blood folate concentrations with NTD-risk in their case-control study [121]. 81 blood samples from women with NTD-affected pregnancies (cases) were compared with 247 blood
samples from women with healthy pregnancies (controls). Plasma and RBC folate concentrations were analyzed using the microbiological assay. The researchers found an inverse dose-response relationship between maternal folate status and the risk of neural tube defects. A logistic regression model applied to the data showed maximal reduction in the risk of NTDs with maternal RBC folate concentrations ≥906nmol/L, and plasma folate concentrations ≥15.9 nmol/L [121]. At and above these blood folate concentrations, the incidence of NTDs was 0.8 in 1000 births, similar to its incidence in the general population [121] (Table 2). Since RBC folate concentration is reflective of long-term folate stores within the body, ≥906nmol/L RBC folate became a universal cut-off adopted by most researchers to reflect blood folate concentrations with optimal protection against neural tube defects.

Table 2. RBC folate concentrations among cases and controls of the Daly et al. (1995) study, in relation to NTD-risk [121].

<table>
<thead>
<tr>
<th>RBC folate (nmol/L)</th>
<th>Number of cases (%)</th>
<th>Number of controls (%)</th>
<th>NTD risk (per 1000 births)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-339</td>
<td>11 (13.1)</td>
<td>10 (3.8)</td>
<td>6.6</td>
<td>3.3-11.7</td>
</tr>
<tr>
<td>340-452</td>
<td>13 (15.5)</td>
<td>24 (9.0)</td>
<td>3.2</td>
<td>1.7-5.5</td>
</tr>
<tr>
<td>453-679</td>
<td>29 (34.5)</td>
<td>75 (28.2)</td>
<td>2.3</td>
<td>1.6-3.3</td>
</tr>
<tr>
<td>680-905</td>
<td>20 (23.8)</td>
<td>77 (29.0)</td>
<td>1.6</td>
<td>1.0-2.4</td>
</tr>
<tr>
<td>≥906</td>
<td>11 (13.1)</td>
<td>80 (30.0)</td>
<td>0.8</td>
<td>0.4-1.5</td>
</tr>
<tr>
<td>Total</td>
<td>84 (100.0)</td>
<td>266 (100.0)</td>
<td>1.9</td>
<td>1.5-2.3</td>
</tr>
</tbody>
</table>

Building on this, Wald and colleagues used data from the Daly et al. (1995) study as well as 13 other studies to establish a relationship between serum folate concentration and the
incidence of neural tube defects, and formulate guidelines for folic acid supplementation [122]. The analysis included 502 women supplementing with folic acid in both placebo-controlled and uncontrolled trials. The authors found that a daily increase in folic acid intake of 0.1mg was associated with an increase in steady-state serum folate concentrations by approximately 2.3nmol/L (1.0ng/mL) in women of childbearing age. This assumption was used to further extrapolate the associated NTD risk-reduction, based on a woman’s baseline folate status and the respective increase in daily dose of folic acid (Table 3) [123]. Overall, the authors showed that doubling serum folate concentrations roughly halved the risk for NTDs, but that the dose of supplementation required was dependent on a woman’s baseline folate status. Based on their results, Wald et al. recommended 5.0mg folic acid daily for women planning a pregnancy, since it was associated with an 85% reduction in the risk of NTDs in contrast to the current recommendation of 0.4mg folic acid daily, which only offered about a 36-46% protection against NTDs [123].

Table 3. NTD-risk reduction modelled associated with different doses of folic acid supplementation, in relation to baseline serum folate status. Adapted from Wald et al. (2001) [123]

<table>
<thead>
<tr>
<th>Increase in daily folic acid dose (mg)</th>
<th>Baseline folate of 2.5ng/mL</th>
<th>Baseline folate of 5.0ng/mL</th>
<th>Baseline folate of 7.5ng/mL</th>
<th>Baseline folate of 10.0ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>23%</td>
<td>13%</td>
<td>9%</td>
<td>7%</td>
</tr>
<tr>
<td>0.5</td>
<td>57%</td>
<td>41%</td>
<td>32%</td>
<td>27%</td>
</tr>
<tr>
<td>1.0</td>
<td>71%</td>
<td>57%</td>
<td>48%</td>
<td>41%</td>
</tr>
<tr>
<td>5.0</td>
<td>91%</td>
<td>85%</td>
<td>80%</td>
<td>75%</td>
</tr>
</tbody>
</table>
Finally, a recent study by Crider et al. (2014) uses a Bayesian model to evaluate the optimal RBC folate status for the prevention of neural tube defects based on population data from two major study cohorts: the Community Intervention Project and the Folic Acid Dosing Trial [124]. The scope and relevance of this study is based on several limitations of the Daly et al. (1995) study, whose results are used as a benchmark for RBC folate concentrations and associated NTD-risk reduction. Within the study by Daly et al., maternal blood folate concentrations were evaluated at a median time of 15 weeks gestation, whereas the neural tube completes closure around 4 weeks gestation (day 28) [121]. Further, considering that the study population within the Daly et al. study was quite homogenous in terms of ethnicity and background within Ireland, it is questionable how generalizable the findings are to populations with different ethnic backgrounds and nutritional habits [124]. Thus, Crider and colleagues used population data of 247,831 women from two previous large-scale study cohorts from China, and evaluated their RBC folate concentrations on embryologic day 28, as well as the associated NTD-risk with certain strata of RBC folate levels. The authors found that a RBC folate concentration between 1000-1300 nmol/L was associated with optimal protection against NTDs that are folate-sensitive (overall lowest feasible NTD risk: 5-6 per 10,000 births) (Table 4) [124]
Table 4. Risk of NTDs based on associated RBC folate concentration among women in the Community Intervention Project in China (n = 228,456). Adapted from Crider et al. (2014) [124]

<table>
<thead>
<tr>
<th>RBC folate concentration (nmol/L)</th>
<th>NTD risk (per 10,000 births)</th>
<th>95% uncertainty interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>340</td>
<td>48.8</td>
<td>36.3-65.6</td>
</tr>
<tr>
<td>500</td>
<td>25.4</td>
<td>20.8-31.2</td>
</tr>
<tr>
<td>900</td>
<td>9.4</td>
<td>7.9-11.0</td>
</tr>
<tr>
<td>1000</td>
<td>7.9</td>
<td>6.5-9.3</td>
</tr>
<tr>
<td>1100</td>
<td>6.7</td>
<td>5.5-8.1</td>
</tr>
<tr>
<td>1200</td>
<td>5.8</td>
<td>4.6-7.1</td>
</tr>
<tr>
<td>1300</td>
<td>5.1</td>
<td>4.0-6.3</td>
</tr>
<tr>
<td>1400</td>
<td>4.5</td>
<td>3.4-5.6</td>
</tr>
</tbody>
</table>

1.1.6 Folic acid and public health initiatives

While the literature confirmed that folic acid was associated with a reduction in the risk of neural tube defects, population blood folate levels still remained low, and even women supplementing with 0.4mg folic acid daily, were at considerable risk of an NTD upon conception [125], and the overall prevalence of NTDs remained high in the mid-1990s. Thus, despite supplementation awareness campaigns in the United States, it was of critical importance to implement a measure that would dramatically increase population-wide folate concentrations in at least 75% of the population [125, 126].
It was under these circumstances that the fortification of staple food products with folic acid was proposed.

**1.1.6.1 Folate fortification**

By 1998, the United States and Canada implemented mandatory folate fortification of cereal and grain products including white wheat flour, cornmeal and enriched pasta [127, 128]. In Canada, 150µg folic acid was added to every 100g of white flour or white flour-based food products, and 200-270µg folic acid was added to every 100g of pasta, to account for folate loss due to cooking [128-131]. This level of fortification was expected to add 100-200µg folic acid daily to the food supply, and increase the overall blood folate concentrations of women of childbearing age, as well as reduce the incidence of NTDs in the population by over 20% without risking overexposure in certain groups among the population [132, 133].

At a worldwide level beyond North America, Costa Rica implemented wheat and corn flour fortification by 1998 [134], and Chile by 2000 [135]. Since its inception as a public health strategy, 53 countries have implemented regulations on the mandatory fortification of flour with folic acid [136]. Several countries, however, have not adopted a folate fortification programme because of concerns associated with population-wide exposure and long-term consumption of folic acid [137].

**1.1.6.1.1 Pre- and post-fortification blood folate concentrations**

In the United States, based on the National Health and Nutrition Examination Survey (NHANES), the mean serum folate concentrations of women prior to fortification (before 1998) were 13nmol/L and reached 29nmol/L post-fortification (by 2002) [138, 139]. Similarly, mean RBC concentrations were 397nmol/L before 1998, and increased to 630nmol/L by 2002, and remained stable till 2004 [138, 139].
In contrast to the United States, where blood folate samples have been collected at a nationally representative scale as part of NHANES, the data available for Canada has been based on specific segments of the larger population, until the recent Canadian Health Measures Survey.

In Canada, Ray et al. found that the mean RBC folate concentrations of women of childbearing age in Ontario prior to fortification were 526.8nmol/L (between 1996-1997) and increased to 740.9nmol/L post-fortification (between 1998-2000) [140]. Similarly, data from 2006 evaluating blood folate concentrations among women in Ontario showed that 40% of women of childbearing age, and 36% of pregnant women had RBC folate concentrations of less than 906nmol/L [141], thus raising concerns about the efficacy of folate fortification among the population that requires it most. Data from 2007-2009, from a nationally-representative sample as part of the Canadian Health Measures Survey showed that the median RBC folate concentrations among women of childbearing age was 1193nmol/L, yet 22% of women had RBC folate concentrations below the optimal 906nmol/L [142].

1.1.6.1.2 Pre- and post-fortification NTD prevalence

Since the ultimate aim of folic acid fortification programmes was to raise blood folate levels in women, and consequently the incidence of folate-responsive NTDs, epidemiological studies investigating the prevalence of NTDs prior to and after fortification provide strong evidence regarding the effectiveness of fortification as a public health strategy.

In Canada, several researchers have reported changing trends in the prevalence of neural tube defects, including the specific incidence of spina bifida and anencephaly. De Wals and colleagues found a 46% reduction in the prevalence of NTDs after fortification, as they analyzed data from seven Canadian provinces (Newfoundland and Labrador, Nova Scotia, Prince Edward Island, Quebec, Manitoba, Alberta, British Columbia) [143]. This was similar to the percent
reduction observed among a cohort of women in Ontario by Ray et al. (48%) [144]. Data from Quebec showed a slightly lower reduction in the prevalence of NTDs [145]. Generally, the magnitude of risk reduction is dependent on the baseline prevalence of NTDs within a particular region. Since the prevalence of NTDs has always been slightly higher in the Eastern Canadian provinces compared to the Western provinces, studies from Nova Scotia and Newfoundland and Labrador demonstrated the highest percentage decrease in the incidence of NTDs – 54% and 78% respectively [133, 146] (Table 5).

Studies also found a greater decrease in the incidence of spina bifida than anencephaly, as a result of folate fortification. Results from De Wals et al.’s seven province-wide study showed 53% decrease in the rate of spina bifida compared to a 38% decrease in the rate of anencephaly [147].
Table 5. Percentage decline and birth prevalence of NTDs (rates per 10,000 births) pre- and post-fortification in Canada. Adapted from Bailey et al. [10]

<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>Pre-fortification prevalence</th>
<th>Optional fortification prevalence</th>
<th>Post-fortification prevalence</th>
<th>% decline</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Wals et al. [147]</td>
<td>7 Canadian provinces</td>
<td>(1993-1997)</td>
<td>15.8</td>
<td>10.9</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>De Wals et al. [145]</td>
<td>Quebec</td>
<td>(1992-1997)</td>
<td>18.9</td>
<td>n/a</td>
<td>12.8</td>
</tr>
<tr>
<td>Ray et al. [148]</td>
<td>Ontario</td>
<td>(1994-1997)</td>
<td>11.3</td>
<td>n/a</td>
<td>5.8</td>
</tr>
<tr>
<td>Liu et al. [133]</td>
<td>Newfoundland and Labrador</td>
<td>(1991-1997)</td>
<td>43.6</td>
<td>n/a</td>
<td>9.6</td>
</tr>
</tbody>
</table>

1.1.6.2 Folic acid supplementation guidelines

In 1993, Health Canada and the Society of Obstetricians and Gynaecologists of Canada (SOGC) recommended that all women of childbearing age, who were planning a pregnancy or capable of becoming pregnant should consume 400µg of folic acid daily [149]. Despite these recommendations, folic acid supplement use in Canada, as well as the overall increase in blood folate levels among women childbearing age remained low. Hence, folic acid fortification was employed as a population-wide public health strategy.

Current recommendations by the SOGC suggest that women who are planning a pregnancy begin supplementing with 0.4-1mg of folic acid per day at least three months prior to
pregnancy. However, data on rates of supplementation among women in Canada suggest that there is still a gap between the applications of this recommendation, as the rates of supplementation among women remain low.

1.1.6.2.1 Rates of Folic Acid Supplement Use

Data from the Canadian Health Measures Survey on folic acid supplement use shows that only 28.2% of women regularly consume folic acid supplements [150]. Similarly, data from the Canadian Maternity Experiences Survey shows that 89.7% women used folic acid during their pregnancy, yet only 57.7% began using folic acid 3 months prior to pregnancy [151], as per healthcare recommendations. These trends are similar across Canadian provinces, where periconceptional folic acid supplementation rates are still significantly lower than folic acid use during pregnancy (Figure 5).

Given the data on risk factors associated with folate status, it is likely that certain sections of the population have even lower rates of folic acid supplementation. These likely include women with low socioeconomic status, obesity, substance abuse, poor nutrition and poor general health.
Figure 5. Percentage of Canadian women who used folic acid supplements 3 months before pregnancy vs. the first 3 months of pregnancy (Data from 2006-2007). Adapted from The Canadian Maternity Experiences Survey [151].

1.1.6.2.2 Social Media for Public Health Messages

In order to specifically target a subsection of the overall population, women of low socioeconomic status, to increase folic acid supplementation awareness, de Walle et al. (1999) used targeted mass media advertising campaigns to raise awareness and influence behaviour regarding folic acid supplementation among this women of low socioeconomic status, and gained much success [152]. The use of social media for the dissemination of public health
messages and health information is based on similar principles, because of its wide-reach across the general population [153, 154].

1.1.6.2.3 Social Media for Recruitment

The use of social media in intervention-based clinical trials has been limited. While it has been previously used with much success in questionnaire-based studies as well as to target community-based populations and study behaviour, its applications towards clinical trial recruitment have been scarce [155, 156]. Given the widespread use of the internet for medical information, social media represents a promising recruitment tool for interventional studies if used in a controlled and regulated fashion.

1.1.7 Recommended daily intake of folate

1.1.7.1 The Dietary Reference Intakes (DRI) for folate

The Dietary Reference Intakes (DRIs) are a set of reference values for nutrient intakes among healthy populations. DRIs include specific intake values for each nutrient, and the requirement associated with each value may vary based on age, life stage and gender. DRIs include reference values for Estimated Average Requirement (EAR), which is the median intake estimated to meet the nutrient requirements of 50% of healthy individuals at a given life stage, Recommended Daily Allowance (RDA), which is defined as the average daily intake sufficient to meet the nutrient requirements of 97%-98% of healthy individuals at a given life stage, Adequate Intake (AI) is the recommended average daily intake which is established when there is insufficient evidence to determine an EAR and RDA, and finally, the Tolerable Upper Intake Limit (UL) is defined as the highest level of daily intake that is likely to pose no risk of adverse health effects for almost all individuals at a given life stage [157]. There are minor differences in intake recommendations based on the governing regulatory agencies worldwide, however, the
recommendations presented here are as per the Institute of Medicine guidelines within the United States and Canada.

### 1.1.7.1.1 Dietary Folate Equivalents (DFEs) and Bioavailability

The Food and Nutrition Board developed Dietary Folate Equivalents (DFEs) to account for the higher bioavailability of folic acid in comparison to naturally occurring food folates. Synthetic folic acid supplements have nearly 100% bioavailability [158]. In contrast, naturally occurring food folates only have about 50% bioavailability [159]. Finally, fortified foods with folic acid are about 85% bioavailable [160]. Thus, each DFE is defined using the following equation [157]:

\[
\text{DFE (µg/day)} = (1.7 \times \mu \text{g folic acid}) + \mu \text{g naturally occurring food folate}
\]

### 1.1.7.1.2 Recommended Daily Allowance (RDA)

For healthy adults between the ages of 19-50 years, RBC folate, serum folate and plasma homocysteine concentrations within the normal range were used as a guideline to establish the EAR for folate. Based on these considerations, folate has an EAR of 320µg/day and an RDA of 400µg/day, after accounting for a 10% coefficient of variation in folate requirements [157].

### 1.1.7.1.3 Tolerable Upper Limit (UL)

While there is no upper limit for naturally found food folates, the Institute of Medicine has established a UL of 1000µg/day for folic acid intake in the form of supplements or fortified-foods [157]. The basis for this guideline is the potential concern of masking vitamin B12 deficiency that is associated with high levels of folate intake.
1.1.7.2 Recommendations in pregnancy

Given that folate requirements increase during pregnancy due to the increased need of folate in cell division and metabolism associated with embryonic and fetal development, as well as the expansion of maternal plasma and tissues, folate DRIs are slightly higher in pregnancy. The EAR for pregnant women is 520µg/day and the RDA is 600µg/day [157]. Women carrying more than one fetus during a pregnancy may require intakes higher than the current RDA [157]. Interestingly, the RDA of folate in pregnancy was established based on data from a controlled metabolic study [161], as well population studies reporting folate intake [162-166], for the prevention of folate deficiency. However, NTD risk reduction has not been considered a basis for evaluating the adequacy of folate intake in pregnant women. Based on the Institute of Medicine guidelines, the UL of folic acid in pregnancy is still 1000µg/day [157].

1.1.8 Assessment of folate status

1.1.8.1 Dietary assessment questionnaires

Dietary assessment questionnaires are used to evaluate nutrient intake or overall food intake. When combined with information on supplement use, dietary questionnaires serve as important tools in characterizing total intake of nutrients. Commonly used intake questionnaires include a food frequency questionnaire (FFQ), 24h dietary recall, and food records [167]. Advantages of these methods include their inexpensive nature and the ease of administration, as they are usually self-administered. They also have several limitations including bias associated with self-reporting, lack of specificity due to their qualitative or semi-quantitative approach, and typically low correlation coefficients between estimated nutrient intake through questionnaires and actual biomarker values (<0.5) [168-170]. However, they represent an important tool in
characterizing the dietary options associated with a particular population in influencing nutrient intake levels within that population [171].

1.1.8.2 Biochemical assessment

Biochemical assessment of nutrients involves evaluating their physiological concentrations. In the case of folate analysis, RBC folate, serum or plasma folate, and plasma homocysteine concentrations are used as biomarkers of optimal folate function within the body. RBC folate concentrations represent long-term tissue stores of folate, while plasma folate concentrations reflect short-term folate status [17]. Total plasma homocysteine concentrations are typically used as a functional indicator of B-vitamin status [169]. The advantages of biochemical assessment of folate status include its ability to capture physiological levels of the nutrient, as well as detect different folate vitamers (depending on the method of assessment). Since physiological concentrations of nutrients translate into their role in health or disease, biochemical assessment is the most reliable measure in determining nutrient levels. However, limitations of this approach include its inability to correlate completely with dietary intake levels of the nutrient (since metabolism and absorption would influence physiological levels), physiological fluctuation in biochemical levels, and technical error associated with laboratory measurement [167, 171].

1.1.9 Analytical methods for the biochemical assessment of folate status

Several methods exist for the analysis of folate in different biological matrices including RBC, plasma, and serum.
1.1.9.1 Microbiological assay

The microbiological assay is based on the principle of folate utilization by a test organism, generally *Lactobacillus rhamnosus* (*Lactobacillus casei*), and uses $^{14}\text{CO}_2$ production or turbidity as marker of bacterial growth, proportional to total folate content [172, 173]. The advantages of this method include its high degree of sensitivity and specificity for biologically active folates, as well as the low cost associated with the method. In contrast, it has been reported that the presence of antibiotics or folate antagonist medications can lead to an underestimation of the folate content using this method, as they may interfere with the growth of the test organism [174, 175]. While commonly used in diverse research settings, the clinical applications of the microbiological assay are limited because of its time- and labour-intensive nature.

1.1.9.2 Immunoassay

The immunoassay or protein-binding assay initially emerged as a simpler and faster alternative to the microbiological assay. Within this assay, the test sample is mixed with a known amount of high-affinity folate binding proteins (FBP), and is based on the principles of competitive or noncompetitive protein binding [176]. The binding of labelled folate is quantified and compared against a standard curve to determine the folate concentration of the test sample. Since protein-binding assays are often automated or semi-automated, they have rapid turnover time and ease of use, which contributes immensely to their clinical utility [177]. The limitations of this method include the variability in folate binding affinity. It has been reported that FBPs from different sources can vary in their binding properties to folate. It has also been found that folate binding affinity to FBPs may vary among folate species, may not be linear with folate concentration, and may change depending on the length of polyglutamate chains [4, 178]. Finally, the presence of contaminants such as folate analogues or folate antagonists can lead to a
displacement of the test folate from FBP, leading to a potential overestimation of folate content [4, 176].

### 1.1.9.3 Chromatographic assays

Contrary to the microbiological assay and immunoassay, which measure total folate, chromatographic assays characterize individual folate species. Separation of folate vitamers can be achieved by reversed-phase high performance liquid chromatography (RP-HPLC) [4, 179]. Folate vitamers are identified they elute through the column, based on their characteristic retention times, and can be characterized using electrochemical detection, microbiological assay, or tandem mass spectrometry [4, 179]. Due to its ability to characterize different forms of folate, it is useful in research settings but not for routine clinical use.

Total folate can also be characterized by gas or liquid chromatography by measuring PABA produced from the acid hydrolysis of folate. The quantity of PABA released from the test samples is measured by mass spectrometry or fluorescence detection, and is compared to the amount of labelled PABA released from a folate standard with known concentration [4]. This method is highly sensitive, and the acidic conductions assist in the liberation of folate from plasma proteins and other specific and non-specific folate binders [4]. However, its limitations include the complex sample-preparation involved, especially in the case of gas chromatography-mass spectrometry (GC-MS), thus increasing the chance of folate degradation. Other limitations also include the variability between folate vitamers in the efficiency of acid hydrolysis, and the potential for interference by PABA derived from non-folate sources, leading to a false overestimation of folate content [4].
1.1.10 **Risks associated with folate overexposure**

Even though folate is a water-soluble vitamin that exhibits low toxicity because of the elimination of excess amounts through urine, there have been concerns regarding certain sections of the population being exposed to unnecessarily high doses. This is especially a concern in countries that have implemented mandatory folate fortification for the prevention of neural tube defects among women of childbearing age. Due to this broad-spectrum strategy, many groups, including children and the elderly, who may consume bread and flour products as staples, are exposed to unnecessarily high intake levels of folate.

Further debate exists because there is no consensus on the minimum blood folate concentrations that cause harm, hence, despite the UL recommendations by the Institute of Medicine (1000µg/day) for adults [157], there is also a lack of consensus regarding the safe maximum concentration of folate intake.

Excess folic acid levels within the body are associated with the theoretical concern of impairment of folate metabolism through competition with reduced, coenzymatic folates for endogenous transporters, binding proteins and folate-dependent enzymes [180-183]. However, there is currently no conclusive evidence to suggest that high levels of folic acid used within a controlled period of time, such as the periconceptional period, are associated with the risk of overexposure or adverse health effects. Nevertheless, some of the concerns associated with high folate intakes include:
1.1.10.1 Masking vitamin B12 deficiency

The most commonly cited concern associated with high folate concentrations is their potential of masking the overt clinical signs of vitamin B12 deficiency, leading to possible neuropsychiatric dysfunction [184]. However, this is rare to find clinically [185].

Thus, of greater concern is the hypothesis that high folate concentrations may exacerbate hematologic symptoms associated with vitamin B12 deficiency. This is supported through the lack of conclusive studies in the literature. Using data from the NHANES (1999-2000), Morris et al. describe the interrelationship of folate and vitamin B12 status as risk factors for cognitive impairment in the elderly. They found that in elderly persons with low vitamin B12 status, high serum folate concentrations were associated with cognitive decline [186]. However, when elderly persons had high serum folate concentrations with normal vitamin B12 concentrations, a protective effect against neurocognitive decline was observed within this study [186]. Similarly, contrary to the understanding that optimal levels of folate, vitamin B12 and B6 are all necessary for neuroprotection, a recent study has associated high levels of folate intake with a reduced risk for Alzheimer’s disease in the elderly, independent of vitamin B12 or B6 status [187].

1.1.10.2 Impaired immune function

A study by Troen et al. among postmenopausal women in the United States demonstrated an inverse U-shaped relationship between folic acid concentrations and natural killer cell cytotoxicity, which is a marker of innate immune function. Within this study, authors found that women with low folate intake (<233µg/day), who took up to 400µg/day folic acid through supplements, had better immune function than women who did not use any folate supplements. However, women whose dietary folate intake was high (≥233µg/day), and used >400µg/day folic acid supplements had impaired natural killer cell function [188]. Further, the authors found a
significant inverse linear relationship between the concentration of unmetabolized folic acid in plasma and natural killer cell cytotoxicity, especially in women older than 60 years of age [188].

1.1.10.3 Pro-carcinogenic potential

Given the role of folate in DNA synthesis, repair and cellular division, much of the literature has focused on in dual-modulatory role in cancer – as it has been implicated in both cancer prevention and promotion. Epidemiologic evidence, in vitro data and animal studies have shown that increased folate concentrations are associated with protection against the development of several types of cancer including cancers of the colorectum, oropharynx, esophagus, pancreas, stomach, lungs, cervix, ovary, breast, as well neuroblastoma and leukemia [189]. However, animal studies on colorectal cancer have demonstrated the importance of the timing and dose of folic acid intervention. If folic acid supplementation is started when cells are normal, prior to the establishment of neoplasms or adenomas (tumour precursors), tumour development and progression are inhibited by folic acid as it maintains normal cell division [189-193]. In contrast, if folic acid supplementation is initiated after the establishment of neoplasms of adenomas, then folate may promote the proliferation of neoplastic cells and support other tumour-promoting mechanisms [190-192, 194]. It is in this case that folic acid may be pro-carcinogenic.

A recent meta-analysis evaluating data from 27 studies has shown that high folic acid intake is not associated with an increased risk of colorectal cancer [195]. In fact, it was found to have a protective effect by decreasing the cancer risk by 8-15%. The authors did note, however, that there was variability within the literature in defining “high folate intake” [195].
1.1.10.4 Epigenetic effects

Folate plays a key role in the methylation of homocysteine to methionine, which is incorporated into proteins and has other important functions associated with methylation reactions [196]. It is also hypothesized that epigenetic mechanism such as DNA methylation and histone modification play an important role in the development of health and disease [197]. The role of folate in influencing epigenetic modifications has been demonstrated through the agouti mouse, where maternal folic acid supplementation has resulted in changes in the epigenetic programming and subsequent phenotype of the offspring [198, 199]. The influence of folate status of DNA methylation in humans is likely to be tissue-, site- and gene-specific [197], yet the implications of maternal folate status and epigenetic programming in infants require further exploration in humans.
1.2 THESIS SCOPE

1.2.1 Overall Aim and Statement of the Problem

The periconceptional period is defined as the period prior to conception leading up to early pregnancy. There is tremendous evidence (The Developmental Origins of Health and Disease) to suggest that exposures, in utero development or physiological changes, as well as the maternal environment experienced during the periconceptional period are all part of a critical window whose effects mediate the health and disease of the fetus, and its later life [95]. Given the important role of folic acid in the prevention of neural tube defects, that may occur early in pregnancy (as neurulation is complete by day 28 post-conception), it is of primary importance that women begin periconceptional folic acid supplementation at least three months prior to conception.

However, this is compromised due to several factors. Given that about 50% of pregnancies are unplanned, it is not always possible for women to begin folic acid supplementation prior to pregnancy. Further, several studies across the world confirm that women’s awareness and use of folic acid during their pregnancy is much higher than their awareness of its benefits and use before conception [200-203]. Despite the breadth of scientific evidence, increasing awareness surrounding periconceptional folic acid supplementation in addition to prenatal supplementation remains a current challenge among women of childbearing age. This thesis addresses several challenges and risk factors associated with the goal of increasing periconceptional folic acid supplementation or folate status in the periconceptional period.
The research approaches adopted in this thesis are diverse in their methodology in an attempt to tackle the multifaceted issue of periconceptional folic acid supplementation. The first study characterizes an approach with the highest internal validity [204], as we conducted a randomized controlled trial to evaluate steady-state folate pharmacokinetics among pregnant women supplementing with either 1.1mg or 5mg folic acid. The second study is based on secondary analysis emerging from the randomized controlled trial, and through time-series analysis, quantitatively demonstrates the recruitment efficacy of social media in clinical studies, while the discussion in the literature surrounding social media has been largely qualitative. The third study critically evaluates the breadth of literature on the subject of oral contraceptive use and folate status through a systematic review and meta-analysis. Finally, the fourth study encompasses the highest degree of external validity [204], as we analyze the folate status of women in Toronto through a population-based observational study.

While the randomized clinical trial (Chapter 2) overtly focuses on folic acid supplementation during the periconceptional period and pregnancy, the systematic review and meta-analysis (Chapter 4) present the question of oral contraceptives possibly lowering blood folate concentrations in women, which may warrant extra folic acid supplementation. Both studies also highlight vulnerable populations that may require higher doses of folic acid. In the first study (Chapter 2), this is expressed through women who would benefit from the 5mg folic acid whereas in the third study (Chapter 4), this is suggested for women who experience a clinically significant folate-lowering effect of oral contraceptives. Both these studies are guided by the principle of monitoring blood folate concentrations so that healthcare professionals are able to adequately characterize vulnerable populations who may require additional folic acid. The theme of monitoring ties into the second study (Chapter 3) evaluating recruitment, where
recruitment trends over a period of time were monitored, and the effectiveness of social media as an intervention was evaluated. Finally, monitoring population levels is embodied by the fourth study (Chapter 5) where the folate status of women in Toronto is evaluated. Both the second study (Chapter 3) and the fourth study (Chapter 5) also link strongly to the analysis of public health initiatives, as social media presents a unique medium for the dissemination of public health messages, and the population-level monitoring of folate status is necessary in evaluating the success or failure of population-wide public health strategies such as, folate food fortification and supplementation awareness campaigns. The overall thematic connections between the studies presented in this thesis are summarized through the schematic below (Figure 6).

Figure 6. Clinical significance and thematic connections of the studies included within this thesis.
1.2.2 **Research Objectives**

This thesis is divided into four different studies which are presented in detail within each chapter dedicated to each study. The objectives associated with each study are outlined below:

I. **To assess the steady-state folate pharmacokinetics in pregnancy among women who supplement daily with 1.1mg (regular dose) vs. 5mg (high dose) folic acid in the periconceptional period (Chapter 2).**

Steady-state pharmacokinetics of folic acid supplementation at the doses of 1.1mg vs. 5mg folic acid have not previously been studied in pregnancy. This study aimed to understand two important folate supplementation effects in the population of pregnant women: a pregnancy-related effect on both dose groups, as well as a dose-related difference between the two dose-groups. A previous study by Nguyen et al. demonstrated non-linear kinetics at 5mg folic acid supplementation in non-pregnant women of childbearing age [205]. It was thus important to investigate whether this trend continued in pregnant women. Furthermore, the time point at which steady-state was achieved despite pregnancy-related changes was of interest to us. While the pharmacokinetics of folic acid have been evaluated at lower doses of supplementation during pregnancy, it was necessary to evaluate the long-term kinetics of folic acid at the high dose (5mg), which is prescribed and recommended to women who may have a high risk of NTDs or low folate status, as it would aid healthcare professionals in determining the timing and dosage of periconceptional folic acid supplementation for these individuals.
II. To compare the recruitment success and efficiency between traditional healthcare-based methods of recruitment vs. social media in a randomized clinical trial on folic acid supplementation in pregnancy (Chapter 3).

Randomized controlled trials and clinical studies typically depend on healthcare-based establishments for patient or participant recruitment. This may include intensive forms of recruitment such as patient recruitment from a particular hospital or clinic setting, or more passive recruitment in the form of clinic referrals, advertisement and bulletin board postings at clinics, or brochures in waiting rooms. Despite recruiting through these traditional healthcare-based methods for over 4.5 years, our randomized clinical trial on folic acid supplementation among pregnant women in Toronto faced marginal recruitment. Social media was employed alongside healthcare-based recruitment strategies to increase the recruitment rate within this study, as well as analyze the overall efficacy of each method of recruitment. Evaluating the efficacy of recruitment methods is an under-studied area within clinical trial methodology, and our findings may benefit other clinicians and researchers in choosing recruitment strategies for special populations, such as pregnant women.

III.a) To systematically review and meta-analyze available clinical literature on oral contraceptive use and its potential effect on plasma and RBC folate concentrations. 

b) To systematically review the data available on the novel folate-fortified oral contraceptive (Beyaz®) in order to evaluate its potential as an alternative to current folic acid supplementation therapy (Chapter 4).

The effect of oral contraceptive use on blood folate status has not previously been systematically reviewed or meta-analyzed within the literature. In fact, the scientific literature surrounding this issue is largely controversial and lacks consensus. Currently, oral contraceptives
are not widely cited as a risk factor for low folate status (and associated increased NTD risk). Approximately 18% of American women currently use oral contraceptives [206]. Similar data exists for Canada, where about 17% of women use oral contraceptive pills [207]. Given that women of childbearing age are recommended at least 400µg of folate daily, a percentage of these women who use oral contraceptives may be at risk of low folate status or NTDs upon conception, due to the potential folate-lowering effect of oral contraceptives. Further research into this effect, its clinical significance, as well as the folate supplementation options available to these women is of immense importance in targeting women within the periconceptional period who may have an unplanned pregnancy or may conceive shortly after stopping oral contraceptive therapy.

IV. To follow-up on previous population-based studies to capture the current folate status of women within the Greater Toronto Area, in order to determine the percentage of women of childbearing age that are still inadequately protected, as well as to analyze population-based trends emerging from the analysis of their folate status (Chapter 5).

Recent population-based data on folate status in Canada is based on the Canadian Health Measures Survey, which presents data from 2007-2009 [142]. Our study aims to update population-based data on folate-status from a cohort within the Greater Toronto Area in 2013. Overall, an assessment of population-wide folate levels will help determine the percentage of women of childbearing age who still need to be targeted to achieve optimal folate status and reduce the risk of preventable NTDs. As well, an analysis of trends within the population would yield insights for further public health-level action. An evaluation of folate levels fifteen years
post-fortification may shed light on the success or failure of existing public health strategies, as well as identify subgroups within the population that may be at risk.

1.2.3 Research Hypotheses and Rationale

I. We hypothesized that the steady-state pharmacokinetics of 1.1mg vs. 5mg folic acid in pregnant women would be similar to the pharmacokinetics of folate observed at these doses among non-pregnant women of childbearing age.

Rationale: Given the findings of a previous study of identical design by Nguyen et al. [205] among women of childbearing age, we anticipated that the pharmacokinetics of folic acid would not be greatly affected by pregnancy, and that steady-state concentrations for RBC and plasma folate would be achieved after 30 weeks of supplementation. In terms of dose-effect, we anticipated that the pharmacokinetics of the high dose of folic acid (5mg) would be different from the regular dose of folic acid (1.1mg). This aspect of our hypothesis was based on two previous studies from our lab group comparing the single-dose and steady-state pharmacokinetics of folic acid at these doses [205, 208]. In the single-dose study comparing the two doses of folic acid, Nguyen et al. found linear pharmacokinetics at both doses, where a 5-fold increase in dose (5mg folic acid) produced a ~5-fold increase in pharmacokinetic measurements (AUC and C_{max}) [208]. In contrast, in the long-term pharmacokinetic study comparing 1.1mg vs. 5mg folic acid supplementation among non-pregnant women demonstrated non-linear kinetics for the high dose of folic acid [205]. Within this study, Nguyen et al. found that repeated supplementation with 5mg folic acid only yielded ~2-fold difference in steady-state RBC folate concentrations despite a 5-fold difference in dose compared to the 1.1mg group.
II. We hypothesized that social media would lead to an increase in recruitment rate based on the breadth of broad-spectrum and pregnancy-specific online sources that were targeted.

Rationale: Within a study analyzing recruitment trends, we would predict that the addition of any additional recruitment methods or sources would contribute to a modest-to-significant increase in recruitment (due to an increase in the number of recruitment sources contributing to overall recruitment). That said, social media was specifically used because of its widespread entrenchment in our daily lives, and its use by health-seeking populations (eg. pregnant women, mothers/parents, individuals seeking healthy nutrition or fitness advice, etc.). Part of our rationale behind adopting this as a recruitment strategy came from the success of social marketing-based recruitment methods. However, a substantial degree of our rationale was influenced by anecdotal or experiential evidence at the Motherisk program. The Motherisk program is telephone counselling program where women who are planning, pregnant or breastfeeding call in to get evidence-based medical advice regarding the safety of drugs and exposures during this period. At Motherisk, a significant degree of pregnant callers questioned the safety of many medications advised by healthcare practitioners because of information they found on the internet. Hence, we adopted social media-based recruitment as an add-on to potentially increase overall recruitment after struggling for 4.5 years with marginal recruitment into a randomized clinical trial.
III. a) We hypothesized that the oral contraceptives likely do not substantially decrease blood folate concentrations.

b) We hypothesized that the folate-fortified oral contraceptive offered an equimolar and equally effective dose of folate (~400µg) for daily supplementation among women of childbearing age.

Rationale: While we did note that the evidence surrounding the effect of oral contraceptive use on folate status was controversial and lacked consensus, most of the later studies suggested that oral contraceptive use did not contribute to a significant decrease in blood folate status [209-211]. Furthermore, despite the fact that the literature characterizing risk factors associated with low blood folate status and increased risk of neural tube defects is extensive, oral contraceptive use is typically not cited as a contraindication or a risk factor warranting extra folate supplementation. In light of both these facts, we predicted that the folate-lowering effect of oral contraceptives was unlikely to be significant. Finally, given that the new folate-fortified oral contraceptive (Beyaz®) uses 451µg of levomefolate calcium (5-methyl-THF), we predicted that this was an equimolar and likely equally effective form of folate supplementation for women of childbearing age who were concomitantly using oral contraceptives.
IV. We hypothesized that a sizeable portion of women within the Toronto area are still suboptimally protected against NTDs, with blood folate concentrations less than 900nmol/L.

Rationale: Our predictions regarding the current folate status of women of childbearing age within Toronto were based on two recent population-based studies. In a study by Bar-Oz et al., using data from 2006, 36% of pregnant women and 40% of women of childbearing age in Toronto were reported to have blood folate levels below 906nmol/L, required for optimal protection against NTDs [141]. Similarly, in a study by Colapinto et al., as part of the Canadian Health Measures Survey, about 22% of women of childbearing age Canada-wide had blood folate concentrations that were suboptimally protective against NTDs [142]. Thus, we predicted that a significant percentage of women of childbearing age within Toronto still not adequately protected against the risk of NTDs.
1.3 REFERENCES


CHAPTER 2.

Pregnancy-induced changes in the long-term pharmacokinetics of 1.1 mg vs. 5 mg folic acid: a randomized clinical trial

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This study has been published:

MS was responsible was updating any changes associated with Research Ethics approval, recruitment of patients within the clinical trial, conducting the clinical trial and associated sample preparation for folate analysis, overall data collection and analysis, as well as writing and submission of the manuscript.

Conflict of Interest: Duchesnay Inc., Blainville, Quebec provided funding and the prenatal multivitamins used in the study. The other authors declare no conflict of interest.
2.1 ABSTRACT

The objective of this randomized clinical trial was to compare steady-state gestational RBC and plasma folate concentrations in pregnant women supplementing daily with 1.1 mg (regular dose) vs. 5 mg (high dose) folic acid. Thirty-seven pregnant women, who were not previously taking folic acid, were enrolled in this open-label, 2-arm, randomized clinical trial after informed consent. Participants were randomly assigned either 1.1 or 5 mg of folic acid-containing prenatals until gestational age (g.a.) 30 weeks. Plasma and RBC folate concentrations were measured at baseline, g.a.6 weeks, g.a.12 weeks, and g.a.30 weeks using a chemiluminescent immunoassay. Results showed sustained significant increase in RBC folate in the 5 mg group between g.a.6 weeks and g.a.30 weeks ($P < 0.001$), and between g.a.12 weeks and g.a.30 weeks ($P < 0.01$), whereas a significant increase in RBC folate concentrations was observed in the 1.1 mg group only between g.a.12 weeks to g.a.30 weeks ($P < 0.05$). Plasma folate increased in both groups from baseline to g.a.6 weeks, and then decreased between g.a.6 weeks and g.a.30 weeks, but this was not statistically significant. Plasma concentrations at g.a.30 weeks in both groups were comparable to their respective baseline concentrations. Thus, physiological changes in pregnancy alter long-term folate pharmacokinetics. Despite supplementation over an extended period of time, steady-state does not seem to be achieved in either dose group within our study.
2.2 INTRODUCTION

Periconceptional folic acid supplementation is associated with a reduction in the risk of neural tube defects (NTD) [1-4]. Pregnancy is especially a time when the body’s folate demands increase to accommodate the rapid cell growth associated with embryonic and fetal development [5]. Folate is a critical contributor to this process through its involvement in one-carbon transfer reactions leading to DNA replication, DNA repair, epigenetic programming and cell division [3, 4].

In 1995, Daly et al. demonstrated that the risk for NTD decreased with plasma folate concentrations ≥ 15.9nM and red blood cell (RBC) folate concentrations ≥ 906nM [1], documenting an inverse dose-response relationship between folic acid dose and the risk of NTD. A study conducted in 2008 showed that 40% of women of childbearing age and 36% of pregnant women in Ontario, Canada had RBC folate concentrations below the recommended 906nM [6]. Data by Colapinto et al. in 2011, suggests similar trends with about 22% women of childbearing age having less than optimal RBC folate concentrations [7]. Despite the increasing awareness about folic acid in pregnancy, many women do not start supplementation until they discover they are pregnant. This is a great public health challenge because the neural tube closes 21-28 days post-conception, at which point many women may not even be aware that they are pregnant. Recent data from Ireland show that although 84% of women took folic acid supplements in their first trimester, only 19% had started before conception, as recommended [8]. Similarly, representative data from Canada shows that even though 77.6% knew about periconceptional folic acid supplementation, only about 57.7% actually began taking it prior to pregnancy [9].
Current guidelines for folic acid supplementation recommend all women of childbearing age to supplement with 0.4mg daily. Women who are planning a pregnancy or pregnant are advised to supplement with up to 1mg folic acid daily. Currently, a dose of 5mg of folic acid is prescribed to women with a history of NTD or NTD-affected pregnancies, women who concurrently use folate antagonist medications, women who are obese or exhibit poor adherence among a broad set of indications by the Society of Obstetricians and Gynaecologists of Canada (SOGC) [10, 11]. Since folate is a water-soluble vitamin, it has relatively low toxic potential because excess folate is excreted in urine. However, chronic folate overexposure has been linked to several risks including: the potential for masking vitamin B12 deficiency, cognitive impairment in the elderly, high levels of unmetabolized folic acid and impaired natural killer cell function, and a dual modulatory role in cancer progression based on animal data [12]. A systematic review and meta-analysis of folate intake during the periconceptional period has failed to show an increased risk for cancer [13], but we would advise women to consult a healthcare professional to determine whether high doses of folic acid would be clinically appropriate for them before beginning therapy.

The objective of this prospective, open-label, two-arm, randomized clinical trial was to assess steady-state folate pharmacokinetics in pregnancy among women who supplement daily with 1.1mg (regular dose) vs. 5mg (high dose) folic acid in the periconceptional period. To the best of our knowledge, this is the first study to examine steady-state pharmacokinetics of folate in pregnancy comparing 1.1mg vs. 5mg folic acid supplementation.
2.3 SUBJECTS AND METHODS

2.3.1 Recruitment

Healthy women 18-45 years of age, who were either pregnant (less than 6 weeks gestational age) or trying to conceive (within the next 3 months), and had not been previously using any form of folic acid supplementation (for at least 3 months prior), nor had any previous history of NTD, were recruited through traditional healthcare-based recruitment sources through medical establishments and social media-based sources in the study [14]. The protocol and all recruitment materials were approved by the Hospital for Sick Children Research Ethics Board, and all participants signed a written informed consent.

Women interested in the study were asked to contact the study coordinator, and after an initial telephone follow-up was conducted, eligible participants were invited to the Hospital for Sick Children to go over formalized written informed consent and proceed further with the study.

The study aimed to recruit 20 women in each arm to detect a difference with a power of 85% and an alpha of 5%, based on a 40% difference in the risk reduction of neural tube defects.

2.3.2 Study Design

This study was a prospective, open-label, 2-arm, randomized clinical trial.

Randomization of participants in the study was managed independently by the Research Pharmacy at the Hospital for Sick Children, Toronto, ON who assigned participants prenatal multivitamins containing either 1.1mg of folic acid or 5mg of folic acid using a randomization software (Research Randomizer). Participants were instructed not to take any additional folic acid supplements concurrently while in the study. Post-randomization, the study coordinator and participants were not blinded to the study drug due to potential ethical concerns. Women in the 1.1mg folic acid group were given the prenatal multivitamin PregVit® (Duchesnay, Laval, QC,
Canada), whereas women in the 5mg folic acid group were given the prenatal multivitamin PregVit-Folic 5® (Duchesnay, Laval, QC, Canada). The tablets or the packaging of the study drug were not altered in any form. Both prenatal preparations were prescribed as 2 tablets/day in the form of a morning pill (pink) and an evening pill (blue). Both prenatal tablets are of the same size and are identical in the quantity of other vitamins and minerals, except folic acid (Table 6).

Table 6. Composition of PregVit and PregVit-Folic 5, taken as morning and evening tablets daily.

<table>
<thead>
<tr>
<th>Morning (AM) tablet</th>
<th>Evening (PM) tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene (2700 IU)</td>
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</tr>
<tr>
<td>Thiamine (3 mg)</td>
<td>1.1 mg – PregVit</td>
</tr>
<tr>
<td>Riboflavin (3.4 mg)</td>
<td>5 mg – PregVit-Folic 5</td>
</tr>
<tr>
<td>Vitamin E (30 IU)</td>
<td>Calcium (300 mg)</td>
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<tr>
<td>Vitamin C (120 mg)</td>
<td>Vitamin B₁₂ (12 µg)</td>
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<tr>
<td>Niacinamide (20 mg)</td>
<td>Vitamin D (250 IU)</td>
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<tr>
<td>Pantothentic acid (5 mg)</td>
<td></td>
</tr>
<tr>
<td>Magnesium (50 mg)</td>
<td></td>
</tr>
<tr>
<td>Iodine (0.15 mg)</td>
<td></td>
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<tr>
<td>Iron: ferrous fumarate (35 mg)</td>
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<tr>
<td>Copper (2 mg)</td>
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<tr>
<td>Zinc (15 mg)</td>
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</table>

PregVit contains 1.1mg folic acid and PregVit-Folic 5 contains 5mg folic acid. Both prenatal multivitamins are manufactured by Duchesnay Inc., Blainville, QC, Canada.

Baseline blood samples of 8 mL were obtained from all participants after a 6-h fast to measure RBC and plasma folate concentrations. Each participant also completed a validated Block Dietary Folate Equivalents food frequency questionnaire (NutritionQuest, Berkeley, CA) to estimate the amount of folate intake through their diets at baseline and at 30 weeks gestation, as well as a general intake form documenting other general, medical, demographic and obstetric information. During the baseline appointment, women received a 3-month supply of their assigned prenatal multivitamin. Prenatal multivitamins were replenished at each clinic visit as necessary. Women were advised to leave each missed tablet within the blister pack, and to bring
all blister packs with them to clinic visits and appointments, so that adherence could be calculated by pill counts. Participants returned to the hospital for blood draws upon pregnancy at 6 weeks gestation (neural tube completes its closure), 12 weeks gestation (organogenesis is complete), and 30 weeks gestation (volume of distribution is the highest at this point in pregnancy).

2.3.3 Sample preparation

Blood samples were collected in 2 Vacutainer® (4mL) tubes containing EDTA after a minimum 6-h fast. The tubes were shielded from light and placed on ice, and samples were processed within 2h of sample collection. Hematocrit readings for each blood sample were obtained by using heparinized capillary tubes and placing them in a centrifuge.

To process the whole blood folate sample (for RBC folate), a 1% wt:vol solution of ascorbic acid and deionized water was prepared, as it acts as an antioxidant and helps in the preservation of RBC folate. 100µL of the ascorbic acid solution was added to each 900µL of whole blood sample. Each sample was incubated at 37ºC for 30min, after which samples were immediately frozen at -80ºC.

The remaining blood sample was centrifuged at 1500g for 4ºC for 20min to isolate blood plasma. Plasma samples were prepared by adding 1% wt:vol sodium ascorbate solution to 500µL plasma, and then were stored at -80ºC.

2.3.4 Competitive Binding Receptor Assay

Whole blood and plasma samples were analyzed to measure RBC and plasma folate respectively, using a chemiluminescent immunoassay (Access Folate, Beckman Coulter Inc., Fullerton, CA). This assay has a lower limit of detection of 0.5ng/mL. Samples above the linear range were diluted to bring them within the linear range of the assay. All of the samples were
assayed at a certified laboratory that runs clinical tests for folate analysis. Thus, stability, quality control and dilution testing were routinely conducted as part of the assay set-up protocol.

2.3.5 Folate Standards

National Institute of Standards and Technology (NIST, Gaithersburg, MD) folate standards were used for plasma folate and the WHO international standard for whole blood folate by National Institute for Biological Standards and Control (NIBSC, Hertfordshire, England), to evaluate the efficacy of the Access Folate assay in quantifying blood folates against the *L. casei* microbiological assay [15].

2.3.6 Statistical Analysis

Patient characteristics in each dose group were compared using GraphPad Prism (version 5; GraphPad Software, San Diego, CA). Continuous variables were compared using unpaired t-test and Mann Whitney U test, where applicable, and categorical variables were compared using chi-square test and Fisher’s exact test, where applicable.

Further statistical analyses were conducted using IBM SPSS (version 18; PASW Statistics, Armonk, NY). A mixed-model repeated measures analysis of variance (ANOVA) was conducted to determine whether there was any interaction between the dose group (between-subjects factor) and time (within-subjects factor) on RBC and plasma folate concentrations. Assumptions of normality of the data were assessed through visual inspection of the Q-Q plots of standardized residuals (to establish linearity) and the Shapiro-Wilk statistic (p>0.05). Homoscedasticity was confirmed through the graphical inspection of residual plots and Levene’s test (p>0.05). Homogeneity of covariances was established through Box’s test of equality of covariance matrices (p>0.05) and sphericity was established through Mauchly’s statistic (p>0.05). The interaction term was investigated for both RBC and plasma folate, and the
statistical significance of between-subjects factors and within-subjects factors (through Bonferroni adjusted pairwise comparisons) was identified. In the case of a non-significant interaction, main effects of time and treatment group were reported.

A mixed-model ANOVA was also used to investigate the interaction between the dose group and time on dietary folate equivalents (DFE) as recorded through the validated Block Dietary Folate Equivalents screener. As above, assumptions were tested and the statistical significance of the interaction was identified.
2.4 RESULTS

87 women who were either planning a pregnancy or were at an early stage of pregnancy (less than 6 weeks) were enrolled and randomized into this study; 45 of these women were randomized to the PregVit (1.1mg folic acid) arm and 42 were randomized to the PregVit-Folic5 (5mg folic acid) arm. Amongst these, 19 women in the 1.1mg folic acid arm and 18 women in the 5mg folic acid arm conceived during the course of the study, contributed to all evaluable data points, and were thus analyzed (Figure 7)

Figure 7. Consolidated Standards of Reporting Trials (CONSORT) patient flow diagram.

Study patient recruitment (n=37) flow chart based on CONSORT guidelines.
No significant differences were observed between patient characteristics of women in the two dose groups (Table 7).

### Table 7. Patient Characteristics.

<table>
<thead>
<tr>
<th></th>
<th>1.1mg folic acid group (n=19)</th>
<th>5mg folic acid group (n=18)</th>
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<tbody>
<tr>
<td>Age (yr)</td>
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<tr>
<td>Weight (kg)</td>
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<td>67.71 ±19.19</td>
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<td>Duration of participation (weeks)</td>
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<td>3</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>Smoking</td>
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</table>

Data are presented as mean ±SD. a= Unpaired t-test performed, b=Mann Whitney U test performed, γ= Chi square test performed, δ=Fisher’s exact test performed. No significant differences (p>0.05) observed between groups.
RBC folate concentrations were measured in 19 women in the PregVit (1.1mg folic acid) group and 18 women in the Folic5 (5mg folic acid). Mean duration of participation in the study was about 52 weeks in the 1.1mg group (28 weeks between baseline and g.a.6 weeks), and 40 weeks in the 5mg group (16 weeks between baseline and g.a.6 weeks).

There was a statistically significant interaction between dose and length of time on RBC folate concentration: F(3,105)=9.471, p < 0.001, partial η² = 0.213. RBC folate concentrations were significantly different between the two dose groups at g.a.12 weeks (p = 0.03) and g.a.30 weeks (p = 0.001). There was also a statistically significant effect of time on RBC folate in both 1.1mg and 5mg dose groups (p < 0.001). In both 1.1mg and 5mg folic acid groups, RBC folate concentrations increased significantly at g.a.6 weeks, g.a.12 weeks and g.a.30 weeks as compared to their baseline concentrations (p < 0.001). In the 1.1mg group, there was a statistically significant increase in RBC folate observed between g.a.12 weeks and g.a.30 weeks (p = 0.044). In the 5mg group, there was a significant increase in RBC folate concentrations between g.a.6 weeks and g.a.30 weeks (p = 0.001), as well as between g.a.12 weeks and g.a.30 weeks (p = 0.002) (Figure 8).
Data are presented as mean ±SEM. Significant interaction effects were observed between time and dosage group at g.a.12 weeks and g.a.30 weeks, as part of the mixed model ANOVA: * indicates p<0.05 and *** indicates p<0.001. Differences between time points in each dose group were compared through Bonferroni adjusted pairwise comparisons as part of the mixed-model ANOVA: * indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001. These are represented using horizontal brackets and are coded based on the dose arm within the study. The absence of horizontal brackets between two time points shows non-significant interactions.

Plasma folate concentrations were generally higher in the 1.1mg group compared to the 5mg group, with significant differences between baseline (p=0.013, Mann Whitney U Test) and g.a.12 weeks (p= 0.0066, Mann Whitney U Test). Plasma folate concentrations increased in both groups until pregnancy, and decreased in both groups over the course of pregnancy, with a faster rate of decrease in the 5mg group between g.a.6 weeks to g.a.12 weeks. There was no statistically significant interaction observed between the dose group and time on plasma folate concentration, F(3,108)=0.905, p=0.44, partial $\eta^2 = 0.025$. The main effect of time showed a statistically significant difference at different time points (p<0.001), and the main effect of group was a significant difference in plasma folate concentrations between the two dose groups (p=0.027). Bonferroni adjusted pairwise comparisons demonstrated a significant difference.
between baseline and g.a.6 weeks (p=0.024), baseline and g.a.12 weeks (p=0.004), but not baseline and g.a.30 weeks in the 1.1mg folic acid group. Similarly, significant differences were observed between baseline and g.a.6 weeks (p=0.037), baseline and g.a.12 weeks (p=0.011), but not baseline and g.a.30 weeks in the 5mg folic acid group (Figure 9).

Figure 9. Plasma folate levels achieved with 1.1mg vs. 5mg folic acid daily supplementation in pregnancy.

Data are presented as mean ±SEM. Differences between time points in each dose group were compared through Bonferroni adjusted pairwise comparisons as part of the mixed-model ANOVA: * indicates p<0.05 and ** indicates p<0.001. These are represented using horizontal brackets and are coded based on the dose arm within the study. The absence of horizontal brackets between two time points shows non-significant interactions. Differences between the dose groups at each time point were compared using the Mann Whitney U test: † indicates p<0.05 and †† indicates p<0.01.
No significant differences in dietary folate equivalents (DFE) consumed through folate-rich food sources were observed at baseline vs. 30 weeks gestation in both groups. A slight decrease in DFE intake was observed at 30 weeks in comparison to baseline in both groups, but this trend was not statistically significant. (Table 8)

Table 8. Dietary folate intake of participants within the study, evaluated through the Block Dietary Folate Equivalents Screener.

<table>
<thead>
<tr>
<th></th>
<th>1.1mg folic acid group (n=19)</th>
<th>5mg folic acid group (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (µg DFE/day)</td>
<td>523.0 ±232.3</td>
<td>556.0 ±241.5</td>
</tr>
<tr>
<td>30 weeks gestation (µg DFE/day)</td>
<td>479.9 ±174.0</td>
<td>489.6 ±274.0</td>
</tr>
</tbody>
</table>

Data are presented as mean ±SD. There was no significant difference (p>0.05) between between dose groups or time, as determined by a mixed-model ANOVA. The Block Dietary Folate Equivalents screener is produced and analyzed by Block Dietary Data Systems, NutritionQuest, Berkeley, CA.

All participants in the study demonstrated very high adherence (96.1% ± 3.3% in the 1.1mg folic acid group and 95.7% ± 2.9% in the 5mg folic acid group) as determined through pill counts. Mean adherence tended to decrease in women who were planning a pregnancy for more than 3-4 months, and increased again upon discovery of a pregnancy, but these trends were not statistically significant.

To investigate the potential effect of weight gain during pregnancy on the decrease in plasma folate concentrations observed in both groups, plasma folate level per dose was calculated. Weight gain in both groups between g.a.6 weeks and g.a.30 weeks was similar (9.2kg gain in 1.1mg group and 10.6kg gain in 5mg group), and hence no significant differences were observed in the plasma folate concentrations per dose in either group by 30 weeks gestation (Figure 10).
Assessment of NIST and NIBSC folate standards by chemiluminescent immunoassay exhibited a close correlation with certified values. The NIST plasma folate standards with certified values of 5.6 nM, 14 nM and 44 nM by NIST yielded values of 6.8 nM, 13.67 nM and 40.95 nM respectively, using the Beckman Coulter chemiluminescent immunoassay. Similarly, the NIBSC hemolysate folate standard of 29.48 nM had a value of 22.6 nM using the immunoassay. This suggested that the values obtained by the immunoassay are comparable with the values of the folate standards derived using the microbiological assay.
2.5 DISCUSSION

In our study, despite aiming to assess steady-state folate concentrations in women who supplement daily with 1.1mg vs. 5mg folic acid, we did not reach a steady-state in either dose-group by 30 weeks gestation. This means that dynamic changes in folate distribution, metabolism and elimination continue up to late pregnancy. The two major outcomes we evaluated were biomarkers of folate status: RBC folate, the more reliable measure of long-term folate stores, and thus, of maternal status, and plasma folate, the indicator of short-term, recent folate intake.

The elimination half-life of RBC folate is about 8 weeks [16-18]. Thus, if folate followed linear pharmacokinetics [19] at 1.1mg and 5mg, we would expect it to achieve a steady state between 4-5 half-lives (32-40 weeks). Even though the mean duration of participants in the study was about 52 weeks in the 1.1mg and 40 weeks in the 5mg group (Table 2), neither dose group achieved steady-state. Instead, a significant increase in RBC folate concentrations was seen in both dose groups between g.a.12 weeks and g.a.30 weeks, and no significant difference was observed between the baseline and g.a.30 weeks concentrations of plasma folate in both dose groups, suggesting non-linear kinetics and that the processes of folate distribution and elimination continue to change till at least 30 weeks of gestation. This is in stark contrast to our previous study on steady-state folate pharmacokinetics in non-pregnant women with 1.1mg vs. 5mg [10] where steady state was readily achieved consistent with the elimination half-life, demonstrating that pregnancy alters folate pharmacokinetics. Thus, we certainly observed gestational age-induced pharmacokinetic changes within this study.

Since RBC folate is a measure of long-term tissue stores, it is possible that the increased RBC folate concentrations over time seen at both dose levels reflect folate accumulation due to
supplementation. Part of the increase in RBC folate during pregnancy can be explained by a 33% increase in RBC production during pregnancy [20], leading to increased incorporation of folate into red blood cells.

In both dose levels (1.1mg and 5mg), there was a significant increase in RBC folate observed between baseline and all other time points in pregnancy. However, the sustained increase in RBC folate concentrations over the course of pregnancy was observed in the 5mg folic acid group starting at an earlier time point (g.a.6 weeks) and with a sharper slope (Figure 2), suggesting this may be the preferred dose of supplementation for women with folate deficiency or disease states associated with folate malabsorption during pregnancy.

A unique finding of our study is that plasma folate decreased in both dose groups (1.1mg and 5mg) over the course of the pregnancy, after significantly increasing until g.a.6 weeks. Plasma folate levels in both dose levels at 30 weeks gestation were comparable with their respective baseline concentrations (Figure 3), despite high adherence exhibited by participants in both dose groups. Long-term studies assessing folate kinetics show that a plateau is achieved in plasma folate concentrations 12-14 weeks after supplementation, independent of dose [21]. Results from a prior study of identical design in non-pregnant women confirm these results, as a plateauing of plasma folate concentrations was observed in both 1.1mg and 5mg folic acid groups by 12 weeks, and this trend continued till 30 weeks of supplementation [10]. Within our study, given that the mean duration of supplementation between baseline and g.a.6 weeks was 28 weeks in the 1.1mg group and 16 weeks in the 5mg group, it is possible that steady-state may have been achieved in between assessments or prior to conception for some participants. However, the significant difference in plasma folate concentrations between baseline and g.a.6 weeks in both dose groups (p < 0.05) does not suggest a plateauing trend.
Multiple factors have been implicated in the decrease of plasma folate concentrations observed during pregnancy [22]. Folate requirements increase in pregnancy due to increases in growth of maternal and fetal tissues, as well as in blood volume, and the increase in cell division associated with embryonic and fetal development [23]. In pregnant women, the need for folate exceeds that of the calculated total fetal and placental folate content at term, indicating that increased folate turnover and one-carbon metabolism take place during pregnancy [24]. Increased urinary excretion of folates and increased folate catabolism in pregnancy have also been associated with the decrease in plasma folate concentrations in pregnancy [25].

Studies reporting increased folate catabolism in pregnancy, especially during the second and third trimesters have shown contradictory findings. While studies by Caudill et al. [26-28] showed no difference in the rate of urinary folate excretion and folate catabolism between pregnant vs. non pregnant subjects, McPartlin et al. [24, 29] found that rates of folate catabolism in pregnancy increased by more than two-fold in comparison to non-pregnant controls. A recent Japanese study [30] also supported increased urinary excretion of folates during pregnancy. Importantly, most of these studies investigated much lower folate doses of 450µg/day or 850µg/day in comparison to the doses of 1mg/day and 5mg/day investigated in our study. Because of the higher doses used in our study, folate catabolism and increased urinary folate excretion may play a significant role in explaining the decrease in plasma folate concentrations seen between 6 weeks gestation to 30 weeks gestation in both dosing groups.

Hemodilution or the general increase in plasma volume during pregnancy [31] may also partially explain the decline in plasma folate concentrations. A report by Kim et al. [32] has shown inverse relationship between BMI and serum folate concentrations in pregnancy, suggesting that weight gain over the course pregnancy may also partially explain the consistently
decreasing plasma folate concentrations between g.a.6 weeks to g.a.30 weeks in both dosing groups.

Our study shows that, similar to non-pregnant women, the five-fold increase in dose resulted in only a twofold increase in RBC folate, which not only shows non-linear kinetics but also strongly suggests saturation in absorption at excessive doses, similar to the way the body handles iron and other micronutrients [33, 34].

While our results are complicated by a between-group statistically significant difference in plasma folate concentrations at baseline, the decrease in plasma folate observed is likely multifactorial and interdependent in its regulation. It may be explained by increased folate delivery to fetal tissues (infants have higher blood folate concentrations at birth than their mothers [35, 36]), increased folate turnover, catabolism, urinary excretion, weight gain and hemodilution.

Further, though non-significant, the differences in duration of supplementation among the two dose groups between baseline and g.a.6 weeks (28 weeks for 1.1mg folic acid vs. 16 weeks for 5mg folic acid) may also partially explain the higher plasma folate concentrations observed within the 1.1mg folic acid arm at g.a.6 weeks, contrary to the dose-effect expected.

One of the limitations of our study is the number of time points at which blood folate concentrations were assessed. While a greater number of time points would have increased our ability to correlate pharmacokinetic changes with the major physiological changes in pregnancy, this was not feasible due to ethical concerns and its potential impact on recruitment and retention. Hence, we chose the three most relevant and significant time points in pregnancy based on steady-state folate pharmacokinetics previously studied in non-pregnant women [10].
Thus, we measured folate concentrations at 6 weeks gestation when the neural tube completes its closure, 12 weeks gestation when organogenesis is complete, and 30 weeks gestation when volume of distribution is the highest in pregnancy. Our study could have also been strengthened by evaluating urine folate catabolite concentrations at each of the above time points over the course of the study to gain a better mechanistic understanding of clearance-related changes. Detailed analysis of folate metabolites through LC/MS methods would also present a better opportunity to hone in on specific changes in folate metabolism that may occur during pregnancy. We used the chemiluminescent immunoassay to analyze total folate concentration since it is routinely used in clinical laboratories and would help make our results more translatable in clinical practice. However, the different approaches suggested here remain avenues for future research.

The results of this study along with several others show that increased folate catabolism may be a major contributor to the decrease in plasma folate concentrations observed in pregnancy. While we do not have direct data to support this conclusion, a study by Higgins et al. [37] showed that folate catabolism peaked in the third trimester – the period associated with maximal increase in fetal mass. The changes in folate catabolism demonstrated in this study could not be explained by normal maternal weight gain or increased renal clearance in pregnancy.

Though the importance of supplementation with folic acid is stressed in the first trimester of pregnancy because of its effect on the reduction of NTDs [38, 39], continued supplementation over the course of the pregnancy is often not emphasized. Although NTD-risk in pregnancy is evaluated through RBC folate as an indicator of long-term folate stores, it is the plasma folate stores that actually supply folate to the developing fetus [19, 21] and are influenced by recent
changes in folate intake and turnover. Thus, the decrease in plasma folate concentrations over the course of pregnancy may have major implications for women who may not be adhering to regular folic acid supplementation, may have a folate-deficient diet, or may have other conditions that impair folate absorption. This is supported by findings of increased pre-eclampsia and prematurity associated with low folate intake [34]. While this decrease in folate concentrations was not clinically significant in our study population composed of women who were already self-motivated, it may have important implications in women who may not have adequate folate status prior to and during pregnancy.

We recommend that these women consider the 5mg dose of folic acid for supplementation under the guidance of a healthcare professional, and that its use should be considered on an individual basis under a broad series of indications [11, 40]. Over-exposure to folic acid in healthy individuals may lead to a potential masking of vitamin B12 deficiency. High concentrations of folate have been linked with cognitive decline in elderly populations, and reduced natural killer cell cytotoxicity among post-menopausal women [12]. Hence, while the 5mg folic acid may have immense clinical utility for women with impaired folate status, its use should be considered on a case-by-case basis for a controlled period of time within the periconceptional period.

Therefore, overall, in light of the gestational age-induced pharmacokinetic changes that occur during pregnancy in decreasing plasma folate concentrations despite continued supplementation, it is pivotal that women not only begin folate supplementation prior to pregnancy, but also continue it over the course of pregnancy. Even though guidelines by the CDC and WHO only recommend folate supplementation during the first trimester of pregnancy
[38, 39], plasma folate kinetics reveal that this is not sufficient and continued supplementation is recommended for all women throughout the periconceptional period.
2.6 REFERENCES


CHAPTER 3.

The Role of Social Media in Recruiting for Clinical Trials in Pregnancy

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This study has been published:

MS conceived and designed the experiments, was responsible for the recruitment of patients within the clinical trial, performed the experiments, overall data collection and analysis, as well as writing and submission of the manuscript.
3.1 ABSTRACT

Background: Recruitment of women in the periconceptional period to clinical studies using traditional advertising through medical establishments is difficult and slow. Given the widespread use of the internet as a source for medical information and research, we analyze the impact of social media in the second phase of an ongoing randomized, open-label clinical trial among pregnant women. This study aims to assess the effectiveness of social media as a recruitment tool through the comparison of diverse recruitment techniques in two different phases of the trial.

Methods: Recruitment in Phase 1 of the study consisted solely of traditional healthcare-based sources. This was compared to Phase 2 of the study where traditional recruitment was continued and expanded, while social media was used as a supplementary source. Yearly recruitment and recruitment rates in the two phases were compared using the Mann Whitney U test. The contributions of each recruitment source to overall recruitment were analyzed, and the impact of potential confounders on recruitment rate was evaluated using a multiple regression and Interrupted Time Series Analysis.

Results: In the first phase of the study, with over 56 months of recruitment using traditional sources, 35 women were enrolled in the study, resulting in a mean rate of ±0.62 recruits/month. In the 6 months implementing recruitment through social media, 45 women were recruited, for a 12-fold higher rate of ±7.5 recruits/month. Attrition rates remained constant, suggesting that social media had a positive impact on recruitment. The Interrupted Time Series Analysis detected a significant difference in recruitment after the intervention of social media (p<0.0001) with an evident increase in the number of recruits observed after the use of social media.
Conclusions: Clinicians and scientists recruiting for clinical studies should learn how to use online social media platforms to improve recruitment rates, thus increasing recruitment efficiency and cost-effectiveness.
3.2 INTRODUCTION

Poor recruitment is a major obstacle to the successful and efficient completion of clinical trials. A recent survey of corresponding authors of randomized trials found that nearly 60% had either failed to meet their recruitment target or required an extended recruitment period [1]. Insufficient recruitment of study participants may result in losing the statistical power of a predictive conclusion, as well as prolonging the time and increasing the cost associated with the study.

The path to recruitment is usually the untold story in randomized clinical trials. While even failed results and conclusions of experiments are reported, inefficient recruitment methods often go unreported. Studies assessing effective recruitment strategies are far too scarce. The few systematic reviews that have addressed this issue stress the lack of generalizability of recruitment methods given the degree of subjectivity with respect to a particular study design, intervention type, and the nature of participation required by volunteers [1-4].

The issue of poor recruitment becomes even more exaggerated when the target of a study is a special population such as women in the periconceptional period or during pregnancy. Risk perception with a clinical intervention during this period is often skewed from ‘actual risk’ to ‘imagined risk’ given this state of vulnerability and fears of coercion [5]. As a result, there is a great need for the assessment of recruitment strategies in special populations, such as women in the periconceptional period, which are not only efficient but also cost-effective.

With the advent of the internet and medical information being available on the internet in recent years, volunteers participating in clinical trials have moved away from being “patients” to “informed health-care consumers” [6]. Many people thoroughly search their symptoms on the internet before they decide to visit a physician who assigns them a diagnosis. About one-third of
American adults access social media for health matters [7]. A survey conducted by the Opinion Research Corporation demonstrated that 59% of adults in the USA use the internet to seek health information [8], making it the most popular option over seeking similar information from a healthcare provider.

The accessibility of medical information on the internet has not only made modern-day patients more aware, but also more involved in their personal healthcare. Thus, using social media to expose clinical trials to a larger subject population seems like an obvious next step in trying to optimize recruitment strategies. Social media is typically an online platform that can enable dialogue among individuals and online communities, serving as a site for information dissemination and discussion. After several years of slow and frustrating recruitment for a randomized, open-label trial on folic acid in pregnancy, we decided to examine the effectiveness of recruitment through social media. We use a broad definition of social media which encompasses social networking sites such as Facebook and Twitter, along with local online city classifieds like Kijiji and Craigslist, as well as online discussion forums and message boards on specific websites (Figure 1). Though skepticism and issues with credibility of information on social media prevail on the minds of both participants and healthcare professionals, we hypothesized that social media may be a valuable tool for recruitment if used in an organized and targeted fashion. The objective of the present report was to compare recruitment success and efficiency between traditional healthcare-based methods of recruitment vs. social media.
Figure 11. What constitutes Social Media?

The various online social media and networking platforms used for the recruitment of pregnant and planning women within the study.

3.3 METHODS

3.3.1 Study

This study is based on secondary, post-hoc analysis emerging from a randomized clinical trial with the objective of comparing the steady-state pharmacokinetics of regular (1.1mg) and high (5mg) folic acid tablets over 30 weeks of pregnancy. The study aimed at recruiting women between the ages of 18-45 years, who were not taking folic acid-containing multivitamins 3 months prior to enrollment, and were either early in pregnancy or trying to conceive. Women
who had a previous history or a previous affected pregnancy with neural tube defects were excluded from the study (Figure 12). Participants who completed the study were granted monetary compensation for their participation. This was pro-rated based on the degree of participation and remained the same over the course of the study.

**Figure 12. Study Design**

An outline of the study design.

### 3.3.2 Recruitment

Originally, recruitment materials such as posters, ads and study information brochures were approved by the Hospital for Sick Children Research Ethics Board for the study in 2007.
In the first phase of the study (from April 2007- November 2011), the primary mode of recruitment focused on recruiting participants amongst healthcare establishments. This was achieved through a variety of different methods. We targeted women who called the Motherisk program. The Motherisk program is a telephone counselling program that provides evidence-based information to healthcare professionals and women about the safety and teratogenic risks associated with drugs, chemicals, radiation, infections and other exposures in the periconceptional period. Due to the large volume of callers every day, study details were presented to women who fit the inclusion criteria by Motherisk counsellors and they were asked for telephone consent and follow-up by the study coordinator if they expressed interest in participating. Motherisk counsellors were trained to briefly explain the main objective, intervention, time course and target population of the clinical trial to callers who expressed interest. Interested callers were then followed up by the study coordinator. Voicemails were left after initial consent if interested callers could not be reached, and were called back up to a maximum of three times. Concurrently, we used notice board postings at Sick Kids and Women’s College hospitals in areas where a general high traffic of families was expected. The notice board postings at both these hospitals were re-posted monthly or as needed after approval from Public Relations department. Furthermore, study materials were presented in the form of brochures and postings before and after clinic appointments, to eligible women at the above hospitals as well as fertility clinics associated with the study. This was achieved through multiple ways. Study brochures were available within waiting rooms and study postings were also posted on bulletin boards within the clinics. Secondary staff, who usually included research coordinators at other clinics or hospitals, closely communicated with the folic acid study coordinator to identify eligible patients arriving at the clinic for the upcoming week. Patients were screened on
the basis of the inclusion criteria, with the major determiners being women who were “early in pregnancy” or “trying to conceive within 3 months” and “were not currently on folate-containing multivitamins”. Eligible patients were flagged based on the review of patient charts each week and upon their clinic visit, study details were presented to them through two possible ways: either through physicians at the clinic during their consultation, or, if this was not possible due to time-constraints or other commitments of an appointment, physicians often directed flagged patients to secondary staff at each clinic, who were trained to briefly outline study information and were able to spend more time with each patient reviewing the objective and demands of the study. In both these cases, upon interest, a women was handed a study brochure outlining study information and the contact information of the study coordinator. Interested women were asked to contact the study coordinator directly via telephone or email, as listed on the brochure. Monthly meetings were held at all clinics between the study coordinator and the secondary study staff to go over recruitment progress and replenish advertisement supplies. All study postings were also revisited monthly or as needed for renewal.

After the study had been going on for 4 years, recruitment strategies were re-evaluated in the second phase of the study (December 2011-May 2012). We continued to actively engage in recruitment approaches based amongst healthcare establishments. These included pregnancy and prenatal community programs and health centers, family doctor’s offices, new immigrants and women’s centers, university health clinics, and midwifery clinics, as well as all of the previously used recruitment sources. As before, women interested in participating could contact the study coordinator via telephone or email, as listed on the advertisement.

However, in parallel to this approach, advertising was expanded to the realm of social media. Study details were posted on the drug sponsor’s website, Duchesnay
(www.duchesnay.com) and the Motherisk website (www.motherisk.org), both of which are largely accessed by women in the periconceptional period. Advertisements were also posted regularly on local online classifieds such as Craigslist (http://toronto.craigslist.ca/) and Kijiji (http://toronto.kijiji.ca) in the “community, volunteers” and “baby items/baby+kids” categories to target women planning families. Ads on these platforms were renewed every 2-3 days to keep them well updated on the front page of each section. A similar approach was used with study postings on pregnancy discussion forums and message boards, such as Baby and the bump (http://babyandbump.momtastic.com/) and Baby on the way (http://www.babyontheway.ca/toronto). Study ads were created as new threads or announcements, and were renewed every month, or as needed. Further, postings detailing study information were also occasionally posted on social media networks such as Facebook (www.facebook.com) and Twitter (www.twitter.com). Posts on all of above online media were limited to placement of study ads, as approved by the Ethics Review Board at the Hospital of Sick Children. Interested participants were asked to contact the study coordinator if they had any questions or concerns, which were addressed via telephone follow-up, as per the study protocol.

Upon approaching the study coordinator with interest in the study, the participants were contacted by a healthcare professional member of the study to discuss study details and confirm consent to participate. Interested participants were then invited to the Hospital for Sick Children for their first appointment to go over formalized informed consent and proceed further with the study (Figure 12).

### 3.3.3 Statistical Analysis

Recruitment from the two phases of the study was divided such that Phase 1 included “traditional” recruitment from healthcare establishments, whereas Phase 2 included “traditional”
recruitment supplemented with “social media”. All data were tested for normal distribution. We compared monthly recruitment between the two phases using the Mann Whitney U test, since the data were not normally distributed. Furthermore, the recruitment rate per year was calculated using the ratio of women recruited over the period of time that recruitment was conducted. Characteristics of the women in the two groups (“traditional” vs. “social media”) were compared by the chi-square test or Student’s t-test, wherever applicable. All of the above analysis were conducted using GraphPad Prism (version 5; GraphPad Software, San Diego, CA).

A multiple regression was conducted using IBM SPSS (version 18; PASW Statistics, Armonk, NY) to analyze the impact of predictors such as recruitment source, age, previous fertility problems and a previous history of chronic illnesses on recruitment rate. Given our sample size of 80 participants, we identified the four most important predictors affecting recruitment rate and used these as covariates within the multiple regression.

As part of the multiple regression, the Durbin-Watson statistic was evaluated, to assess the independence of residuals.

Interrupted Time Series Analysis was conducted using SAS (version 9.3; SAS Institute, Cary, NC) to detect a difference in recruitment after the introduction of social media. Log transformation of the raw values was used to transfer the data counts close to normal distribution. Visual inspection of the series plots, autocorrelation functions (ACF), partial autocorrelation function (PACF), inverse autocorrelation function (IACF) plots, and Dickey-Fuller unit root tests were used to check the assumption of stationarity. Non-significant results showed stationarity of the series; therefore, no differencing was used.

The shifted mean was modelled by creating a shift indicator with the value of “0” or “1” before and after the intervention of social media. Transform function was used to realize it within
the model building process.

The error structure was then fitted with the autoregressive integrated moving average (ARIMA) model. The order of $p$, $q$, and $d$ in ARIMA model was determined by carefully examination of ACF, PACF, and IACF plots. Maximum likelihood method was used for estimation of parameters.

Chi-Square test on the residuals was used to assess if series was left with white noise. Non-significant Chi-Square statistics indicated that our model fitted well. Residual QQ plots were used to test the departure from normality assumption.

A set of candidate models were arrived at, and final model was selected by the lowest akaike information criterion (AIC), significant parameter estimates (indicator and AR/MA lags), Chi-square test on the residuals, residuals diagnosis plots, and forecast plots. Fitted plots were produced in both log and original scale.
3.4 RESULTS

The study started recruitment in April 2007. Between 2007-2011, with over 56 months of recruitment in Phase 1 of the study using traditional sources, 35 women were enrolled in the study, resulting in a mean rate of ±0.62 recruits per month. In Phase 2 of the study, ongoing recruitment from traditional sources was supplemented with active recruitment from social media-based sources. During these 6 months implementing recruitment through social media (December 2011-May 2012), 45 women were recruited, for a 12-fold higher rate of ±7.5 recruits per month (p<0.0001) (Figure 13 and 14).

Figure 13. Success of recruitment strategies over time.

Recruitment through traditional healthcare-based advertising constituted the first phase of recruitment from April 2007–November 2011. Starting December 2011–May 2012, new social media based recruitment strategies were applied along with continued use of traditional healthcare-based recruitment. Yearly recruitment in the two phases was compared using the Mann Whitney U test.
Recruitment through traditional healthcare-based advertising constituted the first phase of recruitment from April 2007–November 2011. Starting December 2011–May 2012, new social media based recruitment strategies were applied along with continued use of traditional healthcare-based recruitment. Recruitment rates in the two phases were compared using the Mann Whitney U test.

Despite the fact that traditional healthcare-based recruitment outlets were expanded, social media generated about 78% of the recruitment during this phase. Amongst these sources, local online classifieds such as Kijiji and Craigslist had the greatest contribution of 58% of total recruitment (Table 9).
The ratio of withdrawals adjusted for total recruitment maintained a constant trend in both phases, as it was 0.34 during the first 56 months, and 0.22 during the 6 months of the second phase (Figure 13 and 14).

The women recruited by the two methods were not different significantly by any of the assessed variables including body weight, age, gravidity, race distribution, marital status, level of education and employment (Table 10).
Table 10. Characteristics of women recruited by traditional methods vs. social media.

<table>
<thead>
<tr>
<th></th>
<th>Traditional Healthcare Establishments (n = 35)</th>
<th>Social Media (n = 45)</th>
<th>p</th>
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<tr>
<td>Time spent using method (mos)</td>
<td>56 mos</td>
<td>6 mos</td>
<td>–</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>31.3±3.85</td>
<td>31.7±4.83</td>
<td>0.73</td>
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<tr>
<td>Weight (kg)</td>
<td>68.9±17.7</td>
<td>63.9±11.6</td>
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<tr>
<td>Gravidity</td>
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</tr>
<tr>
<td>South Asian</td>
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<tr>
<td>Other</td>
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<tr>
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<td>Highest Level of Education</td>
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The multiple linear regression confirmed social media as a significant predictor of recruitment rate (p<0.05) with a Pearson correlation of 0.81 and an $R^2$ value of 0.656. The other predictors including age, fertility issues or a history of chronic illnesses did not statistically significantly predict recruitment rate. Regression coefficients and their standard errors are summarized in (Table 11).
As part of the multiple regression, the Durbin-Watson statistic was evaluated, to assess the independence of residuals. We obtained a Durbin-Watson statistic of 2.56, indicating that the possibility of autocorrelation could not be ruled out. For this reason, time-series analysis was conducted.

After log transformation of the interrupted time series model, stationarity was established, and a shifted mean was determined for both phases of the study. No determined trend or seasonality was found, which may have been due to limited time points. Prior to the intervention of social media, the mean was 0.41, whereas after the intervention with social media, this mean shifted statistically significantly to 6.69 (p<0.0001), as obtained through Maximum Likelihood Estimation. The ARIMA model used had d=0, p=1, q=1, and the chi-square test on residuals demonstrated that the residuals were merely random errors (p>0.05) and thus, the model was a good fit. Thus, the predicted model closely fit the observed data (as both are generally within the 95% confidence interval), with an evident increase in the number of recruits observed after the use of social media (Figure 15).

The final model used is as below:

$$y_t = \mu + \omega_0 \text{Shift}_t + \frac{e_t}{(1 - \varphi e B^6)^{\gamma}} , \text{ where } p = 6, q = 0, d = 0,$$

where $\varphi$ is the autoregressive operator.
Figure 15. Interrupted Time Series: recruitment after the intervention of social media.

Monthly recruitment before and after the intervention of social media, as observed and fitted with a logarithmic time-series model, within a 95% confidence interval. A forecast plot is also applied to predict the upward trend in recruitment observed after the intervention with social media.
3.5 DISCUSSION

Given the limited research on effective recruitment strategies in clinical trials concerning special populations, especially women in the periconceptional period and pregnancy [2,5,10], this paper investigated the introduction of online social media as a targeted recruitment strategy to supplement traditional methods of recruitment. This study is the first to assess the efficacy of social media as the primary recruitment tool and its overall success compared to traditional health-care based recruitment amongst women in the periconceptional period.

In our study, net recruitment through health professionals, clinics, and health centers associated with the Motherisk program over the course of 5 years was 0.62 recruits per month. Although recruitment rate from traditional healthcare-based sources was doubled by adding new sources in Phase 2 of the study, this increase did not explain the significant increase in recruitment. Instead, the introduction of social media was strongly associated with a twelve-fold increase in recruitment rate in Phase 2. Analysis of socio-demographic characteristics between the two groups showed no differences, indicating a homogenous study population, thus alleviating concerns about selection biases based on different recruitment sources. Interrupted time-series analysis strongly endorsed the use of social media as the cause of this increase in recruitment, with a statistically significant increase in mean recruitment (p<0.0001) after the introduction of social media.

While recruitment rate is a key variable in determining how many participants will enroll in a study, assessing attrition rate [3-5] is also critical to ensure that maximum participants who enroll are being retained in the study, and there are a sufficient number of participants to eventually meet the sample size and power requirements for the study. We observed that the rate of withdrawals, adjusted for the total rate of recruitment, was constant over time.
While advertising on mass media platforms through radio and television announcements, as well as newspaper and magazine advertisements have been a recruitment approach used by many clinical trials in the past, these are generally passive recruitment strategies [1, 10] that often involve high costs which may not translate to a similar degree of returns.

The difference between these conventional forms of mass media and online social media seems like a subtle one at first, yet has immense implications. While recruits from other forms of mass media represent people who may have landed upon the research opportunity by chance, and then generated interest in it, recruits from social media are primarily people who have been actively seeking information about a related topic, whose pursuit leads them to a particular ad about a research opportunity. This is reinforced by public surveys that indicate that the internet is the first point of reference on the path to seeking health and wellness information for many people [6]. Hence, though the use of social media has traditionally been classified as a low-cost “passive” recruitment tool [9], we believe that the use of social media as recruitment tool should be redefined to “actively” target a specific population to yield highly efficient and cost-effective results.

In our experience, the key challenge faced in the first phase of recruitment was capturing women who were planning or early a pregnancy, yet had not already begun prenatal supplementation. Women being targeted at healthcare establishments seem to be self-motivated women who are already aware of all the right things to do before pregnancy, and thus many potential candidates did not fit our inclusion criteria if they had already begun taking folic acid.

In contrast, since many women use the internet extensively as a health-seeking source to research information in pregnancy before they are trying to conceive or during pregnancy, it is likely that they may have landed upon our study ad during their search, and we were likely able
to capture eligible women on social media platforms *one step earlier* than we would have found them through traditional sources.

In our study, the social media outlets were chosen based on their wide reach and capacity to target a large volume of people every day. While general platforms such as Facebook, Twitter, Kijiji and Craigslist were chosen for their broad scope, advertising on these portals was streamlined to sections where a high traffic of women or families would be expected. This included the “volunteers” or “baby items” sections on online classifieds, and study postings through pages focusing on women’s health and pregnancy on Facebook and Twitter. Institutional and sponsor’s websites, as well as pregnancy forums and message boards, were similarly chosen because of the high traffic of women in the periconceptional period expected on these platforms.

Because of technological improvements and our consequent increased dependence on social media over time, recruitment for the study in 2007 vs. 2012 cannot be compared solely through time. However, as demonstrated by recruitment rates in Phase 2, even when recruitment through traditional sources was expanded and actively engaged, their contribution to recruitment was modest, while social media contributed significantly to the sharp increase in recruitment rates, thus *increasing the overall efficiency of recruitment*. It is also likely that awareness through social media contributed to general interest in the study, including word-of-mouth referrals as seen in other studies [9, 10], and may have influenced some of the recruitment from traditional healthcare-based sources.

Aspects of our study that may have contributed positively to recruitment, and are consistent with the literature on effective recruitment strategies [1] include: the inclusion of monetary incentive upon completion, a relatively low-risk intervention, as well as a potential previous knowledge or recommendation of the study drug. However, specific aspects that strongly
influenced the positive impact that social media had on recruitment include the fact that our study was a health-seeking study targeting a population—likely young to middle-aged women—who are active users of social media platforms and are often using the internet as the first point in seeking this information. The marked impact of social media may not have been as significant if we were targeting an elderly population or patients suffering from certain chronic disease states. Thus, factors such as age, generational demographics and scope of internet access will inevitably define the reach, relevance and efficiency of social media as a recruitment source.

One of the most common apprehensions against using social media may include suspected bias in the population recruited from these platforms. As monetary incentive was advertised within the posting, there may be concerns that the population recruited may be more interested in money-making endeavours and may not be fully committed to the study, since platforms such as Kijiji and Craigslist (which contributed about 58% of recruits in Phase 2) are often used to find economical deals. However, comparisons of socio-demographic characteristics as well as a previous history of medical conditions and fertility issues between recruits from traditional healthcare-based vs. social media-based sources revealed no differences in our study. Due to our limitations of a small sample size, further research is necessary to investigate potential bias in the population recruited from social media-based platforms. Nonetheless, our results suggested that even though compensation may act as an initial attractor to invoke interest in pursuing a study, since participants were only given compensation upon completing the study, their continued participation indicated commitment to research for benefits beyond short-term monetary gain.

While privacy issues and legal concerns were not a challenge that we personally faced, they pose a great obstacle preventing most researchers from adopting social media-based
recruitment approaches [6,9]. Our approach was hence to use social media solely as a recruitment platform. The study material that was posted on social media sites was limited to advertising, and was identical to material that was disseminated through posters, brochures or postings using traditional healthcare-based recruitment sources. User comments and questions were not engaged with on public fora, but were instead redirected by asking them to contact the study coordinator via telephone or email, to maintain confidential interaction as per the study protocol.

In general, some of the limitations of our study included its small sample size and the small time course over which social media was applied. These were limited within our study because of the small sample size requirements of the clinic trial this is associated with. Yet this sample size was sufficient to demonstrate significant changes due to a large effect size. Further larger-scale research is warranted to explore the applied use of social media as part of larger clinical trials. Also, since the analysis on recruitment within this study was composed of secondary post-hoc analysis, further research is necessary to understand the broader applications of social-media based recruitment in clinical trials.

A white paper by Oglivy Washington and The Center for Social Impact Communication at Georgetown University outlines tenets on the use of social media for public health marketing [7]. Based on our experience in the current study, along with some suggestions modified from the paper above, we propose the following guidelines for using online social media as a primary cost-effective and efficient recruitment tool in clinical trials:

1) **Understanding the target population**

The primary step in the process of transforming the use of social media as a recruitment tool from “passive” to “active” is to thoroughly research the target population. Participants should not
be treated merely as ‘the general population’. It is crucial to identify the defining characteristics and social networks of the population being targeted if it represents a special population (i.e. pregnant women vs. women battling cancer vs. elderly). This will not only help in creating population-specific platforms and goals, but also present early insight into the potential efficacy of recruitment through social media as an approach based on the internet use and medical-information-intensive use of a particular demographic. Even if the study merely aims to recruit “healthy volunteers” from the “general population”, the overall population should be segmented to different demographics, and each should be targeted individually for optimal results. In the age of internet access and the reliance of the population on the internet for medical information, it is crucial that we use online tools of social media to personally target and empower the participant, and move away from mere business and marketing approaches to recruitment where ads are created as ‘one-size-fits-all’ aimed to capture a random portion of the ‘general population’.

To truly recruit individuals who are interested in research for its own sake and the potential benefits it represents to them as well as the society at large, it is critical to approach participants with intentions of honest medical dialogue, equipped to address more complex questions given the plethora of information they are exposed to the internet.

Online social media, if used correctly, epitomizes community and conversation, and is a definite movement away from fears of coercion that may arise with the use of high incentives, direct interaction with the primary caregiver, as well as some other means of recruitment.

2) **Using a combination of passive, broad-spectrum, as well as targeted active recruitment techniques**
Any holistic recruitment strategy should incorporate a combination of active and passive recruitment techniques to maximize chances of success. Traditionally, active techniques have yielded greater participants, yet are also associated with a greater cost and time involvement [9, 10]. Passive techniques may be less efficient yet hold the potential for exposure to a larger population.

Social media incorporates both these characteristics when used in an applied fashion, and thus, may greatly enhance recruitment if used to supplement other recruitment approaches. Posts on social media platforms, depending on how intensively they are updated and the type of platform being used, hold hybrid characteristics of active and passive recruitment methods. In a space where one is targeting a specific demographic, they represent the active attempt to engage that specific subpopulation. However, on larger social networking platforms, they hold great power of dissemination given the large degree of exposure.

3) Monitoring response rates and revising methods based on feedback

The organic and live nature of social media necessitates continual response-monitoring and the revision of strategies based on response. Since any updates on postings or edits literally appear real-time, it is important to evaluate the impact a particular recruitment strategy has on achieving target goals. Oftentimes, participants contact the study coordinator stating their confusion or reluctance with an aspect of the study based on the ad. All feedback should be recorded and appropriately incorporated to keep recruitment postings clear and up-to-date.

It is critical to keep regularly updating ads, ensuring that they are always around the top and have the chance to be most frequently read. If there is an obvious lag in response, potential limitations of the approach should be assessed and revised.
4) Addressing apprehensions, maintaining transparency, and transitioning the participant encounter to a regulated environment

With the breadth of medical information being available on the internet, there is also a breadth of incorrect information. Accordingly, some people may be suspicious of the credibility of a clinical trial attempting to recruit through online platforms that aren’t necessarily regulated by healthcare centers. To address this apprehension, it is crucial to state affiliations to the research institution [6], including contact information, so that contact is not only easy to establish but also secure for the participant.

While social media may serve as a prolific window to recruiting a participant, explanation of the study protocol and the process of informed consent should be executed at the research institution in a secure and regulated setting as per study protocol.

If the study involves online questionnaires or online completion of forms, secure databases should be used to maintain patient confidentiality and ensure security of patient information.

In conclusion, given that the recruitment through traditional medical establishments was intensively employed in both phases, and that the surge in recruitment rate was attributed mainly to social media-based recruitment methods, we can conclude that supplementing with social media-based recruitment strategies increase the efficiency of the recruitment process. Despite potential apprehensions, social media holds great promise as a recruitment tool if applied in a targeted and regulated fashion. Though the magnitude of its impact on recruitment may vary based on the sample size required, the study design, and the nature of its intervention, social media can be effectively incorporated in the recruitment efforts of any study to enhance current recruitment methods.
3.6 REFERENCES


CHAPTER 4.

Association between folate status and use of oral contraceptives: a systematic review and meta-analysis

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This study has been submitted to *Pediatrics* for publication.

**MS** was responsible was part of the conception and design of the study, helped with the systematic search and further selection of articles at each stage, was the primary reviewer at all stages, responsible for overall data collection, extraction and analysis, as well as writing and submission of the manuscript.
4.1 ABSTRACT

Objective: (i) To systematically review and meta-analyze the effect of oral contraceptive use on plasma and RBC folate concentrations.

(ii) To systematically review the efficacy of the folate-fortified oral contraceptive in increasing blood folate concentrations to optimally protective levels against neural tube defects (<906nmol/L)

Methods: MEDLINE, EMBASE, Web of Science and the Cochrane library were searched from inception to June 2013 for human studies evaluating oral contraceptive use and folate status. Case-control, cohort studies and clinical trials were included. Outcomes were meta-analyzed using a random-effects model.

Results: Among the 2831 women in 17 studies included in the analysis whose plasma folate concentrations were available, there was a significant folate-lowering effect of oral contraceptives observed -1.27 (95% CI -1.85, -0.69, p < 0.0001). Similarly, analyzing 1389 women in 12 studies whose RBC folate concentrations were available, significantly lower folate status was observed among oral contraceptive users -59.32 (95% CI -58.03, -23.04; , p < 0.001). Overall, efficacy and bioequivalence data for the folate-fortified oral contraceptive (Beyaz) show that it is at least as effective as folic acid in raising blood folate concentrations, and that the concomitant administration of the folic acid with the oral contraceptive component does not affect its absorption or kinetics.

Conclusions: Given the decrease in blood folate concentrations associated with the use of oral contraceptives, it is pivotal that women of childbearing age continue folate supplementation during oral contraceptive therapy. The folate-fortified oral contraceptive offers one such option
to women, especially those who may be planning a family after the cessation of oral contraceptive therapy.
4.2 INTRODUCTION

The use of oral contraceptives in women of childbearing age has been suggested to be associated with compromised folate status, yet the clinical relevance of this has been controversial. While Shojania and colleagues were the first to report lower serum folate levels in women using oral contraceptives when compared to controls [1], many other groups have been unable to replicate this finding [2-4]. After initial reports, later papers hypothesize that oral contraceptives may affect folate metabolism in a mild way that is not clinically significant [5].

Current data suggest that 45.7% women of childbearing age become pregnant within 3 months of stopping their oral contraceptive [6], and 28.1% take folic acid prior to conception [7]. Folic acid supplementation is associated with a reduction in the risk of neural tube defects that occur between 4 weeks post-conception when the neural crest fails to fuse, at which point, many women may not even know that they are pregnant [8]. Hence, it is critical that women start supplementing with folic acid in the periconceptional period, at least 3 months before becoming pregnant [9].

Beyaz® (ethinyl estradiol 0.02mg/ drospirenone 3mg/ levomefolate calcium 0.451mg), a novel folate-fortified oral contraceptive, has been approved by the FDA in September 2010 for the purpose of raising folate levels in women who are using it as a method of contraception [10, 11].

This systematic review has two objectives. The first is to systematically review and meta-analyze the available clinical literature on oral contraceptive use and its potential effect on plasma and RBC folate concentrations. The second objective is to systematically review the data
available on the novel folate-fortified oral contraceptive (Beyaz), in order to evaluate its potential as an alternative to current folic acid supplementation therapy.
4.3 METHODS

4.3.1 Literature search

The databases MEDLINE (indexed since 1946 + in process/non-indexed), EMBASE (since 1947), Web of Science and the Cochrane Central Register of Controlled Trials, were searched from their inception to June 2013. A health librarian was consulted to develop a comprehensive search strategy using the search terms: *folic acid, folate and derivatives, pregnancy, periconceptional period, oral contraceptives*, and related terms using the exploded versions of subject headings and their associated keywords. No restrictions were placed on the language of the articles, the type of publication or study design, or the study model used (in vitro, animal, clinical).

4.3.2 Inclusion and exclusion criteria

Studies were initially screened through title and abstract for relevance, and included all studies with the combination of the terms “oral contraceptives”, “folate”, “pregnancy” and their associated derivatives, independently by two reviewers. Screening at the second stage required a review of the methods, and case reports, editorials, letters to the editor, and reviews were excluded, independently as well by the two reviewers. Only clinical studies, both observational and interventional studies, were included if they evaluated folate status in a population of women using oral contraceptives vs. those who did not take the pill. Articles were excluded if the potential changes in folate status due to oral contraceptives were not discernable through a directly measured outcome but were based on statistical modelling or secondary data. Conference abstracts showing preliminary results, which were part of a larger published study
were excluded at this stage to avoid double-counting of data. All peer-reviewed publications that met the inclusion criteria, whether an article or a conference abstract, were included.

4.3.3 Data extraction and reporting

Standardized Cochrane data extraction forms were used to collect study details and data from each included article, and were completed independently by two reviewers. No language restrictions were placed during the search. Extracted data included information on study design, setting, sample size, participant characteristics, type of oral contraceptive used, timing and measurement of exposure, blood folate concentrations and methods used to analyze them, a standardized study quality/bias assessment tool, and a summary of key results. PRISMA guidelines were followed throughout all steps within the systematic review and meta-analysis.

4.3.4 Meta-analysis

Summary estimates for mean difference between RBC folate or plasma folate concentrations among those receiving oral contraceptives or not were calculated using the inverse-variance method as part of a random-effects model. Forest plots were used as a visual representation of individual and summary estimates.

For both plasma folate and RBC folate concentration comparisons, the Inverse Variance method was used to calculate mean difference, as part of a random-effects model. Since all of the measured outcomes used the same units (ng/mL), mean difference was utilized as the best measure of continuous data in this case.

The Cochrane Q test and the $I^2$ statistic were used as a measure of between-study heterogeneity. Visual inspection of funnel plots was also used for pooled comparisons to
evaluate the potential for publication bias and heterogeneity, where applicable. All meta-analysis calculations were done using Review Manager (version 5.2; Cochrane Information Management System).
4.4 RESULTS

The pooled searches from all databases resulted in 23,340 citations being extracted. After the removal of 9,897 duplicates, 68 articles were included based on selection for relevance after a review of the title and the abstract as necessary. Amongst these, 33 articles met the final inclusion criteria after a review of the title, abstract and methods. A flowchart of the systematic search strategy and the study selection process is outlined in Figure 16.

Figure 16. Systematic search strategy and study selection process.

Overall, 27 studies were included in the Category 1 of the systematic review assessing blood folate concentrations with oral contraceptive use, and 5 studies were included in Category 2 which evaluated the efficacy of the folate-fortified contraceptive as a source of folate. Since both observational and interventional studies were included, both categories were subdivided based on study design and type of clinical data presented.
4.4.1 Category 1: The effect of oral contraceptive use on blood folate concentrations

27 studies were included in this category, with their key findings summarized in Table 12.

4.4.1.1 Observational cohort studies

Kahn et al. (1970) evaluated blood folate levels in pregnant women of lower and upper socioeconomic status, as well as women taking oral contraceptives from a lower socioeconomic class. Since the evaluation of folate status in oral-contraceptive users was only one of the many objectives of the study, the authors recruited 5 controls and 14 combination-type oral contraceptive users, who were either using Ortho-Novum or Ovral. None of these women were using any other medications or multivitamin supplements, and no dietary information was obtained from patients. Overall, the authors found no significant differences between the two groups, with mean serum folate concentrations of 7.14 ±2.51ng/mL amongst oral contraceptive users, compared to 6.92 ±2.96ng/mL amongst controls. The authors also found that oral contraceptive users had better folate stores than their pregnant counterparts, when recruited from the same site. Serum folate levels were measured using a modified method of the L. casei microbiological assay. Kahn and colleagues also measure tissue stores of folate using a visual inspection method – average lobe score (ALS) of circulating granulocytes, but the differences between the group were not reported [3].

Pritchard et al. (1971), amongst their broader report from the Transactions of the 26th annual meeting of the Society of Obstetricians and Gynecologists of Canada study the effects of oral contraceptives on folate metabolism. They report the plasma folate concentrations of 57 women taking oral contraceptives, including Ovulen, Ortho Novum, Norinyl, Norlestrin, Enovid, Ovral, Oracon and others, vs. 55 controls. The oral contraceptive users had been on oral
contraceptive therapy for at least 9 months. The authors found no significant differences between the plasma folate concentrations of the two groups, and that the levels in pregnant women were lower than those found in women taking oral contraceptives [4].

Shojania et al. (1971) investigated role of oral contraceptives on folate metabolism by evaluating RBC folate, serum folate and urinary formiminoglutamic acid (FIGLU) excretion, as well as blood measures, within a group of 176 women on combination oral contraceptives vs. 140 healthy controls. Dietary folate intake and the use of supplements was evaluated through an interview with participants at baseline, and controls included women who had not taken oral contraceptives for at least a year. No exclusions in either group were made based on diet, and the women were recruited from either private clinics or hospitals. Serum and RBC folate concentrations were measured using the L.casei microbiological assay, and urinary FIGLU excretion was evaluated using and enzymatic method. The authors found that the serum folate concentration of women on the oral contraceptives (mean ±SD was 4.2 ±1.8 ng/mL) was significantly lower (p < 0.001) than controls (mean 6.2 ±2.5 ng/mL). The authors also found a duration of use effect, as they showed that the women who had been on oral contraceptives for >2 years (3.9 ±1.6 ng/mL) had significantly lower mean serum folate than women who had been taking them for less <1 year (5.0 ±2.0 ng/mL), or compared to controls (6.1 ±2.2 ng/mL). The differences observed in serum folate carried over to RBC folate as the authors observed RBC folate concentrations of 130 ±62 ng/mL for 95 women in the oral contraceptive group and 175 ±69 ng/mL for 63 women in the control group (p < 0.001). The authors also found that the urinary 12h FIGLU excretion was significantly higher (p < 0.02) in the oral contraceptive group of 58 women (91 ±84μM) than amongst the 35 controls (51 ±45μM). The authors interestingly found that women who were taking oral contraceptives containing mestranol 0.05mg +
norethindrone 1mg (Ortho-Novum I and Norinyl I) trended towards higher serum folate concentrations than oral contraceptives with mestranol 0.10mg + norethindrone 2mg (Norinyl 2), but the difference was not significantly different, suggesting a dose-effect requiring further study. This group also studied the changes in folate measures after the discontinuation of oral contraceptives, and found that serum folate rises within 3 months in most women who stop taking the pill, with the greatest difference in women who had low serum folate status prior to stopping use of oral contraceptives. Similar trends were also observed for RBC folate concentrations and urinary FIGLU excretion [12].

Stephens et al. (1972) conducted a multi-part investigation to evaluate the general differences in serum folate levels between 43 oral contraceptive users vs. 23 controls, as well as an absorption study to measure the differences in polyglutamate and monoglutamate absorption between the two groups, a study to assess the differences in serum folate concentrations at different stages of the menstrual cycle, and finally, an in-vitro investigation on the mechanism by which oral contraceptives may inhibit intestinal folate absorption. The authors recruited healthy women who had been using oral contraceptives for at least 3 months prior to the study for their observational comparison of serum folate concentrations between OC users and controls, and assayed blood folate concentrations using the L. casei microbiological assay. Overall, they found no significant differences between the two groups (mean 6.36 ±2.43 ng/mL vs. 5.94 ±2.42 ng/mL, p < 0.1), even after measuring concentrations on two different days of each menstrual cycle (day 5 and day 20), except for users of Gynovlar (p < 0.001, serum folate concentration was higher on day 20 than on day 5). They were also unable to find any phasic differences in the serum folate concentrations of controls, when measured during different hormonal phases of the menstrual cycle (day 5 and 20). Finally, the absorption studies testing for pteroylpolyglutamate
absorption, showed that the mean rise in serum folate was significantly lower among OC users vs. non-users, when subjects were not pre-treated with folic acid (p < 0.02). However, this difference failed to reach statistical significance once subjected were pre-treated with folic acid (0.05 < p < 0.10). The in-vitro tests also failed to reveal a mechanism of impaired polyglutamate absorption [13].

Gaafar et al. (1973) conducted an observational study with 40 oral contraceptive users, and 28 matched controls who had never used oral contraceptives in Egypt. Both groups included healthy women from an average Egyptian socio-economic class. Oral contraceptive users used Anovular 21 (norethisterone acetate 4mg+ ethinyl estradiol 0.05mg) long-term for 2-4 years. Folate concentrations in the study were assessed using the L. casei microbiological assay. The researchers found significant differences between the serum folate concentrations among oral contraceptive users vs. controls (mean 8.5 ±2.48 ng/mL vs. 14.8 ±4.47 ng/mL, p < 0.01). They found a similar significant difference in the RBC folate concentrations among users vs. non-users (mean 202 ±17.69 ng/mL vs. 326 ±23.59 ng/mL, p < 0.01). The authors also measured FIGLU urinary excretion for 8 hours following an L-histidine loading dose, and found that FIGLU excretion was significantly increased in oral contraceptive users in comparison to non-users (mean 12.7 ±3.40 µg/8h vs. 7.1 ±3.66 µg/8h, p < 0.01) [14].

Ahmed et al. (1975) conducted a cross-sectional observational study in 18 OC-users and 43 controls to compare RBC folate concentrations with the use of Ovulen-50 or Ovral for 6-12 months, among a similar group of women. They also conducted an intra-individual before-after study where they analyzed 23 women’s RBC folate status at baseline and then at different time points within the first 6 months of oral contraceptive therapy. Within the cross-sectional study, they found RBC folate concentrations approaching a significant different within oral
contraceptive users vs. non-users (mean 118.72 ±5.95 ng/mL vs. 135.99 ±5.46ng/mL, p < 0.1).

In contrast, in the before-after study, authors found significant differences in the between month F-value over 6 months of treatment with oral contraceptives (p < 0.001) [15].

Paine et al. (1975) conducted an observational cohort study to measure the folate status of 279 women using oral contraceptives, compared to 247 controls, all of whom were recruited from a family-planning clinic, and were all of similar age and socioeconomic status. Folate status was assessed using both the L. casei microbiological assay and a radioassay. The authors report that 90% of women in the oral contraceptive group were taking a combination-type pill containing norethindrone 1mg + mestranol 0.08mg, and the mean duration of use in this cohort was 27 months (R: 3-96 months). The authors found that serum folate concentrations were not significantly different amongst oral contraceptive users vs. controls when evaluated by the microbiological assay (mean 6.39 ng/mL vs. 6.91 ng/mL, p > 0.1), as well as by the radioassay (mean 3.78 ng/mL vs. 4.00, p > 0.2). They also found no correlation between serum folate concentrations and the duration of oral contraceptive use, evaluated in women who were using it for up to 59 months [16].

Smith et al. (1975) conducted an observational cross-sectional study to evaluate the impact of oral contraceptive use on a range of nutrient indices, including serum and RBC folate. They recruited women from the Louisiana Family Planning Program who had been using oral contraceptives for at least 2 years, and recruited controls from the same program as women who were using alternate forms of contraception for at least 2 years. Women in both groups were matched for age, parity and percent of ideal body weight. The majority of the women were from a similar socioeconomic status and were black. Oral contraceptive users used Norinyl 1-80 (nortethindrone 1mg + mestranol 80µg). Overall, the authors found significantly lower serum
folate (mean 4.5 ±2.0 ng/mL vs. 5.4 ±2.4 ng/mL, p < 0.02) and RBC folate (mean 172.4 ±56.9 ng/mL vs. 199.4 ±62.3 ng/mL, p < 0.02) concentrations among oral contraceptive users vs. non-users respectively [17].

Prasad et al. (1976) conducted an observational study in a factorial arrangement based on socioeconomic status and different hormonal statutes, to assess the role of oral contraceptive use on vitamin status, including folate status in the USA. They divided their participants based on type of oral contraceptive use (Norinyl – norethindrone 1mg + mestranol 50µg vs. Ovral – norgestrel 0.5mg + ethinyl estradiol 5µg), socioeconomic class (high vs. low), and use of oral contraceptives by women 5 weeks after pregnancy during lactation. There were 130 controls, 140 women using Norinyl and 167 women using Ovral, and finally, 202 women who resumed taking oral contraceptives 5 weeks post-partum. Overall, there was a decrease in serum and RBC folate concentrations of the group with higher socioeconomic status as a result of oral contraceptive intake, and the effects were primarily seen with Ovral use when compared to controls. There was also a significant supplementation effect observed in both groups [18].

Areekul et al. (1977) conducted an observational cross-sectional study among 20 oral contraceptive users and 50 controls in Thailand measuring for a variety of hematological indices including serum folate, RBC folate, and serum folic acid binding protein (FABP) concentrations. Oral contraceptive users had been using them for at least 1 year, whereas non-users had not being using oral contraceptives for at least 1 year. Blood folate concentrations were determined using the microbiological assay, and authors found no significant differences in the mean serum folate concentrations of OC users vs. non-users (mean 7.7 ±2.6ng/mL vs. 8.8 ±3.8ng/mL). In contrast, they found significant differences in RBC folate concentrations (mean 625 ±313ng/mL
vs. 798 ±216ng/mL, p < 0.05), as well as serum FABP concentrations (mean 44.6 ±18.4pg/mL vs. 21.3 ±12.2pg/mL, p < 0.05), between users vs. non-users respectively [19].

Martinez et al. (1977) also conducted an observational study to assess the effects of oral contraceptive use on folate status in pregnant women who had discontinued them within 6 months of conception within the USA. The cohort was subdivided into the summer group (recruited between May-July) and the winter group (recruited between October-January). The experimental summer group consisted of 10 women and the experimental winter group included 19 women, and 31 summer controls with 23 winter controls. The authors observed a seasonal variation in RBC folate values (lower in winter months), but no such variation was observed for plasma folate. Dietary information was also recorded using a 24-hour recall, using estimations of frequency of intake and serving size. Blood samples were obtained at the beginning of pregnancy, in the second and third trimester, and folate status was evaluated using a microbiological assay. The authors found that mean plasma folate concentration was significantly lower in oral contraceptive users vs. non-users (4.3 ±0.5 ng/mL vs. 6.3 ±0.6 ng/mL, p < 0.005). Similarly, RBC folate was significantly lower in oral contraceptive users vs. controls (174.6 ±15.2 ng/mL vs. 232.7 ±14.3 ng/mL, p < 0.001). No difference in frequency of intake were observed between oral contraceptive users vs. controls. 58 women were followed throughout pregnancy, and those who used high-folate supplements were excluded. Amongst this subgroup, it was found that oral contraceptive users had RBC folate and plasma folate values that were lower compared to their respective controls, but these trends were not statistically significant [20].

Pietarinen et al. (1977) conducted an observational study comparing the serum and RBC folate concentrations among 22 women using oral contraceptives for ≥4 months, and 18 controls
who had not used oral contraceptives for at least 6 months prior to the study. Blood folate concentrations were evaluated both on day 5 and day 20 of the menstrual cycle. All subjects were healthy and had previously not been taking folic acid supplements, and dietary folate contribution among participants was evaluated through detailed 3-day dietary records prior to the assessment of each blood sample. Serum folate concentrations were measured using the L.casei microbiological assay, and oral contraceptive users used one of the following oral contraceptives: Ortho-Novum 1/50, Ortho-Novum 1/80, Ortho-Novum 2mg, Ovral, Norlestrin, Demulen. In terms of serum folate, the authors found overall significant differences between oral contraceptive users vs. non-users (mean 5.63 ±2.20 ng/mL vs. 7.84 ±4.04ng/mL, p < 0.05), with statistical significance achieved between the assessments on day 5 (p < 0.05) but not on day 20 (p > 0.05). In contrast, RBC folate concentrations were not significantly different between the two groups, whether measured on day 5 or day 20 (p > 0.05). Dietary folate intake was not significantly different between OC-users vs. non-users, nor between either phase of the menstrual cycle [21].

Hettirarachchy et al. (1983) compared the serum folate levels of healthy non-pregnant women of childbearing age, with levels of women on Ovulen 50, a combination oral contraceptive containing ethinyl estradiol 0.05mg + ethinodiol diacetate 1mg, with levels among pregnant women in Sri Lanka. To evaluate the effect of oral contraceptive therapy on serum folate levels, the authors conducted studies: an observational cross-sectional study where they measured the serum folate concentrations of 93 women who had been on oral contraceptive therapy (Ovulen 50) for a period of 3-18 months, and an interventional longitudinal study where they selected 46 healthy women for oral contraceptive therapy (Ovulen 50), and measured blood folate concentrations during months 3, 6, 12, and 18 of oral contraceptive therapy. Serum folate
concentrations within this study were measured using the radioisotopic protein-binding method. Serum folate concentrations in oral contraceptive users were subdivided based on income level and age, and serum folate concentrations women on OC therapy (mean 3.04ng/mL ±1.78ng/mL) was found to be significantly lower (p<0.001) than the concentrations in controls (mean 4.70ng/mL ±1.36ng/mL), with a significant income effect as 64% of subjects of the lowest income class had folate levels of less than 3.0ng/mL (folate deficiency). Results of the longitudinal study showed that the serum folate levels of oral contraceptive users were lower than folate levels during pregnancy, regardless of income level. From a pharmacokinetic perspective, the longitudinal study also showed that folate levels in the OC-treated group stabilized in the 12th month of treatment after progressively decreasing [22].

In an observational cohort study by Brattstrom et al. (1992), which aimed to study the mechanism of vascular disease in women as mediated by steroid sex hormones (oral contraceptive use), and their effect in men as a treatment for prostatic carcinoma. Amongst comparisons of homocysteine concentrations and other blood indices, RBC folate concentrations were also compared in 17 oral contraceptive users, who had been using them for at least 2 years, and 13 controls, who had been non-users for at least 2 years. OC-users used triphasic oral contraceptives either containing EE 30-40µg+levonorgestrel 50-125µg or EE 30µg+levonorgestrel 150µg. The authors found a trend towards decreasing blood folate concentrations among oral contraceptive users (mean 117.83 ±28.24ng/mL) vs. respective controls (mean 150.04 ±63.99ng/mL), but this trend was not statistically significant [23].

Lussana et al. (2003) conducted an observational cross-sectional study aiming to measure the differences in several hematological indices, especially serum folate, between 60 regular oral contraceptive users vs. 159 controls who had not used oral contraceptives for at least 12 months.
The authors recruited healthy women, and described no significant differences in the dietary habits of participants and measured serum folate concentrations using a radioimmunoassay. None of the participants used any multivitamin supplements for at least 12 months. The authors divided oral contraceptive use based on the generations of combination-type oral contraceptives: 1st generation, 2nd generation and 3rd generation. They found no significant differences between the serum folate concentrations of oral contraceptive users when compared to controls (p=0.51) [24].

McArthur et al. (2013) conducted a longitudinal study that derived its participants from another randomized controlled trial. The aim of this study was to investigate biological variability between the blood concentrations of several B-vitamins, as well as the effect of oral contraceptives on these parameters, including serum and RBC folate concentrations. 9 oral contraceptive users and 13 controls were involved in the comparisons over the course of 12 weeks. Women were excluded if they were formerly using multivitamin supplements, and a validated food frequency questionnaire was used to evaluate the dietary intake of folate among the participants. Fasting blood folate concentrations were measured using an automated immunoassay, and results revealed no significant differences between in the serum and RBC folate concentrations of OC users vs. non-users, over the course of 12 weeks [25].

Sutterlin et al. (2013) conducted a case-control study among 71 oral contraceptive users using a combination-type OC containing 20µg of EE (Eve 20, Leios, Lovelle or Miranova) for at least 3 months, and 170 controls who had not been using OCs for at least 3 months. None of the women recruited in the study were using any vitamin supplements, and their serum folate concentrations were measured using an ion-capture assay. Overall, the authors found no significant differences between the serum folate concentrations among OC users vs. controls.
(mean 9.9 ±2.7 ng/mL vs. 9.8 ±3.8ng/mL, p = 0.72). The authors also did not find any significant differences in the percentages of reduced, normal or elevated folate levels between the two groups [26].

### 4.4.1.2 Pre-post study

Castren et al. (1970) conducted an intra-individual, before-after, observational study measuring the folate status of 30 women pre-oral contraceptive therapy and post-oral contraceptive therapy after 3 months of use. The oral contraceptives used included Primovlar, Delpregnin and Lyndiol 2.5. The authors found no significant differences in the serum folate concentrations of participants after 3 months of oral contraceptive use (mean 6.0 ±2.4ng/mL), when compared to baseline (mean 5.5 ±2.2ng/mL), as measured by the microbiological assay. The authors also followed-up 12 women for long-term use of oral contraceptive use, and after a mean of 7.1 cycles, they found no significant differences between serum folate concentrations in comparison to baseline despite the longer duration of use (mean 6.4 ±1.9ng/mL) [27].

Ahmed et al. (1975) also had a pre-post sub-study, but the results from the paper are reported together under “observational studies” [15].

### 4.4.1.3 Interventional studies

McLean et al. (1969) investigated the serum folate concentrations and incidence of folate deficiency with the use of oral contraceptives among 56 women from a rural population in the USA, with similar socioeconomic background and dietary habits. Within this interventional study, 19 women were administered Ortho-Novum I, 20 women were administered Ovulen, and 17 women were not given any medication (controls) for at least 3 months (mean = 18 months). Serum folate concentrations were assessed through the L. casei microbiological assay. The
authors found no significant differences in serum folate concentrations between OC users vs. non-users. The authors also did not find any significant correlation between length of OC therapy and serum folate concentrations amongst participants [2].

Joshi et al. (1986) conducted a study as part of the World Health Organization Special Programme, on the vitamin status of women taking oral contraceptives amongst malnourished women in urban centers in India (Hyderabad and Bombay) and a rural center in Thailand (Chiang Mai). In this interventional study, women were randomized to either Pill A- containing ethinyl estradiol 30µg + levonorgestrel 150µg or Pill B- containing ethinyl estradiol 50µg + levonorgestrel 150µg, or an injectable progesterone-only formulation (DMPA). Blood folate concentrations were evaluated using the microbiological assay. Folate concentrations of middle-class controls were compared with oral contraceptive users and lactating oral contraceptive users at each center. In Chiang Mai, the RBC folate concentrations increased amongst Pill A, B and DMPA users, with statistically significant increases amongst the Pill B and DMPA groups. Similar results were observed for plasma folate concentrations, as they significantly increased in all groups. In Hyderabad, RBC folate concentrations increased within the Pill A group but not in the Pill B group [28].

A preliminary interventional study conducted by Steegers-Theunissen et al. (1992) suggested the importance of time point within the menstrual cycle during which these indices are assessed. In a group of 11 women taking Marvelon and 15 controls, Steegers-Theunissen and colleagues showed no differences in the RBC and serum folate concentrations measured at day 3 (low-hormonal phase) vs. day 14 or 21 (high-hormonal phase) of the two groups. However, the homocysteine levels of OC users were significantly higher (p<0.01) in the oral contraceptive group vs. non-users when measured during the low-hormonal phase (day 3) [29].
4.4.1.4 Single-dose interventional study

Streiff et al. (1970) published an absorption study evaluating folate kinetics after the administration of a single dose of polyglutamate or monoglutamate, to evaluate whether oral contraceptive use affects folate absorption. 9 oral contraceptive users and 9 controls were included in this study, and were administered 200µg polyglutamic acid or monoglutamic acid (folic acid), with mean rise of serum folate measured 2.5 hours after oral administration of the test dose. Folate concentrations were measured using the L. casei microbiological assay. Overall, the absorption of monoglutamic folate was not affected by oral contraceptives, as there were no differences seen in the mean rise of serum folate between the two groups after the administration of a single-dose of monoglutamtic acid. In contrast, polyglutamate absorption was decreased by about 50% in oral contraceptive users vs. non-users, as determined by the mean rise in serum folate concentrations [30].

Results from the absorption study by Stephens et al. (1972) have been discussed in the “observational studies” section, alongside their other experiments [13].

Continuing on in an attempt to investigate the mechanism by which oral contraceptives affect folate status, Shojania et al. (1973) conducted a study to evaluate the effect of oral contraceptives on folate absorption based on the comparison of means of serum folate rise and area under the curve (AUC) of 22 women on oral contraceptives and 16 controls. First, they evaluated monoglutamate folate absorption between 14 women on oral contraceptives and 19 controls, by administering 5mg folic acid, three times a day for four days, and the folate absorption test was performed three days after the last dose of folic acid. Blood samples were obtained just prior to the test dose and then at 1, 2 and 3 hours after the test dose. The test dose consisted of equimolar amounts (4µg/kg) of monoglutamic folic acid or polyglutamate. Overall,
the authors found no significant difference in the maximum rise of serum folate (mean 10.3 ±3.5 ng/mL vs. 8.8 ±2.1 ng/mL) or the AUC (mean 21.8 ±8.0 cm² vs. 18.6 ±4.4 cm²) of women on oral contraceptives vs. controls, when testing for monoglutamate folate absorption.

Polyglutamate folate absorption was tested in 22 women on oral contraceptives and 16 controls. Overall, there was a significant difference between the mean maximum rise of serum folate in women on oral contraceptives vs. controls (6.9 ±3.0 ng/mL vs. 8.7 ±2.4 ng/mL, p < 0.05), as well as in the mean AUC between the two groups (mean 10.7 ±5.0 cm² vs. 15.2 ±3.9 cm², p < 0.01). However, once these values were adjusted for differences in baseline, there was no significant difference found between both groups. Thus, the authors conclude that folate malabsorption is an unlikely explanation for the impaired folate metabolism seen in oral contraceptive users [31].

Finally, Shojania and colleagues (1975) conducted a study to evaluate the changes in plasma clearance and urinary folate excretion of women on oral contraceptives. Plasma clearance of folic acid was evaluated in a group of 6 women on oral contraceptives and 7 controls. The oral contraceptives being used included Ortho-Novum II (norethindrone 2mg + mestranol 0.10mg) used by 4 subjects, C-Quens (chlormadinone acetate 2mg + mestranol 0.08mg) used by 1 subject, and Secrovin (estradiol 0.1mg + dimethisterone 25mg) used by one subject. The mean rise of serum folates at 5, 15, 30 and 60 min after the 15µg folic acid injection to the oral contraceptives were 77.3, 36.6, 21.2 and 10.5 ng/mL, which were significantly different than the respective levels of controls: 122.7, 64.2, 36.6, 23.5 ng/mL (p < 0.02), even after adjusting for a baseline difference in serum folate. The mean rise of serum folate at 5 min after the folic acid injection was significantly lower in the oral contraceptive group (p < 0.01), but the rate of fall of serum folate beyond that was comparable in both groups. When studying urinary folate excretion in 93 women on oral contraceptives, and 67 controls, researchers found that for any level of
serum folate, the women on oral contraceptives excreted significantly more folates in urine than
the controls (p < 0.02). A similar difference existed when participants were adjusted for RBC
folate concentrations [32].

Steegers-Theunissen et al. (1993) assessed the folate kinetics in 29 oral contraceptive
users (Marvelon) vs. 13 controls through an observational study. None of the women in the study
had previously been using folic acid supplements for at least three months. The participants in
the oral contraceptive group had been using Marvelon for a mean duration of 4.6 years (R: 1-8y),
whereas the controls had never used any oral contraceptives. To evaluate serum folate
pharmacokinetics, subjects took a 5mg dose of folic acid, and blood samples were obtained at
different time points following the single dose ingestion of folic acid. Serum folate
concentrations were evaluated using a radioassay, and the authors found differences in the
median serum folate concentration vs. time curves in the OC-group vs. controls, as it was lower
(p < 0.09). The authors found a statistically significant difference between the profiles of the two
groups at t=210 min (p = 0.04) [5].

4.4.1.5 Interventional study + supplement use

Bamji et al. (1985) conducted an interventional randomized longitudinal study in
Hyderabad to compare the impact of continuous and intermittent vitamin supplementation
schedules amongst oral contraceptive users on RBC folate status. The three study groups
included: 1. OC + placebo (non-supplemented), 2. OC + vitamins (2 pills/day on days 22-28;
intermittent), and 3. OC + vitamins (1 pill daily; continuous), as well as non-OC users as
controls. The supplements included 300µg folic acid and other multivitamins. RBC folate
concentrations were evaluated at baseline, and then 3 months and 6 months after the randomized
intervention. After 3 months of treatment, the authors found significant improvement in the OC+intermittent supplementation and the OC+continuous supplementation group (p<0.05), but not in the non-supplemented group. 6 months after treatment, only the OC+continuous supplementation group was successful in significantly increasing RBC folate concentration (p<0.05) [33].

Mooij et al. (1991) also conducted an interventional study to compare the effects of multivitamin supplementation on vitamin levels among oral contraceptive users vs. controls. Baseline samples were retrieved from 28 oral contraceptive users on a monophasic, biphasic or multiphasic OC containing estrogen 30µg and 31 controls on days 3 and 23 of their menstrual cycles, before beginning supplementation. The supplement given to both groups was Gravitamon, which contains 5 mg folic acid as well as other multivitamins and minerals, and blood folate concentrations were measured for 4 menstrual cycles using a radioassay. There were no significant differences between the baseline RBC and serum folate concentrations between the two groups. Furthermore, after 4 months of supplementation, blood folate concentrations in both groups increased, but no significant differences were found between OC-users vs. non-users. The authors noted that the increase in the serum folate levels was higher in the non-OC group after supplementation [34].
### Table 12. Studies on oral contraceptive use and blood folate concentrations.

<table>
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<tr>
<th>Reference, year, and location</th>
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<th>Timing of outcome evaluation</th>
<th>Supplement use and nutritional status</th>
<th>Summary of Results</th>
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<td>McLean et al., 1969 USA</td>
<td>Intervenational study 29 OC users 17 controls</td>
<td>-Patients attending a rural clinic -Similar socioeconomic class</td>
<td>Ortho-Novum I Ovulen</td>
<td>Serum folate</td>
<td>L.casei microbiological assay</td>
<td>12h fasting blood samples</td>
<td>-Similar dietary habits among participants -No info on dietary records or supplement use</td>
<td>-No sig. differences between OC users vs. controls -No sig. relationship between length of OC therapy and serum folate concentrations</td>
</tr>
<tr>
<td>Castren et al., 1970 Finland</td>
<td>Intra-individual before-after observational study 30 OC users (pre-post)</td>
<td>-Healthy women</td>
<td>Primvolar Deltregnin Lyndiol 2.5</td>
<td>Serum folate</td>
<td>L.casei microbiological assay</td>
<td>No info provided</td>
<td>No info provided</td>
<td>-Serum folate concentrations did not sig. change among women after 3 mos of OC use (3 types of OCs used) compared to baseline levels (control) -12 women were analyzed long-term, and even after 7.1 cycles of OC use, serum folate status did not sig. change compared to baseline</td>
</tr>
<tr>
<td>Kahn et al., 1970 USA</td>
<td>Observational cohort study 14 OC users 5 controls</td>
<td>-Patients attending a gynecology clinic -Low socioeconomic status -Pregnant patients of low vs high socioeconomic status also compared in their 3rd trimester</td>
<td>Ortho-Novum Ovral</td>
<td>Serum folate</td>
<td>Average lobe score (ALS) – tissue stores of folate</td>
<td>2h fasting blood samples collected</td>
<td>No supplements No dietary info recorded</td>
<td>-No sig. differences between OC users vs. controls -OC users had better folate stores than pregnant patients</td>
</tr>
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<td>Streiff et al., 1970 USA</td>
<td>Single-dose interventional study 9 OC users 9 controls</td>
<td>-Healthy women -OC users reqd. use for ≥1 year -Non-users did not use OCs for ≥6 mos.</td>
<td>No info provided</td>
<td>Mean rise in serum folate</td>
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<td>Fasting samples, collected 2.5h after single-dose administration</td>
<td>No info provided</td>
<td>-No sig. differences in the mean rise in serum folate between OC users vs. non-users within the monoglutamate folate absorption study -Sig. lower (50% lower) mean rise in serum folate among OC users in the polyglutamate folate absorption study</td>
</tr>
<tr>
<td>Study Authors, Year</td>
<td>Study Design</td>
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<td>Subjects</td>
<td>OC Users</td>
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<td>Drug Use</td>
<td>Serum Folate</td>
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<tr>
<td>Shojania et al., 1971</td>
<td>Observational cohort study</td>
<td>Canada</td>
<td>176</td>
<td>140</td>
<td>- Patients from private clinic, hospital and OC users - Low vs. high socioeconomic status</td>
<td>Ortho-Novum Ovulen Norinyl C-Quens Enovid Others</td>
<td>Serum folate</td>
<td>RBC folate</td>
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<tr>
<td>Pritchard et al., 1971</td>
<td>Observational cohort study</td>
<td>USA</td>
<td>57</td>
<td>55</td>
<td>- OC use for at least 9 mos. or more - Clinically normal pregnant women vs. anemic women also studied in late pregnancy</td>
<td>Ovulen Ortho-Novum Norinyl Norlestrin Enovid E Ovral Oracon Others</td>
<td>Plasma folate</td>
<td>No info provided</td>
</tr>
<tr>
<td>Stephens et al., 1972</td>
<td>Observational cohort study</td>
<td>UK</td>
<td>43</td>
<td>23</td>
<td>- Multi-part investigation: *Comparison cohort study *Absorption study *In-vitro - Women with regular menstrual cycles - OC users for ≥3mos.</td>
<td>Gynovlar Minovlar Lyndiol 2.5 Norinyl I Minilyn Volidan 21 Ovulen Ovulen 50 Orthonovin 2mg Orthonovin 1/80 Orthonovin 1/50</td>
<td>Serum folate</td>
<td>Max. rise of serum folate</td>
</tr>
<tr>
<td>Shojania et al., 1973</td>
<td>Single-dose interventional study</td>
<td>Canada</td>
<td>14</td>
<td>19</td>
<td>- Normal women of childbearing age - No clinical malabsorption</td>
<td>Norinyl-I Norinyl-II Ortho-Novum I Ovral C-Quens Ovulen I Enovid</td>
<td>Max. rise of serum folate (ΔSFA)</td>
<td>AUC</td>
</tr>
<tr>
<td>Gaafar et al., 1973</td>
<td>Observational study</td>
<td>Egypt</td>
<td>40</td>
<td>28</td>
<td>- Women from birth control centers or hospitals - Average socioeconomic class</td>
<td>Anovular 21</td>
<td>Serum folate</td>
<td>RBC folate</td>
</tr>
</tbody>
</table>

<p>| Notes: | - The mean serum and RBC folate conc. of OC users was sig. lower than controls - OC users for &gt;2 yrs had sig. lower levels than users for &lt;1 yr. - Urinary FIGLU excretion was sig. higher in OC users compared to controls - Folate conc. sig. rise after OC discontinuation (w/ 3 mos) |
| - No sig. differences between OC users vs. controls - OC users had higher folate concentrations than women in late pregnancy |
| - No sig. differences in serum folate concentrations measured on day 5 vs. day 20 of menstrual cycle (except among Gynovlar users) - No sig. differences between serum folate of OC users vs. non users - No sig. differences in max. rise of serum folate between OC users vs. non-users after both were pretreated with folic acid (sig. differences seen with high variability without pre-treatment) |
| - Both mean ΔSFA and AUC yielded no sig. difference between the groups in the monoglutamate absorption study - Both ΔSFA and AUC were sig. lower in OC users, in the polyglutamate absorption study |
| - Sig. differences observed between the serum folate and RBC folate conc. of OC-users vs. non-users (p&lt;0.01). |</p>
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<th>Study (Year, Country)</th>
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<tr>
<td>Shojania et al., 1975 (Canada)</td>
<td>Single-dose interventional study</td>
<td>7 OC users 6 controls</td>
<td>Normal women of childbearing age</td>
<td>Plasma clearance of folic acid (ΔSFA)</td>
<td>Mean rise in serum folate (ΔSFA) after 5min was sig. lower in OC users vs. controls.</td>
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<tr>
<td>Ahmed et al., 1975 (India)</td>
<td>Observational cross-sectional study and intra-individual before-after study</td>
<td>18 OC users 43 controls</td>
<td>Healthy women from low-middle socioeconomic class</td>
<td>RBC folate status</td>
<td>Sig. lower RBC folate concentrations observed with OC use (p&lt;0.1)</td>
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<tr>
<td>Paine et al., 1975 (USA)</td>
<td>Observational cohort study</td>
<td>279 OC users 247 controls</td>
<td>Women attending a family planning clinic</td>
<td>Serum folate</td>
<td>No sig. differences between serum folate status of OC users vs. controls, as measured by the microbiological assay and the radioassay</td>
</tr>
<tr>
<td>Smith et al., 1975 (USA)</td>
<td>Observational cohort study</td>
<td>70 OC users 64 controls</td>
<td>Women part of Louisiana Family Planning Program</td>
<td>Serum folate</td>
<td>Sig. lower serum and RBC folate conc. found among OC users vs. non-users (p&lt;0.02)</td>
</tr>
<tr>
<td>Study Authors and Year</td>
<td>Design Type</td>
<td>Country</td>
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<tr>
<td>Prasad et al., 1976</td>
<td>Observational cohort study</td>
<td>USA</td>
<td>307 OC users 130 controls</td>
<td>Women recruited into factorial arrangement design based on socioeconomic status, type of OC used, normal use or use during lactation. OC users req'd OC use for ≥3 mos.</td>
<td>Norinyl Ovral, Serum folate RBC folate L. casei microbiological assay No info provided Supplementation used as a covariate for subgroup analysis. In the higher socioeconomic status group, OC use yielded sig. lower serum and RBC folate concentrations vs. non-users and women who resumed OC use during lactation. No sig. differences in serum or RBC folate status were observed between OC users vs. controls of the lower socioeconomic status group.</td>
</tr>
<tr>
<td>Areekul et al., 1977</td>
<td>Observational cohort study</td>
<td>Thailand</td>
<td>20 OC users 50 controls</td>
<td>Healthy women recruited in both groups. OC users req'd use for ≥1 year. Non-users either never used OCs or ≥1 year since use.</td>
<td>No data on type of OCs reported Serum folate RBC folate Serum FABP L. casei microbiological assay No info provided No info provided. No sig. differences in serum folate conc. b/w the two groups. RBC folate sig. lower among OC users vs. controls. Serum FABP sig. higher among OC users vs. non-users.</td>
</tr>
<tr>
<td>Martinez et al., 1977</td>
<td>Observational cohort study</td>
<td>USA</td>
<td>29 OC users 54 controls</td>
<td>Middle-class women recruited from prenatal clinic. OC users req'd. OC use for ≥3 mos. Subgroups divided based on season of recruitment, pregnant vs. non-pregnant users. Pregnant users included women who conceived ≤6 mos after discontinuing OCSs.</td>
<td>Ortho Novum Norlestrin Ovulen Norinyl Oracon Plasma folate RBC folate L. casei microbiological assay No info provided No info provided. Mean plasma and RBC folate concentrations were sig. lower amongst OC users vs. non-users. Sig. seasonal difference in RBC folate concentrations observed. No sig. differences in nutritional intake of folate between the groups. Plasma and RBC folate values decreased over the course of pregnancy, with lower levels among OC users compared to controls, but these trends were not statistically sig.</td>
</tr>
<tr>
<td>Pietarinen et al., 1977</td>
<td>Observational study</td>
<td>Canada</td>
<td>22 OC users 18 controls</td>
<td>Healthy women from UBC. Controls were not using OCs for ≥6 mos.</td>
<td>Ortho-Novum 1/50 Ortho-Novum 1/80 Ortho-Novum 2mg Ovral Norlestrin Demulen Serum folate RBC folate L. casei microbiological assay Blood samples of day 5 and 20 of menstrual cycle. Fasting blood samples No folate supplement use among participants 3-day dietary records used for nutritional assessment. Serum folate concentrations were sig. lower among OC users in general (p&lt;0.05), with statistical sig. seen on day 5 but not on day 20. RBC folate concentrations were not sig. different between the two groups, regardless of time during menstrual cycle.</td>
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<tr>
<td>Hettiarachchy et al., 1983</td>
<td>Observational cross-sectional and longitudinal studies</td>
<td>Sri Lanka</td>
<td>Healthy women from rural center (nutritionally vulnerable pop)</td>
<td>Ovulen 50 Serum folate Radioisotopic protein-binding method 3h fasting Folate supplement use was part of exclusion criteria</td>
<td>- Serum folate concentrations were sig. lower in OC users than in non-users, with the lowest levels among women of low socioeconomic status.</td>
</tr>
</tbody>
</table>
- Groups stratified based on income status and age
- Pregnant women also studied
- Controls used non-hormonal IUDs

Intake of green vegetables reported to be irregular
- Regardless of income level, folate concentrations over the course of pregnancy were higher than those among OC users
- Low folate conc. associated with prolonged OC use (>12 mos.)

<table>
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<th>Study</th>
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<td>Randomized, interventional longitudinal study</td>
<td>India</td>
<td>15 OC users 17 controls</td>
<td>Controls used IUDs or had undergone sterilization, Urban women from low socioeconomic class, visiting family planning clinics</td>
<td>Ethinyl estradiol 30µg+levonorgestrel 150µg 1) OC + placebo 2) OC + vitamin (intermittent) 3) OC + vitamin (daily) 300µg folate in each vitamin</td>
</tr>
<tr>
<td>Joshi et al., 1986</td>
<td>Double-blind, randomized interventional study</td>
<td>India &amp; Thailand</td>
<td>314 OC users 92 controls</td>
<td>Controls had undergone sterilization, Urban women from low socioeconomic class, visiting family planning clinics</td>
<td>A – ethinyl estradiol 30µg+levonorgestrel 150µg B – ethinyl estradiol 50µg+levonorgestrel 150µg</td>
</tr>
<tr>
<td>Mooij et al., 1991</td>
<td>Two-arm interventional study</td>
<td>Netherlands</td>
<td>28 OC users 31 controls</td>
<td>Healthy adult volunteers from university hospital, -Supplementation effects in OC users vs. non users (controls)</td>
<td>Mono-, bi- or multiphasic OC containing estrogen 30µg Gravitamon supplement 5µg folate in each vitamin</td>
</tr>
<tr>
<td>Steegers-Theunissen et al., 1992</td>
<td>Two-arm interventional study</td>
<td>Netherlands</td>
<td>11 OC users 15 controls</td>
<td>Healthy adult women, Controls had never used OCs or had not used them at least 1 year prior</td>
<td>Marvelon</td>
</tr>
</tbody>
</table>

- Both intermittent and continuous supplementation sig. improved RBC folate concentrations among OC users, compared to controls (OC users+placebo)
- 6 mos. after the treatment, only the continuous supplementation schedule was successful in increasing RBC folate concentrations
<table>
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<tr>
<th>Study</th>
<th>Study Type</th>
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<td>Brattstrom et al., 1992</td>
<td>Observational cohort study</td>
<td>17 OC users, 13 controls</td>
<td>Healthy women - OC users reqd. ≥2 years of use - Non-users reqd. ≥2 years w/o use</td>
<td>Triphasic OCs: EE(30-40µg)+levonorgestrel (50-120µg) EE 30µg+ levonorgestrel 150µg</td>
<td>No info provided</td>
<td>Fasting blood samples</td>
<td>No info provided - Data was part of larger study in men and women aiming to determine the role of steroid sex hormones (OC use) on vascular disease in women and as a treatment for prostatic carcinoma in men - No sig. differences observed between OC users vs. non-users, but a trend existed towards lower RBC folate in users</td>
</tr>
<tr>
<td>Steegers-Theunissen et al., 1993</td>
<td>Single-dose interventional study</td>
<td>29 OC users, 13 controls</td>
<td>Healthy adult women - Controls had never used OCs</td>
<td>Marvelon Test dose-5mg folic acid</td>
<td>Serum folate</td>
<td>Dualcount solid phase boil radioassay</td>
<td>Blood samples on day 3 of the menstrual cycle - Overnight fast - Sig. difference between the serum folate time course profiles at t=210min, where OC users had sig. lower serum folate concentrations vs. controls - Urinary folate excretion between the two groups showed no sig. difference.</td>
</tr>
<tr>
<td>Lussana et al., 2003</td>
<td>Observational cross-sectional study</td>
<td>60 OC users, 159 controls</td>
<td>Healthy adult women - Controls had not used OCs for ≥12 mos. prior to treatment - Users were on regular OC treatment</td>
<td>OCs described based on generation: 1&lt;sup&gt;st&lt;/sup&gt; gen. 2&lt;sup&gt;nd&lt;/sup&gt; gen. 3&lt;sup&gt;rd&lt;/sup&gt; gen.</td>
<td>Serum folate</td>
<td>Overnight fast</td>
<td>No use of multivitamin supplements for ≥12 mos. - No sig. diff diterary habits b/w participants - No sig. differences observed between the serum folate concentrations of oral contraceptives users vs. non-users</td>
</tr>
<tr>
<td>Sutterlin et al., 2003</td>
<td>Case-control study</td>
<td>71 OC users, 170 controls</td>
<td>Healthy adult women - Controls had not used OCs for the past 3 mos. or more</td>
<td>20µg EE: Eve 20 Leios Lovelle Miranova</td>
<td>Serum folate</td>
<td>Ion-capture assay</td>
<td>No vitamin use for ≥3 mos. before study - No sig. differences in the serum folate concentrations between OC users vs. controls (p=0.72) - % of reduced, normal or elevated folate conc. also did not differ sig. b/w the two groups.</td>
</tr>
<tr>
<td>McArthur et al., 2013</td>
<td>Observational longitudinal study</td>
<td>9 OC users, 13 controls</td>
<td>Healthy women recruited as part of other RCT - Followed up till 12 weeks</td>
<td>Combined OC: EE 30-35µg + drsp 3mg</td>
<td>Serum folate</td>
<td>Automated immunoassay</td>
<td>Fasting blood samples - No supplement use - Validated FFQ used - No sig. differences in serum or RBC folate concentrations b/w OC users vs. controls over 12 weeks</td>
</tr>
</tbody>
</table>

4.4.2 Meta-analysis

4.4.2.1 Oral contraceptive use and plasma folate concentrations

Out of the 27 studies in this category, 21 assessed plasma folate concentrations, and 17 studies were included in the meta-analysis, since they actually reported concentrations rather than only reporting % differences or p-values. Within these 17 comparisons that studied a total of 1359 oral contraceptive users and 1472 controls, we found a significant difference in the plasma folate concentrations of OC-users when compared to non-users (p < 0.0001). The pooled estimate for the change in the mean difference of plasma folate concentrations with the use of oral contraceptives was -1.27 (95% CI -1.85, -0.69; random-effects model; Figure 17). There was also a significant degree of inter-study heterogeneity between the included studies (p < 0.00001, I² = 88%).

Figure 17. Forest plot for plasma folate concentrations among OC users vs. non-users.
4.4.2.2 Oral contraceptive use and RBC folate concentrations

Among the 13 studies that measured RBC folate concentrations, 12 studies actually presented the concentrations in a quantitatively extractable form and were included in the meta-analysis. These studies included 772 oral contraceptive users and 617 controls. Yet again, we found a significant difference in the mean difference favouring oral contraceptive users (p < 0.001). The pooled estimate for mean difference of RBC folate concentrations was -59.32 (95% CI -58.03, -23.04; random-effects model; Figure 18). There was a highly significant degree of inter-study heterogeneity in this meta-analysis (p < 0.00001, I² = 96%).

**Figure 18. Forest plot for RBC folate concentrations among OC users vs. non-users.**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>OC users Mean</th>
<th>SD</th>
<th>Total</th>
<th>Non-users Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>Mean Difference IV, Random, 95% CI</th>
<th>Mean Difference IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omer et al 1973</td>
<td>102 17.95</td>
<td>40</td>
<td>326</td>
<td>108.92</td>
<td>56.95</td>
<td>45</td>
<td>0.9%</td>
<td>-124.00 [-154.32, -113.68]</td>
<td>-</td>
</tr>
<tr>
<td>Ahmed et al 1975</td>
<td>110.72</td>
<td>25.24</td>
<td>18</td>
<td>136.98</td>
<td>56.8</td>
<td>45</td>
<td>9.1%</td>
<td>-17.27 [-33.10, -1.44]</td>
<td>-</td>
</tr>
<tr>
<td>Smith et al 1975</td>
<td>172.99</td>
<td>56.9</td>
<td>70</td>
<td>119.84</td>
<td>62.3</td>
<td>64</td>
<td>9.0%</td>
<td>-26.60 [-49.86, -3.34]</td>
<td>-</td>
</tr>
<tr>
<td>Shojaee et al 1970</td>
<td>130</td>
<td>95</td>
<td>95</td>
<td>175</td>
<td>68</td>
<td>63</td>
<td>9.0%</td>
<td>-45.00 [-68.11, -21.89]</td>
<td>-</td>
</tr>
<tr>
<td>Prasad et al 1976</td>
<td>182.36</td>
<td>84.23</td>
<td>225</td>
<td>256.07</td>
<td>122.13</td>
<td>165</td>
<td>9.0%</td>
<td>-72.81 [-94.45, -51.17]</td>
<td>-</td>
</tr>
<tr>
<td>S-Theunissen et al 1983</td>
<td>101.52</td>
<td>38.71</td>
<td>20</td>
<td>180.64</td>
<td>36.04</td>
<td>13</td>
<td>8.8%</td>
<td>-0.68 [-23.47, 25.23]</td>
<td>-</td>
</tr>
<tr>
<td>Barril et al 1985</td>
<td>83.8</td>
<td>33.5</td>
<td>15</td>
<td>65.0</td>
<td>37.42</td>
<td>17</td>
<td>6.3%</td>
<td>-12.10 [-39.67, 12.47]</td>
<td>-</td>
</tr>
<tr>
<td>Joffe et al 1986</td>
<td>89.61</td>
<td>110.3</td>
<td>154</td>
<td>250.75</td>
<td>167.8</td>
<td>92</td>
<td>8.8%</td>
<td>-181.15 [-208.11, -154.19]</td>
<td>-</td>
</tr>
<tr>
<td>Brodtmann et al 1992</td>
<td>117.93</td>
<td>28.24</td>
<td>17</td>
<td>150.04</td>
<td>63.98</td>
<td>13</td>
<td>8.4%</td>
<td>-32.21 [-46.50, -5.98]</td>
<td>-</td>
</tr>
<tr>
<td>Martinez et al 1977</td>
<td>174.6</td>
<td>76.8</td>
<td>27</td>
<td>232.7</td>
<td>162.1</td>
<td>51</td>
<td>8.2%</td>
<td>-58.16 [-99.98, -17.22]</td>
<td>-</td>
</tr>
<tr>
<td>Pietarinen et al 1977</td>
<td>104.1</td>
<td>71.7</td>
<td>22</td>
<td>204.9</td>
<td>71.8</td>
<td>18</td>
<td>6.1%</td>
<td>-26.80 [-59.50, 23.80]</td>
<td>-</td>
</tr>
<tr>
<td>Areekul et al 1977</td>
<td>62.25</td>
<td>313</td>
<td>20</td>
<td>79.8</td>
<td>21.6</td>
<td>50</td>
<td>3.4%</td>
<td>-173.00 [-322.87, -23.33]</td>
<td>-</td>
</tr>
</tbody>
</table>

Total (95% CI) 772 617 100.0% -58.03 [-83.01, -23.04]

Heterogeneity: Tau² = 3430.92, Chi² = 339.83, df = 11 (p = 0.00001), I² = 98%

Test for overall effect Z = 3.22 (p = 0.002)

4.4.2.3 Funnel plots

To further investigate the heterogeneity observed in both of the above comparisons, funnel plots were created to evaluate the nature of inter-study heterogeneity and publication bias. The funnel plot for the plasma folate comparison (Figure 19) revealed slight asymmetry with a greater number of small-to-medium studies favouring a positive correlation (small-study effect) whereas studies with a larger effect size favoured no correlation.
Figure 19. Funnel plot for plasma folate concentrations comparing OC users vs. non-users.

In contrast, the funnel plot for RBC folate concentrations was symmetrically distributed, but limited because of the small number of included studies and overall sample (Figure 20). All included studies had moderate-to-large effect sizes.
4.4.3 Category 2: Blood folate concentrations with the folate-fortified oral contraceptive

The 5 studies presented in this category are summarized in Table 13.

4.4.3.1 Bioequivalence studies

Wiesinger et al. (2012) conducted a randomized, open-label, three-period, crossover study at a single center in Germany to evaluate the bioequivalence of the new folate-supplemented contraceptive (EE/drospirenone/levomefolate calcium) with its respective OC component (EE/drospirenone) and levomefolate calcium (5-methyl-THF). Within this intra-individual crossover design, each participant was randomized to a treatment sequence including:

1) EE 0.03mg/drospirenone 3mg (Treatment A), 2) EE 0.03mg/drospirenone 3mg/levomefolate calcium 0.451mg (Treatment B) and 3) levomefolate calcium 0.451mg (Treatment C). Each
treatment was started between days 3-6 of the participant’s menstrual cycle, each treatment was administered as a single dose orally, along with extensive dietary restrictions that were standardized across all participants. Each consecutive treatment was separated by a washout period of one menstrual cycle. Food diaries were maintained by participants to record their food consumption 3-days prior to the treatment, and dietary folate intake was estimated by 3-day dietary records. The authors evaluated plasma levels of L-methyl-THF through blood samples at 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 12, 16, 24, 34, 48, 72, 96, 120, 144, and 168h post-treatment, and measured through a validated liquid chromatography/tandem mass spectrometry (LC/MS) method. The authors measured AUC_{∞}/AUC_{last} and C_{max} as primary variables, with all other pharmacokinetic parameters as secondary variables. Amongst 41 women in the per-protocol set, the baseline-corrected pharmacokinetics of L-5-methyl-THF in EE/drospirenone/levomefolate calcium were [geometric mean (geometric coefficient of variation): C_{max} = 51.7 nmol/L (30.6%) and AUC_{last} = 236 nmol•h/L (26.3%), and was closely comparable to the respective values in 43 women administered levomefolate calcium alone: C_{max} = 48.7 nmol/L (30.4%) and AUC_{last} = 239 nmol•h/L (26.5%). Hence, the geometric mean ratios and the 90% confidence intervals for the AUC and C_{max} were between 80-120%, demonstrating that the bioequivalence of 5-methyl-THF was similar after the administration of EE/drospirenone/levomefolate calcium as well as levomefolate calcium alone.

Blode et al. (2012) conducted a second randomized, open-label, three-arm, intra-individual crossover bioequivalence study at a single center in the Netherlands. Healthy volunteers, who had not previously been using folic acid supplements for at least two menstrual cycles prior to the study were recruited. The study aimed to demonstrate bioequivalence for all active ingredients of EE/drospirenone/levomefolate calcium, specifically its levomefolate
calcium component. There were three different treatments used: A) EE 0.02mg/drospienone 3mg, B) EE 0.02mg/drospirenone 3mg/levomefolate calcium 0.451mg, and C) levomefolate calcium 0.451mg. The administration of each treatment sequence was randomized, and each treatment was separated by a washout period of at least 1 menstrual cycle. Each treatment was self-administered as a single oral dose, with extensive standardized dietary restrictions within the protocol, and were started between days 3-6 of their menstrual cycle. Dietary folate intake was recorded through 3-day weighted diet records, and blood samples were collected at -0.5, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 34, 48, 72, 96, 120, 144 and 168h post-administration. Finally, the concentrations of L-5-methyl-THF were analyzed using LC/MS, and the AUC and C\text{max} were the primary variables. Within the per-protocol set, 40 women completed the study, and their baseline-corrected geometric means (geometric coefficient of variation) for EE/drsp/levomefolate calcium vs. levomefolate calcium were respectively: C\text{max} = 44.3 nmol/L (32.7%) and AUC\text{last} = 214 nmol•h/L (28.7%) vs. C\text{max} = 44.2 nmol/L (39.4%) and AUC\text{last} = 217 nmol•h/L (28.1%). Both these measures were comparable between the two groups their point estimates and 90% CIs were within the 80-120% necessary to demonstrate bioequivalence.

4.4.3.2 Efficacy studies

Bart Sr. et al. (2012) conducted a multi-center, randomized, double-blind, active-controlled, parallel-group study within the USA, to measure folate status (RBC and plasma folate) among individuals taking the folate-supplemented oral contraceptive (EE/drsp/levomefolate calcium) vs. a regular oral contraceptive (EE/drsp). Healthy adult women were randomized 3:1 to either EE 20µg/drsp 3mg/ levomefolate calcium 0.451mg (n=196) or EE 20µg/drsp 3mg (n=66) for 24 weeks. The study was composed of three phases: a) screening and baseline b) blinded treatment phase for 24 weeks c) follow-up phase (weeks 26-28). Fasting
blood samples were retrieved at baseline, 4, 8, 12, 16, 20, 24 weeks and then during follow-up, between days 25-28 of each treatment/menstrual cycle. Plasma and RBC folate concentrations were analyzed using a validated microbiological assay, and adherence was evaluated through patient diaries and pill counts. Dietary intake was also evaluated using a short folate food-frequency questionnaire. Mean RBC folate concentrations increased from 990 ±390nmol/L at baseline to 1406 ±440nmol/L at week 24, which were significantly different (p < 0.0001) from the EE/drsp group (1014 ±308nmol/L at baseline to 1024 ±293nmol/L). Similarly, mean plasma folate concentrations increased significantly (p < 0.0001) from baseline in the EE/drsp/levomefolate calcium group from 45.0 ±17.6nmol/L at baseline to 60.8 ±19.9nmol/L, compared to the EE/drsp group whose plasma folate concentrations increased from 43.1 ±16.1nmol/L at baseline to 41.0 ±17.6nmol/L.

Castano et al. (2013) published post-hoc subanalysis from the Bart Sr et al. (2012) study, to incorporate dietary folate data as well as data on supplement use in establishing the efficacy of the folate-supplemented oral contraceptive (EE/drsp/levomefolate calcium) compared to controls. The authors found that dietary folate intake did not differ between the two groups at baseline (p=0.6) or at week 24 (p=0.4). Only 26% of the study participants used folate supplements. Interestingly, the authors also found that when incorporating the folate contribution of the oral contraceptive, along with folate in diet and through supplements, the mean dietary folate intake (in DFE µg) was significantly higher in the fortified OC group (1225.9 ±346.2) compared to controls (500.6 ±361.2) by 24 weeks of therapy. The authors also analyzed the percentage of the study population that achieved adequately protective RBC folate concentrations against neural tube defects (>906 nM) by 24 weeks of use of the oral
contraceptives. They found that 91% of women in the folate-fortified OC group vs. 60% in the regular OC group were optimally protected against NTDs.

Diefenbach et al. (2013) conducted a double-blind, double-dummy, randomized, parallel-group study comparing EE/drsp/levomefolate calcium (folate-fortified oral contraceptive) with EE/drsp+folic acid for 24 weeks (invasion phase) and EE/drospirenone for an additional 20 weeks (elimination phase) at a single center in Germany. The objective of the invasion phase was to study the AUC for plasma and RBC folate, and the authors studied the elimination phase to investigate blood folate concentrations after the cessation of EE/drsp/levomefolate therapy. Healthy women who were not previously taking folic acid supplements or any other medications contraindicated for OC therapy were recruited and randomized 1:1 among the two groups, for the two phases of the study. 75 women within the levomefolate calcium group received EE/drsp/levomefolate+placebo, whereas 75 women in the folic acid group received EE/drsp+folic acid, and the pills were identical between the two groups. Compliance in the study was evaluated through patient diaries and pill counts, and dietary folate intake was estimated through a standardized food questionnaire. Blood samples were collected at baseline and then at biweekly intervals, and blood folate concentrations were measured using the microbiological assay. The geometric mean (% geometric coefficient of variation) were comparable for plasma and RBC folate amongst the two treatment groups in the invasion phase: Baseline-corrected plasma folate AUC_{0-24wk} for EE/drsp/levomefolate calcium = 640 nmol•week/L (29.0%) vs. AUC_{0-24wk} EE/drsp+folic acid = 561 nmol•week/L (32.7%); Baseline-corrected RBC folate AUC_{0-24wk} for EE/drsp/levomefolate calcium = 10 427 nmol•week/L (34.3%) vs. AUC_{0-24wk} EE/drsp+folic acid = 8863 nmol•week/L (24.6%). Plasma and RBC folate concentrations among both EE/drsp/levomefolate calcium and EE/drsp+folic acid plateaued after 8 weeks, and were
comparable in both groups, with slightly higher concentrations in the EE/drsp/levomefolate calcium group. At week 24, plasma folate concentrations were 49.9 ±15.5 nmol/L in the levomefolate calcium group vs. 43.3 ±13.3 nmol/L in the folic acid group. Similarly, RBC folate concentrations in the levomefolate calcium group (1361 ±322 nmol/L) were comparable to those in the folic acid group (1207 ±217 nmol/L) at 24 weeks. With respect to the elimination phase, mean RBC folate concentrations 20 weeks after cessation of treatment (i.e. week 44) were 739.8 ±197.6 nmol/L in the levomefolate calcium group vs. 701.1 ±170.6 nmol/L in the folic acid group. Plasma folate concentrations also decreased at similar rates between the two groups. The authors report that the median time after cessation of EE/drsp/levomefolate calcium therapy after which RBC folate concentrations decreased below 906nM was 10 weeks (i.e. week 34).
Table 13. Studies on the novel folate-fortified oral contraceptive.

<table>
<thead>
<tr>
<th>Reference, year, and location</th>
<th>Study design and sample size</th>
<th>Patient characteristics</th>
<th>Intervention/ Drug</th>
<th>Outcomes</th>
<th>Assessment of folate status</th>
<th>Timing of outcome evaluation</th>
<th>Supplement use and nutritional status</th>
<th>Summary of Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blode et al., 2012 <strong>Netherlands</strong></td>
<td>Randomized, open-label, intra-individual crossover study 40 women</td>
<td>-Healthy adult female volunteers with regular menstrual cycles -Supplement use was part of exclusion criteria</td>
<td>Single dose ingestion: A) EE 0.02mg/ drsp 3mg B) EE 0.02mg/drs 3mg/levomefolate Ca 0.451mg C) levomefolate Ca 0.451mg</td>
<td>AUC-, C&lt;sub&gt;max&lt;/sub&gt; Other pharmacokinetic variables</td>
<td>LC/MS</td>
<td>Treatments started b/w day 3-6 of menstrual cycle 1 cycle washout period</td>
<td>Extensive dietary restrictions 3-day weighted diet records</td>
<td>-Bioequivalence was demonstrated for all components: EE, drsp and levomefolate calcium, since the GMRs and CIs were within 80-125% for AUC- and C&lt;sub&gt;max&lt;/sub&gt; values of each component</td>
</tr>
<tr>
<td>Wiesinger et al., 2012 <strong>Germany</strong></td>
<td>Randomized, open-label, intra-individual crossover study 41 women</td>
<td>-Healthy women with regular menstrual cycles -Excluded if formerly used supplements</td>
<td>Single dose ingestion: A) EE 0.03mg/ drsp 3mg B) EE 0.03mg/drs 3mg/levomefolate Ca 0.451mg C) levomefolate Ca 0.451mg</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; AUC Other pharmacokinetic variables</td>
<td>LC/MS</td>
<td>Treatments started b/w day 3-6 of menstrual cycle 1 cycle washout period</td>
<td>Extensive dietary restrictions 3-day dietary records</td>
<td>-The GMRs and CIs were between 80-125% for AUC and C&lt;sub&gt;max&lt;/sub&gt; values, thus demonstrating bioequivalence for EE, drsp and levomefolate calcium -No sig. effects on the rate and extent of absorption of L-5-methyl-THF following concomitant administration with EE and drsp.</td>
</tr>
<tr>
<td>Bart Sr et al., 2012 <strong>USA</strong></td>
<td>Randomized, double-blind, parallel-group study 196 EE/drsp/levomefolate 66 EE/drsp</td>
<td>-Healthy women from urban and rural centers, as well as diverse ethnicities included -3:1 randomization scheme</td>
<td>EE 0.02mg/ drsp 3mg/ levomefolate Ca 0.451mg vs. EE 0.02mg/ drsp 3mg</td>
<td>RBC folate Plasma folate</td>
<td>L.casei microbiological assay</td>
<td>Blood samples b/w day 25-28 of menstrual cycle Folate supplement use recorded but not reported Short folate frequency questionnair e</td>
<td>-No sig. difference in demographic characteristics or treatment compliance between the two groups. -RBC folate conc. were sig. higher in the EE/drsp/levomefolate group compared to EE/drsp (p&lt;0.0001) after 24 weeks of supplementation -Plasma folate conc. were also sig. different between the two groups by week 24 (p&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Diefenbach et al., 2013 <strong>Germany</strong></td>
<td>Randomized, double-blind, parallel-group study</td>
<td>-Healthy women recruited from the local population -Excluded if regularly used supplements</td>
<td>EE 0.03mg/ drsp 3mg/ levomefolate Ca 0.451mg + placebo vs.</td>
<td>RBC folate Plasma folate</td>
<td>Microbiological assay Fasting blood samples Supplements not used Standardized food questionnair e</td>
<td>-Mean dietary folate intake and compliance was similar between the two groups. -The GMRs for the AUC of plasma and RBC folate were comparable between the two groups.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design Details</td>
<td>Participants</td>
<td>Folate Assay Details</td>
<td>Follow-up Details</td>
<td>Results</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Castano et al., 2012</td>
<td>Secondary analysis from randomized, double-blind study</td>
<td>196 EE/drsp/levomefolate, 66 EE/drsp</td>
<td>EE 0.02mg/ drsp 3mg/ levomefolate Ca 0.451mg vs. EE 0.02mg/ drsp 3mg</td>
<td>Dietary folate equivalents (DFEs) based on diet, supplement use and OC contribution, % of pop. with optimal NTD protection</td>
<td>Same as Bart Sr et al. (2012) 26% of participants reported supplement use Short food frequency questionnaire (Columbia university) -No sig. difference in DFEs when accounting for diet and supplement use between the EE/drsp/levomefolate vs. EE/drsp group -Sig. differences between groups by 24 weeks when accounting for folate contribution by OC -After 24 weeks of supplementation, 91% in the fortified-OC group vs. 60% in the regular OC group had protective RBC folate conc. against NTDs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(University of Bonn)</td>
<td>-Mean conc. of plasma and RBC folate were similar between groups by week 24, but were slightly higher in the EE/drsp/levomefolate group. -In the elimination phase, plasma and RBC folate conc. were similar in the two groups after 20 weeks of stopping tx, with slightly higher conc. in the EE/drsp/levomefolate group.</td>
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</table>
4.5 DISCUSSION

Overall, results from the above systematic review and meta-analysis suggest that oral contraceptive use is associated with a statistically significant decrease in blood folate concentrations.

While Shojania et al. (1970), Castren et al. (1970), Pritchard et al. (1971), Gaafar et al. (1973), Paine et al. (1975), Prasad et al. (1976), Martinez et al. (1977), Pietarinen et al. (1977), Hettiarachchy et al. (1984), and Joshi et al. (1985) showed positive associations with oral contraceptive use and a subsequent decrease in plasma folate concentrations, studies by Kahn et al. (1970), Steegers-Theunissen et al. (1992) and Lussana et al. (2003) were unable to demonstrate this association.

Similarly, in the case of RBC folate, Shojania et al. (1970), Gaafar et al. (1973), Ahmed et al. (1975), Prasad et al. (1976), Martinez et al. (1977), Pietarinen et al. (1977), Joshi et al. (1985), and Bamji et al. (1985) demonstrated a positive correlation between oral contraceptive use and a lowering of folate concentrations, but Steegers-Theunissen et al. (1992) were unable to find this effect.

There are several factors that might have influenced the differences in observed effects and can further explain the controversy in the literature.

4.5.1 Potential confounders

4.5.1.1 Study design

The studies included in this review vary substantially in terms of study design, cohort size, populations studied and covariates accounted for within the study. The majority of the included studies were observational cohort studies comparing two groups: oral contraceptive
users vs. non-users. However, even among the cohort studies, there are differences in what constitutes an oral contraceptive user. In some studies, 3-month use was sufficient [18, 20], whereas others required at least 6 months [21] or 1 year [29] of oral contraceptive use. Similarly, the quality of controls differed between the studies. While some studies merely included women from the same population source who were not using oral contraceptives for 6 months [21] or 1 year [24, 29], others defined them more strictly as those who had never used oral contraceptives [5, 14, 15]. Some studies matched their controls for contraceptive methods [16, 17, 22, 33], thus improving the quality of the controls, whereas most did not, and matched them for some external covariate such as socioeconomic status [18, 22, 28] or season of recruitment [20, 28].

Given that the majority of the studies included within this systematic review and meta-analysis are observational studies, it is necessary to account for the variability in study design and execution methods. Pre-post studies can be advantageous because each subject is their own control, however have limitations in comparing different time points and if the effects of the exposure is not acute – as would likely be the case with oral contraceptives. Similarly, the plethora of cohort studies included are useful in comparing exposed vs. unexposed groups efficiently, but their ability to correct for other confounders associated with differences between groups may be problematic. The high heterogeneity observed with the summary estimates within the meta-analysis can be attributed to the inclusion of these different designs.

4.5.1.2 Length of oral contraceptive use

Several studies investigated the relationship between duration of oral contraceptive use and the associated effect on blood folate concentrations [2, 15, 16, 22, 27, 35]

McLean and colleagues (1969) found no significant relationship between duration of therapy with Ovulen or Ortho-Novum vs. controls on serum folate concentrations. Similarly,
Castren et al. (1970) found no significant differences in serum folate concentrations over long-term use of Primovlar and Lyndiol. Paine et al. (1975) found no relationship between duration of OC therapy and serum folate concentrations. Shojania et al. (1971) found that serum folate concentrations were significantly decreased with continued use of oral contraceptive therapy, especially that OC users for ≥2 years had significantly lower serum folate levels than those who had been using them for ≤1 year, and long-term users had a higher percentages of suboptimal folate status. Ahmed et al. (1975) found significantly decreased RBC folate concentrations among Ovulen 50 and Ovral users who were followed up for 6 months, in comparison to their baseline levels. Hettiarachchy et al. (1983) found that prolonged OC therapy (>12 mos) led to low serum folate levels.

Thus, even the literature regarding the length of oral contraceptive therapy yields mixed results, but in many cases, this could be attributed to differences in the length of the study period or other pre-existing factors that may influence the difference. For example, among the negative studies, Castren et al. (1970) had a mean follow-up period of 7.1 months, whereas McLean et al. (1969) and Paine et al. (1975) followed up women between 40 months and 59 months.

4.5.1.3 Type of oral contraceptive (formulation, dose)

In trying to establish a cause-effect relationship, many studies further analyzed the composition of oral contraceptives to determine if certain types of combination-OCs had a higher chance of being associated with lowered folate status than others. McLean et al. (1969) found no relationship between estrogen dose and serum folate levels among the two OCs they used (Ortho-Novum and Ovral), even though their doses of mestranol varied.

Given that oral contraceptives have evolved in the type of progestins, as well as in the doses of estrogens used, some researchers focused on whether this difference may be responsible
for the differences in effects within the literature. Many of the previous studies referred to oral contraceptives containing 35-50µg of EE, whereas OCs nowadays contain lower doses of EE [26], and it is important to investigate whether this folate-lowering effect could still pose a concern. Steegers-Therunissen et al. (1992, 1993), found a decrease in serum folate concentrations after a single-dose kinetic study within users of sub-50 (<50µg EE) oral contraceptives but not within their longer-term study of regular use of the same OCs. Similarly, Sutterlin et al. (2003) focused on OCs with 20µg EE, but found no differences in serum folate concentrations between oral contraceptive users vs. non-users.

4.5.1.4 Hormonal status during blood sampling

Several studies also aimed to evaluate if the changes in hormonal status over the course of a woman’s menstrual cycle, whether through endogenous or exogenous sex hormones, could be responsible for variations in folate status. Stephens et al. (1972) were the first to incorporate different stages of the menstrual cycle in their experiments, by comparing folate concentrations among their subjects on both day 5 (low hormonal phase) and day 20 (high hormonal phase), yet found no significant differences among controls or oral contraceptive users on the two days. However, among their subgroups, users of Gynovlar had significantly higher serum folate concentrations on day 20 vs. 5 (p < 0.001), which the authors were unable to explain but attributed it to the potentially high progesterone content of the oral contraceptive [13]. Pietarinen et al. (1977) also conducted a similar comparison on day 5 and 20, and found statistically significant differences in serum folate between OC users vs. controls on day 5, but not on day 20. Mooij et al. (1991) evaluated the differences in folate status between oral contraceptive users and controls on day 3 and 23 of the menstrual cycle, but found no significant differences based on day of measurement. Similarly, Steegers-Theunissen et al. (1992) made this comparison on day 3
and day 21 among both groups, but found no significant differences in the blood folate concentrations of participants in either group.

Most studies included in this review did not maintain any consistency in sampling all of their subjects on the same day within their menstrual cycle, or failed to report this. Nevertheless, studies by Ahmed et al. (1975), Joshi et al. (1986) and Steegers-Theunissen et al. (1993) did maintain some consistency, and either sampled all of their subjects on a particular day within each woman’s menstrual cycle, or within a constant range of days. Thus, data from these studies controls for hormonal status during one’s menstrual cycle as a potential confounder.

4.5.1.5 Population differences – socioeconomic, nutritional, demographic

Folate’s nutrient status in an individual may be strongly predicted by their socioeconomic class [3], which is why many of the earlier studies either characterized their study population through socioeconomic differences, or analyzed them as a covariate within their studies. Given that many of the earlier investigations were specifically concerned about the possibility of oral contraceptives inducing macrocytic anemia or folate deficiency [1], populations with a low socioeconomic status were targeted as high-risk populations and studied extensively. Kahn et al. (1970) found better folate status and general health among their participants from a higher socioeconomic status. Shojania et al. (1971) found better folate concentrations among their patients with the higher socioeconomic class (private clinic patients) as well, but their differences were not statistically significant and could be attributed to the differences in sample size between the two groups. After a breadth of studies emerging from the Western world, Ahmed et al. (1975) were the first to investigate differences in folate status in a low-middle income group in India. Prasad et al. (1976) were the first to actually incorporate socioeconomic status as a variable within the factorial design of their study, and found a significant income effect (p <
0.001), even though folate concentrations among low vs. high socioeconomic groups were not significantly different at baseline. Hettiarachchy and colleagues (1983) studied a nutritionally vulnerable and low income population in Sri Lanka, and found significant differences between oral contraceptive users vs. non-users, along with generally lower folate levels. Overall, they attributed some of the differences seen within their subjects to the high prevalence of folate depletion among them, which was potentiated by factors such as improper cooking habits or nutrition, lower levels of education (about proper diet) and income [3, 22]. Joshi et al. (1986) compared a variety of hematological indices among a rural center in Thailand (Chiang Mai), and two poor urban centers in India (Bombay and Hyderabad), and found different issues among each population, yet did not maintain a uniform methodology in their analysis of data from all centers, because of which they could not be truly compared.

Even though only one study explicitly demonstrated an income effect within their population [18], several studies highlight it as a potential confounder when analyzing differences within their populations. It is also worth noting, while comparing the outcomes from various studies in this review, that they represent very different populations. Thus, apart from study design and factors that may influence each study’s internal validity, the differences in effects observed among the studies could be attributed to population differences.

Most studies included women from similar demographic and socioeconomic backgrounds, and differences in demographic characteristics of patients were not studied in relation to differences in folate status. Nonetheless, given that this review includes women from several different countries, the pooled data is likely to have high generalizability in comparison to each of the individual cohort studies.
4.5.1.6 Population – pregnancy/lactation

Several studies included women in the periconceptional period, who had either conceived shortly after the cessation of oral contraceptive therapy or had resumed their use while breastfeeding. These women highlight the population that may be at the greatest immediate risk from the potentially folate-depletive effect of oral contraceptives if they conceive shortly after OC therapy. For example, within their factorial design, Prasad et al. (1976) included a subgroup of women who had resumed oral contraceptive use within 5 weeks after pregnancy, during lactation. They found that this group had significantly higher folate concentrations compared to general oral contraceptive users of Norinyl and Ovral (p < 0.001) [18]. Martinez et al.’s (1977) study cohort consisted of women who had conceived within 6 months of using oral contraceptives, and their folate status was evaluated within the first trimester of their pregnancy. They found significantly lower serum and RBC folate concentrations among former oral contraceptive users in the first trimester, but as some of these participants were followed up in the second and third trimester, this difference became non-significant alongside the decrease in folate status with the progression of pregnancy [20].

In contrast, some studies compared how the folate-lowering effect during oral contraceptive use with what is seen during pregnancy. Kahn et al. (1970) found that the last trimester of pregnancy had a significantly greater folate-lowering effect on tissue stores compared to oral contraceptive use. This was supported by Pritchard et al. (1971) who found that oral contraceptive users had higher folate concentrations than women in late pregnancy. Contrastingly, Hettiarachchy et al.’s (1983) longitudinal study showed higher serum folate levels among their pregnant population (at both high and low income levels) vs. the women on oral contraceptives.
Hypotheses regarding mechanism of effect

The mechanism of how folate handling is altered during oral contraceptive use is multifaceted, with several hypotheses surrounding it. The overall effect is likely multifactorial, and incorporates many of these effects to different degrees. Initially, researchers implicated impaired folate absorption as a potential cause. Streiff et al. (1970) found polyglutamate folate absorption to be impaired by 50% among his oral contraceptive users in comparison to non-users, but found that monoglutamate absorption was not significantly impaired. Stephens et al. (1972) found that their results agreed with Streiff et al. when their subjects were not pre-saturated with a loading dose of folic acid, but the difference became non-significant after presaturation. They also conducted an in vitro experiment to test if intestinal pteroylpolyglutamate hydrolase was impaired by synthetic steroid sex hormones, as its impairment would explain the impaired absorption of polyglutamates across the intestine. However, they were unable to detect any inhibition of enzyme activity, ruling this out as a major possibility. Shojania et al.’s (1973) absorption study was also unable to find major differences in folate absorption between groups after adjusting for baseline levels. In light of their other data [12], they implicated impaired folate metabolism through the influence of steroid sex hormones as a greater contributor to this effect, since increased estrogen levels have a stimulant effect on purine metabolism and may thus influence increased folate requirements [36]. Yet, the impairment in metabolism was likely minor because liver function tests did not show any significant abnormalities in oral contraceptive users [12, 14]. Shojania et al.’s clearance study revealed that women on oral contraceptives had a significantly higher urinary excretion of folate, thus involving increased urinary clearance as part of the effect [32]. In parallel to this, some researchers also found increased levels of serum folic acid binding protein (FABP) among oral contraceptive users,
suggesting increased protein binding could also be playing a role in reducing serum folate concentrations [16, 19, 37].

### 4.5.3 Oral contraceptives and folate status

Thus, overall, the literature suggests that oral contraceptive use alters folate pharmacokinetics, without inducing clinically significant folate deficiency. While the rate and type of response may vary based on many of the confounders examined in this review, such as, duration of oral contraceptive therapy, baseline folate status, general socioeconomic and nutritional status, type of oral contraceptive use, etc., it is evident that slight changes in folate handling do occur with the use of oral contraceptives.

Given that 50% of pregnancies are unplanned, women who are planning a pregnancy shortly after stopping oral contraceptive therapy represent a special population who may be at risk for low folate levels upon conception. Blood folate concentrations >906nmol/L are associated with optimal protection against neural tube defects, which may occur before a woman even realizes she is pregnant [8]. Data suggest that 21.1% of women become pregnant one menstrual cycle after stopping OC therapy, and 45.7% conceive within 3 cycles after OC therapy cessation [6]. Thus, it is imperative that women achieve optimal folate levels upon conception either through continued folate supplementation or through a newer option, the folate-fortified oral contraceptive.

### 4.5.4 Efficacy and clinical utility of the folate-fortified oral contraceptive

Overall, the data from the bioequivalence studies associated with Beyaz [38, 39] demonstrates bioequivalence for each of the components associated with it (ethinyl estradiol, drospirenone and levomefolate calcium). This confirms that the concomitant administration of
the oral contraceptive component (EE and drsp) does not alter the pharmacokinetics of the folate component (levomefolate calcium), and vice versa. Similarly, the efficacy studies associated with Beyaz [40, 41] demonstrate that the folate-fortified oral contraceptive significantly increased blood folate concentrations over 24 weeks in comparison to a regular oral contraceptive (identical without the folate component) [40]. While this study was well-controlled, and did analyze dietary folate intake as a potential influence on blood concentrations, it employed an unequal randomization scheme (3:1) without citing a rationale for this. Such an extreme unequal allocation is associated with a loss of power for treatment comparison, and should be noted when considering the significance of the positive effect cited by the authors.

Diefenbach et al. (2013) compared concomitant supplementation with an equimolar dose of folic acid and the folate-fortified oral contraceptive in their two-arm randomized clinical trial, and found comparable RBC folate levels in both groups (with slightly higher levels in the Beyaz group) after 24 weeks of use, suggesting that the folate-fortified OC is at least as effective as folic acid in raising blood folate concentrations. Interestingly, through the elimination phase of their study, they found that upon not using folate supplementation, RBC folate levels fell below 906nmol/L by 10 weeks [41].

Thus, the folate-fortified oral contraceptive is comparable to supplementation with 400µg folic acid in women of childbearing age. However, it also offers several advantages over regular supplementation:

4.5.4.1 Compliance

Recent data from the Canadian Health Measures Survey shows that only 28.2% of women use folate supplements [7]. Given this low rate of supplement use, the folate-fortified oral contraceptive offers an appropriate vehicle as compliance to oral contraceptives is generally
high because of the severity of adverse consequences associated with missing a pill. Studies cite between 92.6%-95.7% compliance for oral contraceptive use [42], thus making it an ideal delivery method for daily folate intake among women.

4.5.4.2 Target population

Unlike broad-spectrum strategies like fortification, the folate-fortified oral contraceptive actually targets the population that needs it most – women of childbearing age. Thus, in the event of an unplanned pregnancy, it offers optimally protective blood folate levels against neural tube defects [11, 40]. And even without pregnancy, it offers the appropriate daily folate dose for women to maintain good health.

4.5.4.3 L-5-methyl-THF vs. folic acid

Finally, the use of levomefolate calcium (5-methyl-THF) within the oral contraceptive offers several advantages compared to folic acid, given that it is at least as effective as folic acid in raising blood folate levels. Since it is the metabolically active form of folate in the plasma, it does not need to be metabolized like folic acid, and is beneficial for women who may have mild to severe deficits in folate metabolism. Further, it is less likely to mask symptoms of vitamin B12 deficiency, which is a theoretical concern with folic acid supplementation [43]. As well, it would decrease the amount of unmetabolized folic acid found in the blood as a result of folic acid supplementation.
4.6 CONCLUSIONS

This is the first systematic review to comprehensively review the available literature on oral contraceptive use and folate status, including the novel folate-fortified oral contraceptive. However, despite the broad coverage of this review, it is not without its limitations. Due to the inclusion of all clinical study types within this review, the pooled data on the outcomes evaluated in the meta-analysis should be taken with a grain of salt, as many of the outcome comparisons come from very different study types. In light of the analysis of the potential confounders associated with the studies in this review, it should also be noted that many of the earlier studies, because of the nature of reporting during that time, did not report outcomes as robustly in terms of methodology or design as the later studies. Finally, even though we have comprehensively attempted to include all of the relevant clinical data on the subject, we did exclude letters to the editor and editorial commentaries, which were sometimes used by earlier authors to report clinical data [1, 44]. In some cases, this preliminary data was added to a bigger study later [1, 35], but in others, this was not evident. We also noted that some of the authors of earlier studies used thematic titles that did not always capture all of the comparisons made within the studies. These studies would likely have been overlooked during our title and abstract review stage if they did not include “folate” and “oral contraceptives” to some effect. We have attempted to
capture as many of these articles as we could through hand searches, but cannot undermine the possibility of having missed some such studies.

The pooled data from our systematic review and meta-analysis suggests the oral contraceptives do have a folate-lowering effect on blood folate concentrations. It is thus imperative that women continue supplementation during oral contraceptive therapy. The folate-fortified oral contraceptive, Beyaz, offers one such option for women of childbearing age.

4.7 REFERENCES


5  CHAPTER 5.

Folate status of women in Toronto:
implications of folate fortification and supplementation

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This study has been submitted to \textit{Can. J. Public Health} for publication.

\textbf{MS} was responsible was part of the conception and design of the study, helped with data analysis, as well as writing and submission of the manuscript.
5.1 ABSTRACT

**Objective:** To assess the population RBC folate concentrations in a cohort of women from the Greater Toronto Area, to evaluate the percentage of women of childbearing age below optimally protective folate levels against neural tube defects (<906nM).

**Methods:** 1035 anonymous whole blood samples from a clinical laboratory were assessed for RBC folate concentrations using a chemiluminescent immunoassay. Folate analysis was requested by healthcare professionals as part of clinical care, and available data included age, gender and RBC folate concentration. Descriptive statistics were used to characterize the percent of women who had suboptimal blood folate concentrations, and a multiple regression was used to analyze determinants of folate status.

**Results:** Our data from 2013 shows that only 7% of women of childbearing age (15-45 years) had RBC folate concentrations below 906nM, substantially lower that our 2006 study (40%) and the 2010 Canadian Health Measures Survey (22%). Results from the multiple regression showed that age is a significant positive predictor of higher RBC folate status (p < 0.001).

**Conclusions:** Compared to earlier data, the low current percentage of women of childbearing age with suboptimal folate concentrations is indicative of the effectiveness of public health efforts in this area – including the reach of fortification and the increased awareness of folic acid supplement use. Future efforts should focus on the need of higher folate doses in women falling into high risk groups for NTDs.
5.2 INTRODUCTION

Folic acid supplementation during the periconceptional period is associated with a reduction in the risk of neural tube defects. A case-control study by Daly et al. was the first to describe the RBC folate concentration of 906nM or above, as being optimally protective against the risk of neural tube defects [1]. This has been accepted as the RBC folate concentration necessary to optimally reduce the incidence of NTDs [2].

Neural tube defects are major malformations of the central nervous system occurring at about 4 weeks of pregnancy, when the neural tube completes its closure [3]. Given that 50% of pregnancies are unplanned, inadequate maternal blood folate concentrations pose a significant risk to this population as an NTD may occur before a woman even knows that she is pregnant. Hence, public health efforts such as mandatory fortification and increased awareness of periconceptional supplementation are important in ensuring that women of childbearing age achieve protective blood folate concentrations.

Beyond neural tube defects, low folate intake during pregnancy has been associated with an increased risk of oral clefts, congenital cardiovascular defects, certain pediatric cancers, as well as overall adverse pregnancy outcomes [4-7]. Though folate generally has low potential for toxicity because of its water-soluble nature, overexposure to folate has been suggested to be associated with the potential masking of vitamin B12 deficiency, colorectal cancer based on animal data, as well as cognitive decline in the elderly [8, 9].

Canada introduced mandatory fortification of grain and cereal products (including white flour, cornmeal and enriched pasta) in 1998, with the goal of increasing the average intake of folic acid among women of childbearing age by 30-70% [10]. Overall, folate-fortified products
provide an additional 100-200µg of folic acid daily [10]. The incidence of neural tube defects has decreased by 46% across Canada after the implementation of folate fortification strategies (1.58 per 1000 births pre-fortification to 0.86 per 1000 births post-fortification) [11].

Recent data from the Canadian Health Measures Survey showed that only 28% of women routinely take folic acid supplements [12], and data from the Canadian Maternity Experiences Survey documented that 57.7% of women began taking folic acid supplements at least three months prior to pregnancy [13], suggesting that there are still sections of the population that may benefit from a more targeted approach to supplementation awareness.

Data derived from the nationally-representative Canadian Health Measures Survey in 2007-2009 showed that 22% of women of childbearing age had blood folate concentrations considered suboptimal in preventing the risk of neural tube defects (<906nM) [14]. Similarly, a previous study from our group analyzed the folate status of women within the GTA, and based on data from 2006, the authors found suboptimal blood folate levels in 40% of women of childbearing age, and 36% of pregnant women [2].

The objective of the present study was to follow-up on previous population-based studies to capture the current folate status of women within the GTA, in order to determine the percentage of women of childbearing age that are still inadequately protected, as well as to analyze population-based trends emerging from the analysis of their folate status. The population cohort in this study is obtained from the same clinical laboratories and uses the same method as our previous study [2].
5.3 METHODS

5.3.1 Sample collection
Anonymous convenience samples were retrieved from clinical requisitions by healthcare practitioners in the Greater Toronto Area, Ontario, Canada for folate testing, at different time points in 2013. Laboratory tests were requested by physicians as part of clinical care, and a sample of these results were anonymously used as representative samples to assess city-wide folate status. Data associated with the samples included age, gender and RBC folate concentration. No data on clinical diagnosis, reason for requisition of tests, dietary or supplementation habits, or other patient characteristics were available. All samples were analyzed at the same clinical laboratory.

Blood samples were collected as per standard laboratory procedures for medical tests within the GTA, using two lavender Vacutainer® tubes containing EDTA. Test analysis included both RBC folate and assessment of hematocrit.

5.3.2 Sample preparation and analysis
Whole blood samples were analyzed to measure RBC folate concentrations, after adjusting for hematocrit, using an automated chemiluminescent immunoassay (Access Folate, Beckman Coulter Inc., Fullerton, CA). This assay has a lower limit of detection of 0.5ng/mL. The linear range of the assay had an upper limit of 2623nM. Samples that were beyond the linear range of the assay had readings as “>2623”, which were imputed as 2623nM for during data analysis.

Whole blood samples were first analyzed for hematocrit and then hemolysed by combining each sample with a mixture of Access RBC folate Lysing Agent, which consists of a
0.15% ascorbic acid solution. The mixture was left to equilibrate at room temperature for 90 min, and the hemolysate was then assayed within 1.5h.

RBC folate concentrations were evaluated using the formula below, and were converted to nmol/L using a conversion factor.

\[
\text{RBC folate (ng/mL)} = \frac{\text{hemolysate folate x 21}}{\text{hematocrit/100}} \\
1 \text{ng/mL} = 2.266 \text{ nmol/L}
\]

Quality control testing including stability and dilution testing for this assay was part of standard setup protocol.

### 5.3.3 Statistical analysis

Descriptive statistics (frequencies, means, percentiles) were used to characterize the population, based on blood folate concentrations, age and gender. 906nM was used as the cut-off for optimal RBC folate concentrations. All of the descriptive analyses were conducted using GraphPad Prism (version 5; GraphPad Software, San Diego, CA).

A multiple regression analysis was conducted to analyze whether age or gender were significant predictors of RBC folate concentrations. The model was tested for basic assumptions including independence of residuals, normality, and homoscedasticity prior to analysis. These analyses were conducted using IBM SPSS (version 18; PASW Statistics, Armonk, NY).
5.4 RESULTS

Overall, among 1035 samples, about 40% of the individuals were male (n = 407) and 60% were female (n = 628). 235 women of ages between 15-45 years met the criteria for women of childbearing age. The descriptive statistics for the population cohort are presented in Table 14.

Table 14. Descriptive characteristics of population cohort.

<table>
<thead>
<tr>
<th></th>
<th>Males (n = 407)</th>
<th>Females (n = 628)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ±SD</td>
<td>56.67 ±19.79</td>
<td>52.98 ±21.79</td>
</tr>
<tr>
<td>Mean RBC folate (nM)</td>
<td>1695 ±510.9</td>
<td>1710 ±561.7</td>
</tr>
<tr>
<td>Min RBC folate (nM)</td>
<td>256</td>
<td>370</td>
</tr>
<tr>
<td>Max RBC folate (nM)</td>
<td>3072</td>
<td>3468</td>
</tr>
</tbody>
</table>

Among the 235 women of childbearing age, only about 7% had blood folate concentrations less than 906nM. The frequency distribution of RBC folate concentrations among women of childbearing age is presented in Figure 21.
Figure 21. Percentage of women of childbearing age, 15-45 years, with suboptimal folate status (<906nmol/L).

As part of the multiple regression, the assumptions for normality, linearity, independence of residuals and homoscedasticity were met (p > 0.05). The multiple regression demonstrated that age was a statistically significant predictor of RBC folate concentrations, with increasing levels with age (p < 0.001). Regression coefficients and their standard errors are presented in Table 15.

Table 15. Summary of Multiple Regression analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE_(B)</th>
<th>(\beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1477.889</td>
<td>78.451</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>3.308</td>
<td>0.834</td>
<td>0.129***</td>
</tr>
<tr>
<td>Gender</td>
<td>32.960</td>
<td>36.232</td>
<td>0.030</td>
</tr>
</tbody>
</table>
5.5 DISCUSSION

Following up on studies presenting population-level data on folate status through biochemical assessment [2, 14], our study presents a city-wide representative sample from the Greater Toronto Area to evaluate the current folate status among women of childbearing age. In comparison to our data from 2006 which showed that 40% of women of childbearing age had suboptimal blood folate concentrations [2], as well as national data from 2007-2009, which showed that 22% of these women were still inadequately protected [14], our current data from 2013 show further improvement in this vulnerable population, as only 7% of women were found to have RBC folate concentrations below 906nM. This 5-fold and 3-fold reduction respectively in the proportion of population at risk marks a remarkable success of public health strategies surrounding folic acid intake among women of childbearing age.

After the introduction of mandatory folic acid fortification of cereal products in Canada, several studies have shown an evident decrease in the incidence of neural tube defects across Canada post-fortification [15-18]. Traditionally, the incidence of NTDs was higher in the eastern provinces compared to the western ones [18], however, recent data indicate that the previous geographical differences have been eliminated in light of the overall effect of folic acid fortification on NTDs [11].

The previous studies [2, 14] are appropriate comparators because of the nature of their design as well as the immunoassays employed in the evaluation of blood folate concentrations. Our data are especially comparable with Bar-Oz’s et al. study because our population cohort was obtained from the same clinical laboratory, and utilized the same chemiluminescent immunoassay for blood folate analysis. In the Bar-Oz et al. study, the mean RBC folate
concentration was 1048nM among 1537 participants. Colapinto et al.’s nationally-representative study showed mean RBC concentrations of 1248nM among 5248 participants, whereas our current study shows much higher average blood folate concentrations (mean 1795nM ±581.5nM) among 1035 participants. While rising folate levels have been successful in reducing the proportion of women of childbearing age who may be at risk of a pregnancy with a neural tube defect because of suboptimal blood folate concentrations, they have potentially become more prevalent in the elderly – a population that is at potential risk of folate overexposure.

One of the strengths of our study is the large sample size representative of the city-wide population. In our study, RBC folate concentrations served as an apt biomarker of folate status because they are representative of long-term, tissue stores of folate. In contrast, plasma or serum folate are indicative of more recent folate intake, and may be susceptible to fluctuation. However, our study was limited by the information available to us (age, gender and RBC folate concentration), which impaired our ability to further analyze any associations between important covariates that comprise clinical data. Similarly, because we did not have the reason for requisition of folate testing available to us, we were unable to make any predictions about the population with regards to whether a test was requested to determine folate deficiency, overexposure or general biomonitoring.

We suspect that our population was biased towards older patients, since the mean age among individuals was much higher (mean age 54.43 ±21.09) than women of childbearing age (15-45 years). Thus, despite our large sample size and broad coverage, the representativeness of our sample may be questionable. Given that folate testing is no longer routinely done in Ontario, the women included within our sample were likely those who were suspected to have medical issues associated with folate status, as determined by the clinical judgement of healthcare
practitioners who requested these tests. However, the fact that even within this sample, which represented those at a higher risk of low folate status, we demonstrated such a low percentage of suboptimally-protected women makes our sample highly applicable in terms of folate-based public health goals. The bias affecting our study would decrease this percentage even more if a more broadly-representative sample from the general population was included. Thus, our results have immense public health implications.

Our finding that age was a statistically significant predictor of increased RBC folate concentrations (p < 0.001) raises a potential concern, as the elderly are noted to be a vulnerable population with respect to folate overexposure [19].

High folate concentrations among the elderly have been associated with a potential for masking vitamin B12 deficiency and cognitive decline [20], however the direction of effect is not only dependent upon folate status but also B12 status. Data from NHANES show that among the elderly, high folate with low B12 status was associated with anemia and cognitive impairment, however, high folate status with normal B12 levels was actually protective against cognitive impairment [21]. A recent study also showed that folate levels in the elderly are protective against Alzheimer’s [22]. In contrast, other studies bring attention to the high risk of folate deficiency among the elderly, especially because many have concurrent medical conditions or use other medications that may impair folate absorption [23, 24]. Further research is necessary for a better understanding of the long-term consequences of folate overexposure among the elderly, as well as how other vitamins involved in one-carbon metabolism affect its homeostatic balance. Continued biomonitoring of folate status among the elderly is necessary, especially if they continually exhibit high blood folate concentrations.
Recent nation-wide data show that folic acid supplement use is the highest in women above the age of 70 (~36%) [12], instead of the population that requires it most – women of childbearing age. This suggests that the directions of our public-health initiatives need to be re-evaluated and that high folate levels amongst the elderly require close monitoring. Given that the elderly are already a medically vulnerable population with a high risk of comorbidities, polypharmacy and impaired liver and renal function, it is pivotal that they are advised on a case-by-case basis in light of generally rising blood folate concentrations.

Apart from fortification, nation-wide data from 2007-2009 shows that only 28.2% of women used folic acid-containing supplements [12] even though women of childbearing age are advised to take 400µg folic acid per day. Similar low rates of supplement use are observed among women in the periconceptional period as only 57.7% women began taking folic acid at least three months prior to pregnancy within the Canadian Maternity Experiences Survey [13]. These statistics show that even though fortification has demonstrated population-wide benefit in decreasing the incidence of neural tube defects, women within the periconceptional period may not be adequately supplementing with folic acid. This is especially important since secondary analysis of data from the Canadian Health Measures Survey reveals that folic acid supplement use is the most significant predictor of folate status among women of childbearing age [25]. The authors also found a significant income effect that influenced folic acid supplement use and blood folate concentrations [12, 25], suggesting that there may a subsection of the population that may not be adequately protected and may require more specifically-targeted public health initiatives.

While women from higher socioeconomic status may be adequately aware of supplement use, women from lower socioeconomic status often have a breadth of concomitant issues that put
them higher risk of inadequate folate intake: poor diet, poor compliance with medications or supplement use, possible teratogenic substance use (e.g. alcohol), high BMI, etc. [26]. These women may currently be inadequately protected and may require higher doses of folic acid (>1mg) under the guidance of a healthcare professional [26], to ensure that they are optimally protected against neural tube defects.

The substantial public health success reflected in our results suggests that future efforts should focus on the need of higher folate doses in women falling into high risk groups for NTD. Specific ethnic communities could also be targeted through intensive community outreach and awareness programs, given that Sikh, Celtic and women from Northern China have a higher risk of neural tube defects [26]. Similarly, women with a previous child with NTD, those treated with antifolate medications, obese women, smokers should be targeted [26].

In conclusion, these results from our city-wide representative analysis of folate status mark the effectiveness of long-standing public health initiatives with a 3-5 fold decrease in the percentage of women of childbearing age who are suboptimally protected against neural tube defects, in comparison to most recent data [2, 14]. On the other hand, they show a significant association with increasing age and higher folate concentrations, which is a cause for potential concern because the elderly are already a vulnerable population with respect to folate overexposure. In combination with data on prevalence of supplement use among Canadians, continued monitoring of folate status among vulnerable populations and targeted public health initiatives for folate supplementation are recommended.
5.6 REFERENCES


6 CHAPTER 6.

General Discussion, Conclusions, and Future Directions

6.1 Discussion and Significance of Research Findings

The four studies presented in this thesis analyze the issue of periconceptional folic acid supplementation through several different perspectives, including pharmacokinetic, qualitative, population-level, intervention-based methodologies. While each of the studies are intimately connected as they further scientific understanding towards the clinical aim of optimizing periconceptional folic acid supplementation, they each uniquely contribute to an aspect of the scientific literature that has not previously been explored. The present chapter discusses the major conclusions of each of the studies presented in the preceding chapters, and highlights their clinical significance with respect to periconceptional folic acid supplementation.

I. Assessing the steady-state folate pharmacokinetics in pregnancy among women who supplement daily with 1.1mg (regular dose) vs. 5mg (high dose) folic acid in the periconceptional period (Chapter 2).

While research exists on the long-term kinetics of low doses of folic acid supplementation in pregnancy, to the best of our knowledge, no previous studies have evaluated the changes in folate pharmacokinetics among pregnant women supplementing 1.1mg vs. 5mg folic acid. Studies on long-term pharmacokinetics present important results regarding the dose and timing of supplementation, in relation to the achievement of steady-state. These are not only necessary in a research setting, but findings from these studies can be extrapolated to form better-informed recommendations for supplementation among pregnant women, that is actually guided by a
mechanistic understanding of folate kinetics. Current prenatal recommendations are based on studies in non-pregnant women of childbearing age, and given the physiological and general pharmacokinetic changes that occur during pregnancy, it is important that recommendations be re-assessed to account for altered pharmacokinetics in the pregnant state. Results from this study included:

- A dose-dependent effect on folate kinetics. The 5mg folic acid group exhibited non-linear kinetics, as a 5-fold increase in dose resulted in about a $\sim 2\text{-fold}$ increase in RBC folate concentrations.
- A pregnancy-induced effect on folate kinetics. Despite supplementation over 40-52 weeks, steady-state was not achieved in either dose group.
- Adherence to folic-acid containing prenatal supplements was high in both dose groups (95-96%).
- Plasma folate concentrations trended towards decreasing over the course of pregnancy, despite high adherence to supplementation in both dose groups.
- RBC folate concentrations continued to increase over 40-52 weeks of supplementation, without reaching steady-state.

Overall, these results are important in defining the alterations in folate pharmacokinetics as a result of dose and the state of pregnancy. The non-linear nature of folate kinetics at high doses warrants further research since this is the recommended dose for women who may have a broad series of conditions that contribute to low folate status and/or increased risk of NTDs. It is necessary that healthcare professionals account for the timing of folate supplementation, because even at the 5mg folic acid dose, long-term kinetics demonstrate that a 5-fold increase in dose on yields about a 2-fold increase in blood folate concentrations [1]. This is in contrast to the short-
term kinetic data, which shows that even the 5mg dose of folic acid exhibits linear kinetics after a single oral dose ingestion [2]. Hence healthcare professionals should base their recommendations for supplementation regimen with 5mg folic acid on steady-state data. Further research is required on determining the exact limiting mechanism that contributes to non-linear pharmacokinetics of folic acid at high doses, though the saturation of absorption is a potential contributor to this observation.

Within our study, we implicated several physiological and pharmacological changes that occur in late-pregnancy to explain the decreasing plasma folate concentrations observed over the course of supplementation in pregnancy. These included increased folate requirements due to the expansion of maternal and fetal tissues, increased blood volume or hemodilution, increased folate catabolism, increased folate clearance and weight gain. Given that plasma folate is actually the bioavailable form of folate that the fetus is exposed to, the late pregnancy-dependent decrease in plasma folate levels may have clinical implications for women with poor folate status. Folic acid supplementation then, is not just important in the first trimester of pregnancy as per many healthcare recommendations [3, 4], but it is critical to continue supplementation throughout pregnancy to maintain adequate folate levels for optimal health and the positive pregnancy outcomes associated with folic acid.

II. **Comparing the recruitment success and efficiency between traditional healthcare-based methods of recruitment vs. social media in a randomized clinical trial on folic acid supplementation in pregnancy (Chapter 3).**

Studies analyzing recruitment within clinical trials are scarce. Some of the systematic reviews or qualitative reviews that do exist on the subject of effective recruitment strategies highlight their lack of generalizability across different study designs, populations or interventions
This problem becomes even more augmented when studying a special population such as pregnant women [7]. After struggling with 4.5 years of marginal recruitment using traditional healthcare-based recruitment methods, we employed social media as a supplementary intensive recruitment strategy while still engaging in healthcare-based recruitment for a clinical trial on folic acid supplementation. Our rationale behind using social media was the widespread use of the internet amongst individuals to access health-related information. After applying social-media based recruitment strategies for 6 months, we found:

- A 12-fold increase in recruitment. Our recruitment rate with the use of traditional healthcare-based recruitment sources in Phase I of the study was 0.62 recruits/month over the span of 56 months. This increased to 7.5 recruits/month over the course of 6 months, with the use of social media in addition to healthcare-based recruitment in Phase II of the study.
- Attrition rate, when adjusted for total recruitment, was not significantly different between the two phases of the study.
- The population of women recruited from social media and healthcare-based establishments were not significantly different in our study cohort in terms of age, employment, education, marital status, fertility problems, medical conditions, gravidity, weight or ethnicity.
- Using Interrupted Time Series Analysis, we showed that the use of social media led to a significant increase in overall recruitment independent of periodicity, seasonality or autocorrelation effects.

Our study was the first to demonstrate the intensive use of social media as an effective tool for the recruitment of pregnant women in randomized clinical trials. While other studies have
used aspects of social media as part of their study interventions in the periconceptional period, its intensive use as a recruitment strategy has not been evaluated in this population. The results from our study highlighted social media as a promising tool for recruitment, and based on our experience with its use as well as the limited literature on social media and social marketing, we developed guidelines for other researchers to optimize its use in clinical trial recruitment. Future studies need to apply social media as a modified recruitment tool for different populations including children, parents, youth, etc., as well as different areas of research including pregnancy, nutrition and exercise, mental health, cancer, etc. to further understand and define the parameters of its maximal effectiveness as a recruitment tool. It will also be necessary to define its limitations and the breach of security and confidentiality that may occur if it is linked with sensitive patient information.

III.  a) Systematically reviewing and meta-analyzing available clinical literature on oral contraceptive use and its potential effect on plasma and RBC folate concentrations.

b) Systematically reviewing the data available on the novel folate-fortified oral contraceptive (Beyaz®) in order to evaluate its potential as an alternative to current folic acid supplementation therapy (Chapter 4).

The literature on oral contraceptive use and folate status first emerged through cases of folate deficiency observed in women on oral contraceptive therapy, in a manner that was similar to pregnancy[8]. The reports on the reduction of blood folate levels as a result of oral contraceptive use have lacked consensus, but much of the later studies show no such effect [9-11]. Our systematic review was the first to collectively and systematically review the existing literature on oral contraceptive use and folate status, as well as quantitatively meta-analyze the results from
the studies through a random-effects model. Given that many researchers suggest that impairment of folate metabolism observed during oral contraceptive use is similar to pregnancy because the synthetic hormones in oral contraceptives mimic a pseudo-pregnancy state, the investigation of this association was important from both a mechanistic perspective and based on its potential clinical impact on women using oral contraceptives prior to conception. In parallel to this, was the emergence of recently FDA-approved folate fortified oral contraceptive (Beyaz) approved for use among women of childbearing age. The data on the folate-fortified oral contraceptive was reviewed to determine its efficacy in increasing blood folate concentrations, and achieve optimally protective blood levels against NTDs, and to review bioequivalence data to investigate whether the oral contraceptive was an appropriate vehicle for the delivery of the folate component. The results from our systematic review and meta-analysis included:

- A significant decrease in plasma folate concentrations associated with oral contraceptive use.
- A significant decrease in RBC folate concentrations associated with use of oral contraceptives.
- Despite the significant effect observed within our meta-analysis, there are several confounders associated with many of the observational studies on the subject.
- The folate-fortified oral contraceptive containing 0.451mg of levomefolate is at least as effective as the equimolar dose of folic acid in raising blood folate concentrations.
- Bioequivalence data on the folate-fortified oral contraceptive indicates that the concomitant administration of the folate component and the oral contraceptive component does not impair the absorption of either.
Results from this systematic review are important in addressing another potential risk factor to impair folate status among women of childbearing age within the periconceptional period – the use of oral contraceptives. Despite the lack of consensus in the literature, our meta-analysis suggests that oral contraceptive use is likely associated with a significant lowering of blood folate status. The extent to which this effect is influenced by length of oral contraceptive use, specific oral contraceptive compounds, and other pre-existing factors within an individual still requires further research. Similarly, even though the clinical efficacy of this folate-lowering effect may be questionable in countries where folate fortification programmes have been applied and where population folate levels are generally high, it may be a concern in nutritionally vulnerable populations or in individuals who have other risk factors associated with lower folate status and increased NTD-risk.

IV. **Determining the current folate status of women within the Greater Toronto Area, in order to define the percentage of women of childbearing age that are still inadequately protected, as well as analyzing population-based trends emerging from the analysis of their folate status (Chapter 5).**

Current national-level data on population folate status comes from the Canadian Health Measures Survey, with data collected between 2007-2009 [12]. A previous study conducted by our lab, with data from 2006, measured the folate status of women of childbearing age within the Greater Toronto Area, and found that 40% of these women had blood folate levels that were suboptimal with respect to NTD-prevention upon conception [13]. Our study represented a follow-up on city-wide representative data on folate status, to determine the percentage of women who were adequately protected against NTDs fifteen years after mandatory folate
fortification in Canada. We also aimed to analyze population trends associated with folate status. Overall, we found:

- Only about 7% of women of childbearing age had suboptimally protective red blood cell folate levels against NTDs (<906nmol/L).
- Significantly higher folate levels were associated with increasing age.
- Mean red blood cell folate concentrations among our population cohort (mean 1795nmol/L ±581.5nmol/L) were higher than levels observed in the Canadian Health Measures Survey (mean 1248nmol/L).

The results of our study suggest the success of public health initiatives associated with raising the blood folate levels among the population – including food fortification and supplementation awareness, at least within Toronto. Despite this progress, there may still be vulnerable populations with poor folate status who may benefit from more targeted approaches, and future efforts should focus on identifying and adequately counselling these populations. While folate toxicity has not been reported due to supplementation in the periconceptional period, during pregnancy and postpartum, or among women of childbearing age, continued monitoring of population folate status is necessary to target the individuals that require extra supplementation and prevent the risk of overexposure among certain groups. Given the breadth of data associating elevated folate levels and cognitive decline among the elderly [14], it is especially important to continue to monitor vulnerable populations for risks associated with folate overexposure or deficiency.
6.2 Overall Conclusions

The pharmacology of folic acid supplementation during the periconceptional period is a multifaceted problem with nutritional, public health, biochemical, health counselling and awareness, and pharmacological aspects and implications. This thesis aims to address diverse questions associated with periconceptional folic acid supplementation using diverse study methodologies ranging from those with high internal validity to high external validity. Our pharmacokinetic study reveals that dose-dependent and gestational age-dependent changes in folate kinetics should be considered for dose and timing adjustment during clinical periconceptional and prenatal supplementation recommendations. Similarly, we demonstrate that oral contraceptive use should be considered a risk factor for potentially impaired folate status, given its association with lower blood folate concentrations through a systematic review and meta-analysis. Our population-wide study reveals that although the vast majority of women of childbearing age in Toronto have achieved optimally protective blood folate levels against neural tube defects, continual monitoring will benefit in identifying vulnerable populations in terms of low or high folate status, as they may require modified healthcare recommendations. Finally, through our experience using online social media for patient recruitment within a clinical trial, we realized its potential not merely as a recruitment tool, but also as a medium that can be adapted for population-level health surveys on folate status, or for folic acid supplementation awareness campaigns targeting special populations such as adolescent women, women from low socioeconomic status, women using oral contraceptives, etc. Thus social media as a platform has wide and emerging applications within the research, clinical and public health setting – and it can serve as a novel methodology both for monitoring population levels and for the dissemination of supplementation recommendations. Despite research confirming the importance of early folic
acid supplementation since the neural tube completes closure on day 28 post-conception, population supplementation data reveals that there is still a translation gap between women’s understanding of the importance of folic acid during pregnancy: Healthcare recommendations need to continue to emphasize the importance of periconceptional folic acid supplementation (prior to and during pregnancy) rather than merely prenatal folic acid supplementation (during pregnancy).
6.3 Future Directions

While this thesis sheds light on many problems surrounding periconceptional folic acid supplementation, future research is necessary to address several unanswered questions on this subject. Further research is necessary on the timing of steady-state achievement at high doses of folic acid, or during pregnancy states. A pharmacokinetic and mechanistic understanding of the changes in folate absorption, metabolism, distribution and elimination that occur in these circumstances will help in establishing the grounds for modified prenatal recommendations, especially if a woman has other risk factors that impair her folate status. This is an important consideration because the clinical trial within this study included healthy adult pregnant women for supplementation of both 1.1mg and 5mg folic acid, whereas the 5mg is currently prescribed to women with medical conditions or lifestyle factors that increase the risk of poor folate status or neural tube defects (not generally considered healthy). This theoretical re-assessment of recommendations based on pharmacokinetic understanding will help tailor the dose and timing of folic acid supplementation on a case-by-case basis.

While current healthcare recommendations and public health guidelines are based on blood folate levels necessary for the optimal prevention of neural tube defects, research investigating similar cut-offs of blood folate concentrations for the other positive benefits that folic acid confers during pregnancy is limited (eg. Reduction in the risk of orofacial clefts, congenital heart defects, pregnancy complications, etc.). Thus, research still needs to answer the questions of how much folic acid is enough, and the optimal dose or blood concentrations required for the prevention of other birth defects or pregnancy complications.

A significant aim of this thesis was to investigate some of the risk factors associated with impaired folate status or inadequate supplementation. While the results of our meta-analysis
demonstrated a significant folate-lowering effect in blood folate levels with the use of oral contraceptives, further research is necessary in confirming the exact mechanism of this effect as well as identifying women in whom this effect may have clinically significant implications. While it is likely that women who have pre-existing risk factors may have be at a higher risk of impaired folate status upon the use of oral contraceptives, this requires further validation. It will also be interesting to evaluate its implications in nutritionally vulnerable populations or among populations where food fortification with folic acid has not been implemented.

We also found diverse applications for social media while using it as a targeted tool for recruitment in our clinical trial. Some of the emerging literature and small studies have focused on using online social media as a platform for the dissemination of public health messages. Given its widespread use on day-to-day basis, and that a large percentage of the population uses the internet as a resource for health information, it has immense potential to be used for different types of and during different stages of clinical research. While our experience was limited to recruitment, it is possible to use online media to conduct population-wide surveys on folate or health status, which may increase response rates and overall coverage of the population. Thus social media can serve as an important tool for monitoring, research and also public health campaigns surrounding supplementation awareness and targeting vulnerable populations. These applications can be explored through future research, while simultaneously working on the security and confidentiality issues that surround internet-based research.
6.4 References


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Appendix A – Research Consent Form

Name: ____________________________________________

Date of birth: _______ /_______/_______ Version date: January 1, 2012
(day) (month) (year)

Title of research project:

Optimizing Periconceptional and Prenatal Folic Acid Supplementation

Title of sub-study:

Measuring Red Blood Cell and Serum Folate Concentrations Among Planning/Pregnant Women: PregVit-Folic 5® vs. PregVit® Daily Supplementation

Principal investigator:

Gideon Koren, MD
Director, Motherisk Program, Hospital for Sick Children
Telephone: 416-813-5781

Study coordinator:

Mahvash Shere, HBSc
Graduate student, Department of Pharmaceutical Sciences, University of Toronto
Division of Clinical Pharmacology and Toxicology, The Hospital for Sick Children
Telephone: 416-813-7283
**Purpose of research:**

We wish to measure red blood cell and serum folate concentrations among women planning a pregnancy or early in pregnancy (< 6 weeks gestation), who do not practice multivitamin supplementation, before and after implementing daily multivitamin supplementation. We want to compare folate measurements between PregVit® and PregVit-Folic 5®. This may be important information for planning or pregnant women who need folic acid which has been shown to reduce the risk of neural tube defects and potentially other malformations.

**Description of research:**

This is a two-arm comparison study: PregVit-Folic 5® (arm 1) contains 5 mg folic acid and PregVit® (arm 2) contains 1.1 mg folic acid. All other vitamin and mineral doses are identical between the 2 supplements. Both supplements are taken as 2 tablets daily, one tablet in the morning (am) and one tablet in the evening (pm). Both multivitamins are appropriate for periconceptional, prenatal, and post-partum supplementation.

The 2 arms are in equipoise, which means they are considered equal and it is unknown which arm is better, hence the study is being conducted to determine if there is a difference.

This is a randomized study, which means that a randomization process was used to assign, by chance alone, which group each participant belongs to. Thus, you were assigned by chance to the □ PregVit-Folic 5® / □ PregVit® group.

The following flow chart outlines the steps of participation:

Research coordinator and potential participant discuss the study.

◇

Enrolment into study through Motherisk Program.

◇

Written consent and randomization.

◇

Participant comes to research site after a 6 hr fast.

◇
5 mL blood sample will be drawn before multivitamin supplementation is initiated.

(Measure baseline folate and vitamin B12 blood concentrations)

Dietary folate questionnaire.

Pick up first supply of multivitamin (with prescription) from Hospital for Sick Children pharmacy.

(For planning women, supply renewal will occur every 3 months).

Start taking the multivitamins everyday, at approximately the same time every day.

Once participant becomes pregnant or as pregnancy progresses…..

Return to research site at 6 weeks gestation to draw one blood sample.

Pick up next supply of multivitamins (hospital pharmacy).

Return to research site at 12 weeks gestation to draw one blood sample.

Pick up next supply of multivitamins (hospital pharmacy).

Return to research site at 30 weeks gestation to draw last blood sample.

Participation in the study is complete. Return all blister packs.

Continue multivitamin supplementation for the remainder of the pregnancy.

The total volume of blood that will be taken is approximately 20 mL (4 teaspoons).

Each appointment will be scheduled according to when you are available.

To monitor self-administration of the multivitamins, we need you to return the PregVit-Folic 5® blister packs and if possible, maintain a diary of pill intake (will be provided).

Potential harms or discomfort:

High doses of folic acid can mask vitamin B12 deficiency. However, this is generally not a concern for healthy individuals, with no chronic medical conditions. One study has shown that
vitamin B12 deficiency can still be detected even with high folate blood concentrations. PregVit® and PregVit-Folic 5® both contain vitamin B12, thus it is being supplemented. Furthermore, vitamin B12 blood concentrations will be measured alongside folate blood concentrations to monitor for deficiencies.

The needle poking may not be pleasant. We will offer you a cream named EMLA® to massage on your arm, which takes away much (sometimes all) of the pain of poking. An alternative that can be used is a gel named Ametop®. There may be a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness that will usually disappear in a few days.

**Potential inconvenience:**

There is a time commitment associated with participation in this study. This includes the time required to get to and from the research site and the duration of each visit to the research site. We will schedule your appointments according to when you are available.

**Potential benefits:**

We will be able to tell you your folate blood level. Results can be disclosed in person or mailed. Daily multivitamin supplementation can improve vitamin and mineral concentrations.

☐ I would like to know the results of the folate and vitamin B12 blood measurements.
   ☐ In person   ☐ By e-mail    ☐ By mail

**Alternatives to participation:**

You are being asked to volunteer for this study. There are no consequences if you do not participate.
Confidentiality:

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law requires us to do this. For example, the law requires us to give information about you if you have an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

Sick Kids Clinical Research Monitors, employees of the funder or sponsor of the study, Duchesnay Inc., or the regulator of the study may see your health record to check on the study. For example, people from Health Canada Health Products and Food Branch, U.S. National Institutes of Health, or U.S. Food and Drug Administration, if necessary, may look at your records.

By signing this consent form, you agree to let these people look at your records. We will put a copy of this research consent form in your patient health records. We will give you a copy for your files.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study, the data will be kept as long as required and then destroyed as required by Sick Kids policy. Published study results will not reveal your identity.

Reimbursements:

We will provide you with some compensation, $250, in recognition of your time and effort upon your completion of the study protocol. If you stop taking part in the study, compensation will be pro-rated according to the degree of participation.
**Participation:**

It is your choice to take part in this study. You can stop at any time. The care you get at Sick Kids will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

In some situations, the study doctor or the company paying for the study may decide to stop the study. This could happen even if the medicine given in the study is helping you. If this happens, the study doctor will talk to you about what will happen next.

If you become ill or are harmed because of study participation, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The study staff, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

**Sponsorship:**

The sponsor of this research is Duchesnay Inc. (Laval, Quebec).

**Conflict of interest:**

Duchesnay Inc. supports the *Nausea and Vomiting of Pregnancy (NVP) Healthline* at the Motherisk Program. Dr. Koren, the principal investigator for this study, is the director of the Motherisk Program and a medical consultant for Duchesnay Inc.
**Consent:**

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.
7) I have read and understood pages 1 to _____ of this consent form. I agree, or consent, to take part in this study.

_______________________  ___________  __________________________________________
Printed name of subject  Age  Subject’s signature & Date

_______________________  __________________________________________
Printed name of person who explained consent  Signature & date

_______________________  __________________________________________
Printed witness’ name (if the subject / legal guardian does not read English)  Witness’ signature & date

If you have any questions about this study, please call or e-mail:

**Mahvash Shere**  at  **(416) 813-7283**

mahvash.shere@sickkids.ca

If you have questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manager at 416-813-5718.
Appendix B – Enrolment Intake Form

Patient No.  Study Participation:

Consultation Date:  Part 3. Measuring Serum and Red Blood Cell
Folate Levels Among Planning/Pregnant Women

Study Co-ordinator:  PregVit® Supplementation
Mahvash Shere  PregVit-Folic 5® Supplementation

Patient Information

Patient Name __________________________________________________________
Home Phone __________________ Work/Cell Phone __________________
Address ______________________________________________________________
City __________________ Province __________ Postal Code ___________
E-mail (optional) _____________________________________________________
Date of Birth ______________________ Weight __________ kg / lbs
LMP __________________________ Regular?  □ Yes _____ days  □ No
G _____  P _____  SA _____  TA _____  ectopic _____  molar _____

Part 3:  Planning a pregnancy □ Yes  □ No
Early in pregnancy □ Yes  □ No
  GA _______ wks
  EDC _____________________
### Demographics

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### Medications and Exposures

☐ None

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<th>Stop</th>
<th>Dose/Route</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
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220
### Substance Use

- None

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<th>Dose/Frequency</th>
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<tr>
<td>☐ Alcohol</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>☐ Tobacco/Nicotine</td>
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<td></td>
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</tr>
<tr>
<td>☐ Marijuana</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>☐ Other</td>
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</table>
**Medical History**

- No medical conditions, healthy overall

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<thead>
<tr>
<th>Category</th>
<th>Condition</th>
<th>Notes</th>
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<tr>
<td>Thyroid</td>
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<td></td>
<td>Other</td>
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<tr>
<td></td>
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<td>_________________________</td>
</tr>
<tr>
<td>GI</td>
<td>Crohn’s disease</td>
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</tr>
<tr>
<td></td>
<td>Ulcerative colitis</td>
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</tr>
<tr>
<td></td>
<td>Peptic/duodenal ulcer</td>
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</tr>
<tr>
<td></td>
<td>Irritable colon</td>
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<tr>
<td></td>
<td>IBS</td>
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</tr>
<tr>
<td></td>
<td>GERD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NVP</td>
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</tr>
<tr>
<td></td>
<td>Heartburn, reflux</td>
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</tr>
<tr>
<td></td>
<td>Indigestion</td>
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<tr>
<td></td>
<td>Abdominal discomfort</td>
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</tr>
<tr>
<td></td>
<td>Constipation</td>
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<tr>
<td></td>
<td>Diarrhea</td>
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</tr>
<tr>
<td></td>
<td>Other</td>
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<td></td>
<td></td>
<td>_________________________</td>
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<tr>
<td>Organ</td>
<td>Heart disease</td>
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<td></td>
<td>Liver disease</td>
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</tr>
<tr>
<td></td>
<td>Kidney disease</td>
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<td></td>
<td>Other</td>
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<td></td>
<td></td>
<td>_________________________</td>
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<tr>
<td>Miscellaneous</td>
<td>Hypertension</td>
<td>Diabetes</td>
</tr>
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<tr>
<th>Psychiatric</th>
<th>Anxiety</th>
<th>Depression</th>
<th>Bipolar</th>
<th>Other</th>
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</table>

| Other        |         |             |         |       |

Patient No. __________  Prenatal Multivitamin: □ PregVit-Folic 5®   □ PregVit®

Patient Name______________________________________________

Planning?  □ Yes  □ No  Early in pregnancy?  □ Yes  □ No
(pregnancy information already documented in enrolment intake form)

Obstetrical Information (for those enrolled as “planning” who are now pregnant)

□ Not applicable

LMP __________________________  Is cycle regular?  □ Yes  □ No

Length of cycle ________ days  EDC __________________________

Gravidity: G ___  P ___  SA ___  TA ___  Other __________________________

Method of confirming pregnancy:  □ home pregnancy test ( # ______ )
 □ blood work with doctor
 □ ultrasound
 □ other __________________________
Any new medications/exposures (since enrolment)?  ☐ Yes  ☐ No

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Start</th>
<th>Stop</th>
<th>Dose/Route</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>

**Obstetrical Information cont’d**  ☐ Not applicable

Any new medical conditions or symptoms since enrolment? (fill in table, if applicable)

<table>
<thead>
<tr>
<th>Condition/Symptom</th>
<th>Start, Description, Treatment, Other Medical Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>
**Blood Sampling: Blood Concentrations of Folate**

Patient No. ___________  Prenatal Multivitamin: ☐ PregVit-Folic 5®  ☐ PregVit®

<table>
<thead>
<tr>
<th>Date</th>
<th>Time of sampling</th>
<th>Week No.</th>
<th>Serum folate (__________)</th>
<th>RBC folate (__________)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>GA _____</td>
<td>wks</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(if &lt; 6 weeks)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 weeks</td>
<td>gestation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 weeks</td>
<td>gestation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 weeks</td>
<td>gestation</td>
<td></td>
</tr>
</tbody>
</table>

**NOTES:**

**Medical Information Update for Part 3**

(Photocopy this page and complete this form at each time of blood withdrawal)

Patient No. ___________  Date ______________________

Prenatal Multivitamin: ☐ PregVit-Folic 5®  ☐ PregVit®
**Medications/Exposures**

- None

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Start</th>
<th>Stop</th>
<th>Dose/Route</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

**Medical Conditions or Symptoms** (fill in table below, if applicable)

<table>
<thead>
<tr>
<th>Condition/Symptom</th>
<th>Start/Frequency</th>
<th>Description (i.e. mild, severe)</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ nausea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ vomiting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ gagging/dry heaving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ Heartburn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ Acid Reflux</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>□ Indigestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>□ Constipation</td>
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<td></td>
<td></td>
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<tr>
<td>□ Diarrhea</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>□ Abdominal pain</td>
<td></td>
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</tbody>
</table>

**NOTES:**
Appendix D - Block Dietary Folate Equivalents Screener  
*(First write-up before filling in the Scantron)*

<table>
<thead>
<tr>
<th>Patient No. _________________</th>
<th>Study Participation:</th>
</tr>
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<tbody>
<tr>
<td>Scantron ID No. _____________</td>
<td>Part 1. Measuring Serum Folate Levels Before and After Single Dose Administration of PregVit-Folic 5®</td>
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<tr>
<td>Consultation Date: ___________</td>
<td>Part 3. Measuring Serum and Red Blood Cell Folate Levels Among Planning/Pregnant Women</td>
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</tbody>
</table>

- [ ] PregVit® Supplementation
- [ ] PregVit-Folic 5® Supplementation
- [ ] PregVit® Supplementation
- [ ] PregVit-Folic 5® Supplementation
About how often do you eat each of the following foods?

<table>
<thead>
<tr>
<th>Food Description</th>
<th>1x per month or less</th>
<th>2 – 3x per month</th>
<th>1 – 2x per week</th>
<th>3 – 4x per week</th>
<th>5 – 6x per week</th>
<th>Everyday</th>
<th>2+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any cold breakfast cereal.</td>
<td></td>
<td></td>
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<tr>
<td>Any cooked cereals (i.e. oatmeal, cream of wheat, grits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Eggs (including in breakfast sandwiches)</td>
<td></td>
<td></td>
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<tr>
<td>Rolls, bagels, muffins, hamburger buns</td>
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<tr>
<td>Bread slices (i.e. sandwiches, toast)</td>
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<tr>
<td>Meal replacement drinks (i.e. Ensure, Carnation Instant Breakfasts)</td>
<td></td>
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<tr>
<td>Orange juice or oranges</td>
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<tr>
<td>Tea (brewed or iced tea) (not including herbal tea)</td>
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<tr>
<td>Crackers/cookies</td>
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<tr>
<td>Doughnuts, pastries, sweet rolls, cake, pan dulce, etc.</td>
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<tr>
<td>Tortillas (including burrito, enchilada, or other dish)</td>
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<tr>
<td>Beans (i.e. pinto, red/black beans, refried like in burrito)</td>
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<tr>
<td>Rice (or dishes with rice)</td>
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<tr>
<td>Spaghetti, pasta, macaroni, noodles</td>
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<tr>
<td>Pizza</td>
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<tr>
<td>Green salad (i.e. lettuce, raw vegetables)</td>
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<tr>
<td>Spinach, chard, collards, mustard greens</td>
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<tr>
<td>Any other vegetables (i.e. string beans, peas, corn, broccoli, etc.)</td>
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