

Micronutrient Intake during Pregnancy: Effects of Excessive Folic Acid on Placental Health and Function

Tasfia Ahmed

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University of Ottawa

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Abstract

Background: In addition to a diet including fortified dietary staples, the use of prenatal multivitamin supplements among women has been shown, in some cases, to lead to excessive micronutrient intake levels for nutrients such as folic acid (FA). It was therefore hypothesized that prenatal vitamin supplementation, in addition to a standard Canadian diet, would place pregnant Canadians at risk for excessive FA intake. With little available research on the potential negative impact of excess FA intake in pregnancy, it was further proposed that high concentrations of FA may adversely affect placental health and function. Thus, the aim of the current study was three-fold: 1) To determine micronutrient intake in a large Canadian cohort of pregnant women; 2) To determine the extent to which FA intake in this cohort may exceed the tolerable upper intake level (UL) after prenatal supplementation; and 3) To determine the effects of excessive FA exposure on placental health and function *in vitro*.

Methodology: Second trimester 3-day food records of pregnant women (N=216) were analyzed for micronutrient intake using ESHA Food ProcessorTM. Nutrient intake values were compared to established Dietary Reference Intake (DRI) values. In a series of experiments, the effects of exogenous folic acid (2-4000 ng/ml) on placental health and function were examined in two placental cell lines [HTR-8/SVneo (N=3) and BeWo (N=3)], and a human placenta explant model (N=6). Following a 48-hour incubation period, the effects of excessive folic acid exposure on placenta cell proliferation, viability, and apoptosis were determined, along with evaluation of placenta cell function via cell invasion and β -hCG hormone release assays.

Results: Through dietary sources alone, most pregnant women studied were consuming adequate levels of most micronutrients. However the majority of examined women (>50%) demonstrated a risk of dietary inadequacy for vitamin D, vitamin E, folate, and iron. In the examined cohort, 83% of study participants reported prenatal supplement usage. *In vitro* exposure of human placenta cells and explants to excessive FA concentrations resulted in no significant differences in cellular proliferation, apoptosis, invasion, or β -hCG hormone production. However, decreased cell viability was observed in BeWo cells at increased FA concentrations (200-2000 ng/mL).

Conclusion: Food sources alone do not appear to provide women in Canada with adequate intake of all micronutrients recommended for a healthy pregnancy. Though a prenatal supplement containing FA may be necessary for most women, current FA levels in many prenatal supplements may lead to excessive FA intake above the established UL. Yet, as measured in this study, high FA concentrations do not seem to adversely affect most primary indicators of placental cell health or function.

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. . . Thank you all!

Dedication

To my Well-Wishers

فَإِنَّ مَعَ الْعُسْرِ يُسْرًا ۖ
إِنَّ مَعَ الْعُسْرِ يُسْرًا ۖ

“So verily, with every difficulty, there is relief.

Verily, with every difficulty, there is relief.”

(Surah Al-Inshirah, 94:5-6)

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List of Abbreviations

Abbreviation	Term
5-MTHF	5-methyltetrahydrofolate
β -hCG	Human Chorionic Gonadotropin, β subunit
BM	Basal Membrane
BMI	Body Mass Index
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine Serum Albumin
CCHS	Canadian Community Health Survey
CHU	Centre Hospitalier Universitaire
CI	Confidence Interval
CK18	Cytokeratin-18
CNF	Canadian Nutrient File
CT	Cytotrophoblast
ddH ₂ O	Double-distilled water
DRI	Dietary Reference Intake
DFE	Dietary Folate Equivalent
DMEM	Dulbecco's Modified Eagle Medium
EAR	Estimated Average Requirement
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EVT	Extravillous Trophoblast
FA	Folic Acid
FBS	Fetal Bovine Serum

FFQ	Food Frequency Questionnaire
FR α	Folate Receptor α
g	Gravity
GDM	Gestational Diabetes Mellitus
HCl	Hydrochloric acid
IOM	Institute of Medicine
IRNPQEO	Integrated Research Network of Perinatology in Québec & Eastern Ontario
LOAEL	Lowest-Observed-Adverse-Effect Level
MVM	Microvillus Plasma Membrane
NHANES	National Health And Nutrition Evaluation Survey
NOAEL	No-Observed-Adverse-Effect Level
NTD	Neural Tube Defect
NE	Niacin Equivalent
OHRI	Ottawa Hospital Research Institute
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCFT	Proton-Coupled Folate Transporter
P/S	Penicillin-Streptomycin Solution
RAE	Retinol Activity Equivalent
RBC	Red Blood Cell
RCF	Relative Centrifugal Force
RDA	Recommended Dietary Allowance
RE	Retinol Equivalent

REB	Research Ethics Board
RFC	Reduced Folate Carrier
RPM	Revolutions per Minute
RPMI-1640	Rosewell Park Memorial Institute 1640
SACD	Subacute Combined Degeneration
SGA	Small-for-Gestational Age
SOGC	Society of Obstetricians and Gynaecologists of Canada
THF	Tetrahydrofolate
UL	Tolerable Upper Intake Level
USDA	United States Department of Agriculture
WST	Water Soluble Tetrazolium salts

Thesis Outline

- Chapter 1** Fundamental concepts are presented, followed by a summary of the rationale, hypothesis and specific research objectives of the project.
- Chapter 2** The first and second research objectives are presented in its entirety. Assessment of micronutrient intake during pregnancy through dietary sources are defined for pregnant Québec women in a large Canadian cohort. The results for this section are presented and followed by an in depth discourse addressing our findings.
- Chapter 3** The third research objective is presented in its entirety. The effects of excessive folic acid on the placenta are explored: first, using two placental cell lines (HTR-8/SVneo and BeWo cells); and second, using human placental explants. The results from these experiments are presented and followed by our interpretation and analysis of our findings.
- Chapter 4** A summary of the study findings is offered and insight into future directions are presented.

Chapter 1

Theoretical Foundations

In this chapter, the aim is to deliver a brief overview on folate metabolism and requirements; the Dietary Reference Intakes and the Risk Assessment Model; prevalence of folic acid supplementation during pregnancy; the available literature on high micronutrient intake; evidence for adverse outcomes due to high micronutrient intake levels; as well as the importance of the placenta and how high micronutrient intake may impact this vital organ of pregnancy. This overview will be followed by the rationale for the study, the overarching hypothesis and specific research objectives.

1.1 Folate and Folic Acid

Folate is the generic term for the water-soluble vitamin B₉, which is critical for one-carbon metabolism and is found in a range of chemical forms [1–3]. Naturally occurring folates (pteroylglutamate) are polyglutamates containing a string of one to six glutamate molecules, and are found in foods such as dark green leafy vegetables, liver, and citrus fruits [4, 5]. Before folate can be absorbed by the body, enzymes in the small intestine must remove all but one glutamate to produce folic acid [6–8]. Folic acid (pteroylglutamic acid) is the monoglutamate form of folate (folylmonoglutamate) and is the form used in fortified foods and vitamin supplements [3, 9]. Once taken up by enterocytes, folylmonoglutamate is hydrolyzed into 5-methyltetrahydrofolate (5-MTHF), the metabolically active form of folate found in circulation [10, 11].

Estimates suggest that about 50% of the folate naturally found in food is absorbed; whereas the bioavailability of folic acid is almost double that of folate due to the unnecessary requirement for enzymatic removal of excess glutamates [12, 13]. To account for differences in folate and folic acid bioavailability, the Dietary Reference Intakes (Section 1.2) represent units of folate as dietary folate equivalents (DFE), where 1 DFE = 1 µg food folate = 0.6 µg of folic acid from fortified food or as a supplement consumed with food = 0.5 µg of folic acid from a supplement taken on an empty stomach [12]. Thus, the source of folate is an important consideration when determining consumption levels in a population.

Acting as a methyl donor in one-carbon metabolism, folate derivatives are necessary for proper DNA, RNA, and protein methylation, as well as DNA synthesis and maintenance

(Figure 1) [14]. Once folylmonoglutamate is converted into 5-MTHF in the enterocytes with the help of enzymes and B-vitamin cofactors, 5-MTHF is transferred to peripheral tissues where vitamin B₁₂-dependent methionine synthase transfers the methyl group from 5-MTHF to methylate homocysteine to produce methionine and tetrahydrofolate (THF) [10]. Methionine is activated by ATP and adenosylmethyltransferase to produce SAM, which functions as a subsequent methyl-donor by methylating DNA, RNA, lipids, and proteins [10]. Alterations in methylation patterns due to improper folate intake, may adversely affect epigenetic regulation of gene expression [15, 16]. On the other hand, THF can be converted to 5,10-methyleneTHF by vitamin B₆-dependent serine hydroxymethyltransferase (SHMT) which simultaneously converts serine into glycine [10]. It is 5,10-methyleneTHF and its derivative 10-formyl-THF that is used in the process of DNA synthesis to respectively produce pyrimidines and purines [10]. Hence, it is understandable that adequate folate intake is essential throughout pregnancy for proper establishment of methylation patterns and DNA synthesis as required for rapid fetal growth and development.

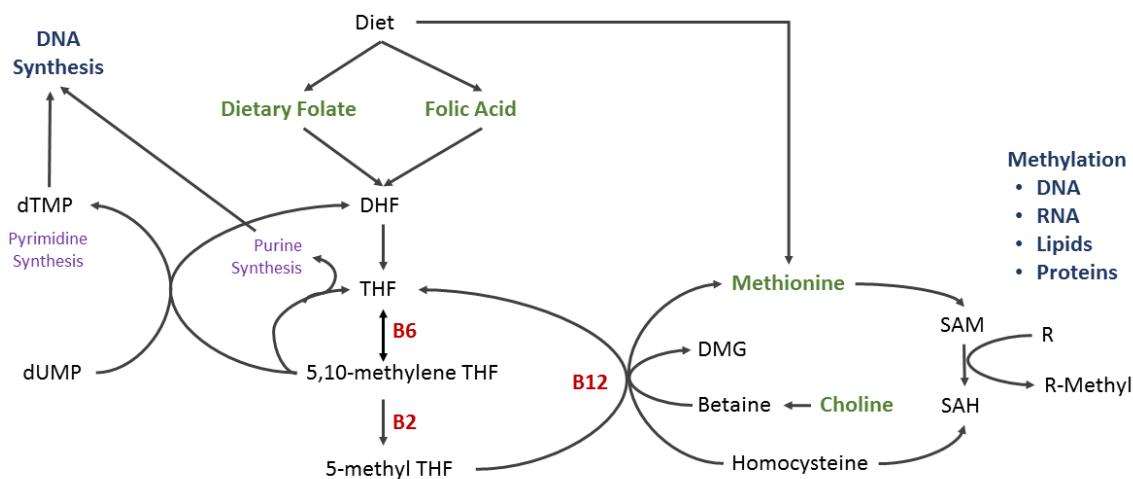


Figure 1. Folate metabolism. An outline of key players in the one-carbon metabolism pathway. In green, nutrients obtainable from dietary sources. In red, B-vitamin cofactors required for proper enzyme function in folate metabolism. Folate derivatives play a role in DNA synthesis as well as methylation of DNA, RNA, proteins, and lipids.

1.1.1 Folic Acid Supplementation within the Context of Pregnancy

Neural tube defects (NTDs), such as spina bifida and anencephaly, are a group of heterogeneous anomalies of the central nervous system caused by incorrect closure of the neural tube during embryogenesis. Initially, folic acid became implicated in NTDs when Thiersch (1952) used aminopterin, a folic acid antagonist, for therapeutic abortions in 12 women and observed fetal malformations in 2 cases of live birth where spontaneous abortion did not occur [17]. At the time, folate-deficiency was known to have a role in megaloblastic anemia, but eventually, Hibbard (1964) suggested that folate-deficiency may also have a role in NTDs [18]. In 1976, a landmark study by Smithells *et al.* [19] officially attributed nutritional deficiencies to be an important contributing factor in congenital defects, specifically finding that babies born to folate-deficient mothers are more likely to have NTDs. This led to increased investigation to determine the protective effects of folic acid supplementation to prevent NTDs.

In 1991, the Medical Research Council (MRC) published results from an international randomised double-blind prevention trial, which demonstrated that when women, who had already had an NTD-affected child from a previous pregnancy, were given 4 mg of periconceptional folic acid, 72% of NTDs were prevented in the second child [20]. In a subsequent large randomised controlled study, periconceptional 0.8 mg folic acid-containing multivitamin intake was shown to be significantly effective in preventing first NTD occurrence [21]. However, other studies have shown that doses as low as 0.36 mg [22] to 0.4 mg [23] of daily folic acid supplementation may be effective in NTD prevention.

Since the discovery of folate's role in helping prevent NTDs [20, 21, 24], 76 countries, including Canada, have instated nationwide fortification programs in which at least one grain product (wheat flour, maize, or rice) is fortified with folic acid [25]. This is a unique example of a public health policy, in which the entire population is exposed to folic acid fortified foods to ensure adequate folic acid intake in women of child-bearing age. In 1996, voluntary fortification of white flour, cornmeal, and enriched pasta was permitted in Canada [26]. However, since 1998, Canada implemented mandatory folic acid fortification for all white flour, cornmeal, and enriched pasta [27]. This program has been effective, resulting in a 46% decrease in the rate of NTDs among Canadians [28]. However, Health Canada additionally recommends women who are pregnant or planning to become pregnant to also take a daily multivitamin supplement containing 0.4 mg of folic acid [29], while the Society of Obstetricians and Gynaecologists of Canada (SOGC) recommends up to 1 mg of additional

folic acid [30]. This discrepancy in recommended folic acid intake warrants further research to determine the appropriate folate intake level during pregnancy.

Health Canada has also identified a high risk population of women, who are recommended to take higher than normal doses of FA throughout pregnancy [31]. This high risk population includes women with a personal or family history of NTDs, women with pre-gestational or gestational diabetes, low dietary folate intake, Hispanic ethnicity, obese women, and women who consume alcohol during pregnancy [32–34]. At-risk women are recommended to take 1-5 mg of folic acid supplementation (in combination with vitamin B₁₂) for 3 months prior to becoming pregnant as well as during the first trimester [35]. This increased dosage was established based upon findings of a reduced recurrence of NTDs following this FA supplementation regimen [20, 36].

Despite the numerous recommendations promoting folic acid supplementation in pregnancy, there has been little work done in regards to the safety of high FA intake from supplementation on the developing fetus. Due to the important role of folate in biological process such as DNA replication and methylation, it is critical that a comprehensive evaluation of safe and efficacious therapeutic windows of FA supplementation is conducted in order to ensure that clinical recommendations provided to pregnant women are safe to them and their unborn children.

In Section 1.2, below, key definitions and calculations used to define micronutrient adequacy, deficiency, and excessive intake will be described and applied to the case of folate and folic acid.

1.2 Dietary Reference Intakes (DRIs)

Dietary Reference Intakes (DRIs) are the most recent set of nutrient intake standards developed by the Institute of Medicine's Food and Nutrition Board to aid proper diet assessment and planning in Canada and the United States [37]. All DRI values were determined by considering indicators of good health, prevention of chronic disease, and observations of adverse health outcomes due to excessive nutrient intake. Depending on data availability, each nutrient has a set of DRIs including an Estimated Average Requirement (EAR) and either a Recommended Dietary Allowance (RDA) or an Adequate Intake (AI) that indicate the recommended daily nutrient intake, based on physiological requirements, for healthy individuals [37]. Additionally, using the Risk Assessment Model, a Tolerable Upper Intake Level (UL) has been recognized for many nutrients and identifies a nutrient

concentration above which adverse health outcomes may become apparent [37]. These DRI values are used to determine the risk of inadequacy, adequacy, and excessive intake in a population (Figure 2). Key terminology is described below and summarized in Table 1 (p.12).

The ***Estimated Average Requirement (EAR)*** is the median nutrient intake value that is estimated to meet the physiological requirements of 50% of healthy individuals in a particular life stage (defined by biological sex, age, and life events such as pregnancy and/or lactation). The remaining 50% of individuals would not meet the nutrient requirements. The EARs for different nutrients were determined by Expert Nutrient Review Panels for each nutrient (ex. The Panel on Folate, Other B Vitamins, and Choline) and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes (i.e. The DRI Committee) at the Food and Nutrition Board, Institute of Medicine [12].

Considerations used to determine the DRI values include nutrient bioavailability, nutrient-nutrient interactions, evidence for risk of developmental abnormality or disease reduction, as well as the available data from the literature involving metabolic, observational, and epidemiological studies [12]. The quality of the available studies were also considered by taking into account the study design, method for intake measurement, and any other confounding factors [38]. In the case of folate, the EAR, in dietary folate equivalents (DFE), was determined to be 0.32 mg DFE/day for both men and women over the age of 19 [12]. Though other factors were considered, this value was based largely on a 1995 study by O'Keefe *et al.* [39], which identified folate intakes deemed to be sufficient to maintain normal blood concentrations of erythrocyte folate, serum folate, and plasma homocysteine over a period of 70 days in 50% (2-3 women) of the 5-6 non-pregnant women in each group in the study.

In pregnant women, there is increased need for folate to support DNA synthesis due to uterine enlargement, placental development, increased maternal erythrocyte count, and fetal growth [40]. Preferential fetal folate usage, over maternal usage, has been suggested by findings of increased circulating folate levels in fetuses and newborns compared to the maternal circulation [41, 42]. To determine the EAR for the pregnant population, population-based studies using the erythrocyte folate count [43–45], reflecting tissue stores of folate, and serum folate levels [46–50] were used as the primary indicators for adequacy among healthy pregnant women. A diet-controlled metabolic study [51] and the urinary excretion of folate breakdown products [52] using healthy pregnant and non-pregnant women were

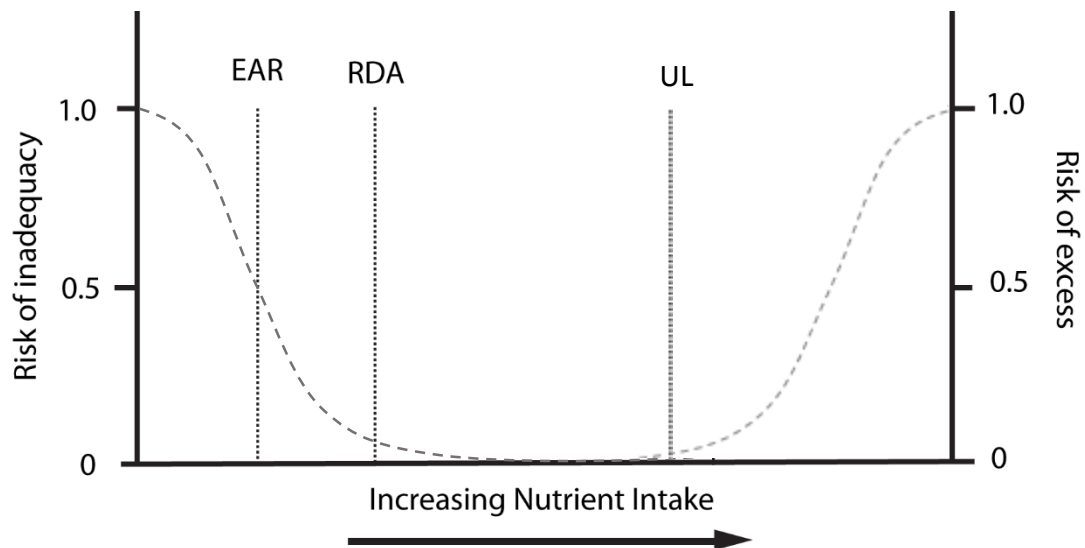


Figure 2. Dietary Reference Intakes (DRIs). Graphical representation of the relationship between the different Dietary Reference Intake (DRI) values. The Estimated Average Requirement (EAR) refers to the nutrient intake level at which risk of nutrient inadequacy is 0.5 (50%) for an individual in a sex-specific life stage group. The Recommended Dietary Allowance (RDA) refers to the nutrient intake level at which the risk of nutrient inadequacy is minimal (2-3%) for an individual in a sex-specific life stage group. Nutrient Intake levels that exceed the Tolerable Upper Intake Level (UL) are deemed to have associated increasing risk of nutrient excess and possible adverse health outcomes.

Source: © All rights reserved. Using the Dietary Reference Intakes. Health Canada, 2010 [38, 320]. Adapted with permission from the Minister of Health, 2014.

also used as evidence to determine the EAR [12]. Since studies demonstrated that 0.2 mg DFE/day is insufficient to support the average folate status in pregnant women [46–50], this intake level was simply added to the established EAR of 0.32 mg DFE/day for non-pregnant women to ensure adequate intake. Thus, the EAR for pregnant women was established to be 0.52 mg DFE/day to support maintenance of maternal tissue stores of folate, despite high fetal demand, in order to prevent megaloblastic anemia [12]. It is important to note, that at the time of EAR determination, limited data was available and a more robust analysis may be necessary.

The EAR is used to calculate the **Recommended Dietary Allowance (RDA)**. The RDA refers to the average daily dietary intake level that is enough to satisfy the required nutrient intake of 97-98% of healthy individuals in a particular sex-specific life stage group. If the EAR is normally distributed and the standard deviation (SD) is available, the EAR value is used to determine the RDA as follows: $RDA = EAR + 2SD_{EAR}$. However, due to limited data on the variability of requirements for folate, a coefficient of variation of 10% was used instead. Based on evidence used to establish the EAR, the RDA for folate in men and non-pregnant women over 19 years of age is 0.4 mg DFE/day, and is 0.6 mg DFE/day for pregnant women [12, 39, 51, 52].

When there is not enough scientific evidence available to determine the EAR and calculate the RDA values, the **Adequate Intake (AI)** is determined. The AI is based on observed or experimental estimates of nutrient intake by a group (or groups) of healthy individuals. For example, scientific evidence is scarce for the determination of an EAR, and hence an RDA, for pantothenic acid, manganese, potassium, and sodium [12, 53, 54]; thus, for these micronutrients, an AI is instead determined. Due to limited data used to determine the AI, the risk of inadequate intake is only determined for micronutrients with an EAR.

Finally, the **Tolerable Upper Intake Level (UL)** is the maximum daily nutrient intake that is unlikely to pose a risk of adverse health effects to almost all individuals in the general population. Chronic nutrient consumption above the established UL increases the risk for adverse health outcomes (Figure 2) [38]. The Risk Assessment Model, developed by the National Research Council, has been a crucial tool in the determination of the UL for many nutrients by the Subcommittee on Upper Reference Levels of Nutrients (i.e. the UL Subcommittee) at the Food and Nutrition Board, Institute of Medicine (IOM) [12, 38, 55]. This method of deriving the UL will be explored in further detail while highlighting a case study on the determination of the UL for folic acid (1 mg) in Section 1.2.1.

1.2.1 The Risk Assessment Model: Identifying the UL for Folic Acid

The Risk Assessment Model was developed by the National Research Council and involves characterizing the nature and likeliness of adverse effects resulting from human exposure to *agents* in the *environment* [55]. For the purposes of the current research project, the agents considered are micronutrients (i.e. folate), while the environment includes food, water, nutrition supplements, and other pharmacologic preparations.

To identify the likeliness of adverse effects for specific micronutrients, the Risk Assessment Model for Nutrient Toxicity involves using the available literature to identify any potential hazard due to nutrient consumption, analyse dose-response assessments to determine any association between nutrient intakes (dose) and the incidence or severity of the effect (response), and also assess the state of a population's general nutrient intake level [38]. Based on these factors, the level of risk is identified and a UL is established.

In most cases, ULs were established based on data examining total nutrient intake from multiple sources, such as food, water, and supplements [38]. However, some ULs were established based solely on evidence related to nutrient intake from supplements and/or fortified food alone. This difference in adverse health effects across different nutrient sources is likely the result of differences in bioavailability and the chemical form of the nutrient, the timing of intake, and/or the amount consumed each time [38]. These differences can affect nutrient-nutrient interactions and result in alterations in absorption, metabolism, excretion, transport, storage, and/or the function of other nutrients.

For example, when determining the DRIs for folate in 1998, there was no evidence indicating adverse health effects from excessive consumption of naturally occurring folate from food sources [38, 56]. However, at the time, evidence suggested that excess folic acid consumption from supplements and fortified foods could result in adverse health effects, particularly neurological damage by masking vitamin B₁₂ deficiency [57–65]. Thus, a UL for naturally occurring folate from food was not identified, but was instead, established for folic acid only.

Using the Risk Assessment Model, it is assumed that there is no risk of adverse effects unless chronic nutrient intake surpasses a threshold level [38]. This threshold level for each nutrient is identified as the Tolerable Upper Intake Level (UL) to protect most of the population. However, in most cases, acute intake in the short-term does not pose a threat [38]. Ideally, the ***no-observed-adverse-effect level (NOAEL)***, which is the highest intake of a

nutrient that does not seem to result in adverse health effects, is used to calculate the UL [38]. However, when there is not enough information to determine a NOAEL, the ***lowest-observed-adverse-effect level (LOAEL)*** may be used instead [38]. The LOAEL is the lowest intake level of a nutrient where an adverse effect on health has been observed.

The UL is derived based on the NOAEL or LOAEL along with any identified uncertainties, referred to as **Uncertainty Factors (UFs)** [38]:

$$UL = \frac{NOAEL \text{ (or LOAEL)}}{UF}$$

UFs arise due to incompleteness of the data. They take into consideration uncertainties identified during the risk assessment when establishing the UL. For example, in the absence of sufficient data for nutrient intake in children, data may be extrapolated based on proportional body weight from information available for adults. Furthermore, in many cases, inferences are made from experiments using lab animals. Based on the number and the degree of uncertainties, a numerical value (1.0 – 10.0) is assigned and referred to as the UF for the particular nutrient. Though somewhat arbitrary, lower UFs indicate a smaller degree of uncertainty; whereas higher UFs indicate a higher degree of uncertainty of the adverse health effects on the general population at a given intake.

To determine the UL for folic acid, the UL Subcommittee, with collaboration from the DRI Committee and nutrient experts in the Food and Nutrition Board (IOM), reviewed existing literature describing the hazardous health effects of high folic acid intake [38]. The health effects specifically examined included: neurological effects [57–65], reproductive and developmental effects [21, 36, 66–73], carcinogenicity [74, 75], hypersensitivity [76–78], and intestinal zinc absorption [56, 79–83]. Based on the data available and analysed, it was determined that the main risk factor to consider in the establishment of the UL for folic acid was neuropathy resulting from folic acid-masked vitamin B₁₂ deficiency [38].

The studies used to determine the reproductive and developmental health effects of high intake of folic acid, specifically evaluated the effects of 0.4–5.0 mg/day of periconceptional folic acid supplementation on the prevention of NTDs [21, 36, 66–73]. Though the sample sizes were large in many of these examined studies (ranging from 81 – 5,502 women per study), they were not designed with a primary outcome of determining risk of adverse short- and/or long-term health outcomes for mothers or the infants. The authors for the folic acid risk assessment admit lack of systematic evidence eliminating

adverse reproductive and developmental effects of excessive folic acid intake and advised further research [38]. Nonetheless, since no adverse reproductive outcomes were specifically reported at the time, the focus in the determination of a UL for folic acid remained on the ability of high folic acid intake to mask vitamin B₁₂ deficiency resulting in neurological deficits.

Pernicious anemia, due to vitamin B₁₂ deficiency, can be treated by folic acid therapy to improve symptoms of anemia. Though this is a cure for the anemia in the short-term, the vitamin B₁₂ deficiency remains and if the deficiency is continued over the long-term, it can result in serious neurological damage such as subacute combined degeneration (SACD) of the spinal cord degeneration and eventual death [84]. Therefore, to prevent masking a vitamin B₁₂ deficiency with excessive folic acid intake, both animal and human studies were used to establish the UL for folic acid [38].

The animal studies, using monkeys and fruit bats, demonstrated that vitamin B₁₂ deficient animals developed symptoms of neuropathy earlier than controls if supplemented with folic acid [57–59]. Across 23 human studies analysed by the UL Subcommittee at the Food and Nutrition Board (IOM), 142 individuals (N=250) reported neurological complications following intake of 5 mg/day or more of folic acid [38]. Each study reported the effects of different doses (5-500 mg/day) of folic acid among 1-48 individuals (including both males and females), over varying time periods (22 days – 10 years) [38]. In an additional 8 human studies, 13 individuals (N=17) reported neurological problems at folic acid intake levels less than 5 mg/day (0.33 – 4.5 mg/day) [38]. Using these studies, there was insufficient data to derive a NOAEL; thus, a LOAEL of 5 mg of folic acid/day was determined [38].

Based on the available data, the uncertainty factor of 5.0 (where 1.0 is the lowest degree of uncertainty on a scale of 1.0 - 10.0) was assigned due to increased risk of severe neurological effects, lack of clear evidence for all patient populations, and using the LOAEL instead of the NOAEL to derive the UL. With an uncertainty factor of 5.0, the UL was determined to be 1 mg of folic acid/day for all individuals over the age of 19 using the following calculation:

$$\begin{aligned} \text{UL}_{\text{folic acid}} &= \text{LOAEL}/\text{UF} \\ &= 5 \text{ mg}/5.0 \\ &= 1 \text{ mg} \end{aligned}$$

It is important to note that one third of the 31 studies were individual case studies [85–94], while only seven had 15 or more study participants [62, 63, 93, 95–98]. Furthermore, all 31 of these studies, except one in 1990 [61], were done between 1947-1961, prior to official fortification of grains in Canada [27]. At the time of UL establishment, since there was no evidence for adverse effects in pregnant and lactating women due to folic acid intake, the same UL of 1.0 mg/day was kept for pregnant and lactating women as well as the general adult population [38]. For children, the UL was adjusted based on relative body weight and no UL was established for infants due to lack of data [38]. Thus, interpretation of the UL, for example in the establishment of clinical guidelines for supplement usage, must be performed with caution, as the established ULs are calculated and established using a degree of ambiguity.

For some nutrients, such as thiamine, riboflavin, pantothenic acid, and vitamin B₁₂, a UL has not been recognized due to insufficient data. Nevertheless, risk of adverse effects due to high intake is still possible though the UL has not been established. Of considerable relevance to the current project, pregnancy was not a priority when ULs were determined for many micronutrients. It could certainly be argued that micronutrient intake levels found to be safe for the general population may not be safe for pregnant women, where the health and safety of a developing fetus must also be considered.

The dated studies and ambiguous nature of the currently established UL for folic acid, particularly in relation to the pregnant and lactating population of women is reason for concern. Currently, only approximately 1% of the adult Canadian population demonstrate metabolic vitamin B₁₂ deficiency [99], suggesting it is no longer of primary concern with relation to high folic acid intake during pregnancy. Furthermore, at present, prenatal multivitamins supplements, such as *Materna*TM, contain formulations that consist of many micronutrients including both folic acid and vitamin B₁₂ to help prevent masking any vitamin B₁₂ deficiency (Table 2). As a result, the UL for folic acid may need to be revised to additionally consider the post-fortification dietary intake of folic acid among pregnant women. Furthermore, the effects of excessive folic acid during pregnancy on fetal health and development in the short and long-term requires further investigation.

Table 1. DRIs and Risk Assessment: Key Definitions

Abbreviation	Definition
DRI	Set of nutrient standards developed by the IOM.
EAR	Median nutrient intake value that is estimated to meet the daily physiological requirements of 50% of healthy individuals in a particular life stage group.
RDA	Average daily dietary intake level that is enough to satisfy the required nutrient intake of 97-98% of healthy individuals in a life stage group.
AI	Recommended average daily nutrient intake level based on observed or experimentally determined estimates of nutrient intake in a life stage group.
UL	Maximum daily nutrient intake that is unlikely to put almost all individuals in a population at risk of adverse health effects.
NOAEL	Highest intake of a nutrient that does not seem to result in adverse health effects.
LOAEL	Lowest intake level of a nutrient where an adverse effect on health has been observed.
UF	Uncertainty factor are considered when determining the UL.

1.3 Folic Acid Supplementation during Pregnancy

As mentioned in Section 1.1, the benefits of folic acid in preventing NTDs has led to fortification of all white flour, cornmeal, and enriched pasta in Canada, as well as the recommendation to take a folic acid-containing supplement (0.4-1.0 mg/day) for women who are planning to become or currently are pregnant [27, 29]. In this section, an overview of the literature pertaining to micronutrient supplementation in the pregnant population will be discussed with particular attention focused on the prevalence of excessive folic acid supplementation in this population.

Prenatal multivitamin supplementation, in addition to diet, leads to micronutrient intake levels that exceed the UL for several vitamins and minerals [100]. A survey performed on the micronutrient intake of 222 pregnant women in the United States showed that on average, women consumed 2-5 times the RDA for 10 vitamins and 5 minerals and had exceeded the UL for iron due to dietary sources and supplementation [100]. Furthermore, the total intake of folate and niacin from dietary and supplemented sources also surpassed the established UL [100], though only intake from supplemental sources pose a risk. Baker *et al.* [101] demonstrated that pregnant American women had hypervitaminemic circulating levels (above the reference range using non-pregnant, non-supplemented women) for folate, biotin, pantothenate, and riboflavin due to supplementation in all three trimesters of pregnancy. Taken together, these studies demonstrate a common denominator where pregnant North American women are consuming high levels of folate after supplementation. Thus, current dietary and supplementary micronutrient intake in the Canadian population requires detailed investigation to support adequate micronutrient intake, particularly folate, needed for a healthy pregnancy.

*Materna*TM is one of the most common prenatal multivitamins taken by pregnant Canadian women [102]. One daily *Materna*TM supplement is able to provide 100% of the RDA for most vitamins and minerals in the supplement (Table 2). Specifically, following a comparison of the RDA for folic acid for healthy singleton pregnancies (0.6 mg DFE/day) and the folic acid present in one daily *Materna*TM supplement (1 mg folic acid), and considering foods fortified with folic acid, there is growing concern for excessive folic acid intake in the pregnant population. Considering the bioavailability of folic acid, this supplement alone is able to provide pregnant women with 278% of the RDA (in DFE). Interestingly, the recommendations promoting prenatal folic acid supplementation for pregnant women do not include consideration of the diet of the individual mother, including her consumption of

Table 2. Nestlé® Materna™ vs. Recommended Dietary Allowance (RDA)

Nutrient	Nestlé® Materna™	RDA	UL	Percent of RDA (%)
Vitamin A (µg RAE) ^a	425	770	--	94
Vitamin A (µg RE) ^b	300	--	3000	--
Thiamine (mg)	1.4	1.4	--	100
Riboflavin (mg)	1.4	1.4	--	100
Niacin – NE (mg) ^c	18	18	35 ^e	100
Pantothenic Acid (mg)	6	6*	--	100
Vitamin B ₆ (mg)	1.9	1.9	100	100
Vitamin B ₁₂ (µg)	2.6	2.6	--	100
Vitamin C (mg)	85	85	2000	100
Vitamin D (µg)	10	15	100	67
Vitamin E (mg) ^d	13.5	15	1000 ^e	90
Folate (ug DFE)	--	600	--	278
Folic Acid (µg)	1000	--	1000 ^e	--
Calcium (mg)	250	1000	2500	25
Copper (mg)	1	1	10	100
Iron (mg)	27	27	45	100
Magnesium (mg) ^f	50	350, 360	350 ^e	14
Manganese (mg)	2	2*	11	100
Selenium (µg)	30	60	400	50
Zinc (mg)	7.5	11	40	68

^a As retinol activity equivalents (RAEs); where 1 µg RAE = 1 µg retinol RE = 12 µg β-carotene. The RAE for preformed vitamin A is the same as RE.

^b As retinol equivalents (RE); where 1 µg RE = 1 µg preformed vitamin A, which is used to establish the UL.

^c As niacin equivalents (NE); where 1 mg NE = 1 mg of niacin = 60 mg of tryptophan.

^d As α-tocopherol.

^e The ULs for niacin, folic acid, and vitamin E apply to synthetic forms obtained from supplements, fortified foods, or both.

^f The EAR and RDA values listed for magnesium are from food sources for pregnant women ages 19-30 and 31-50 years old respectively. The UL for magnesium applies only to the supplemented form found in medication (pharmacological agents) and dietary supplements.

* Adequate Intake (AI) values. RDAs have not been established for these micronutrients.

Sources: Nestlé® Materna™ label [185] and the Dietary Reference Intakes (DRIs) established by the Institute of Medicine's Food and Nutrition Board. DRIs for calcium, phosphorous, magnesium, vitamin D established in 1997; thiamin, riboflavin, niacin, vitamin B₆, folate, vitamin B₁₂ in 1998; vitamin C, vitamin E, selenium in 2000; vitamin A, copper, iron, and zinc in 2001; and calcium and vitamin D DRIs were updated in 2011. Values listed are established for women between the ages of 19-50 years old during pregnancy.

folate-rich and/or folic acid fortified foods. With a higher flour fortification level in Canada (0.15 mg/100 g) than in the United States (0.14 mg/100 g) [27, 103], but with both countries recommending at least an additional 0.4 mg of folic acid from supplements [29, 104], the concern for excessive folic acid intake in the pregnant population is pertinent.

Sufficient consumption of folic acid enriched foods place specific populations of individuals, particularly children, at risk of exceeding the UL for folic acid [105]. Using data collected by the National Birth Defects Prevention (NBDP) Study in the United States, factors determined to be associated with preconceptional folic acid-containing supplement usage include ethnicity, education, maternal age, employment, and income [106]. Women of Caucasian descent with higher socioeconomic status, a later-in-life or subsequent pregnancy, and higher levels of education and income are more likely to take a folic acid-containing supplement [107–109]. Following Health Canada's recommendations, it has been determined that the majority of Canadian women (80-97%) of higher socioeconomic status in the urban cities of Ontario and Alberta are compliant in taking a prenatal supplement with folic acid during their pregnancy [102, 107–109].

However, conflicting results using data from the nationally-representative 2004 Canadian Community Health Survey (CCHS) revealed that, in general, a low percentage (15-29%) of Canadian women of reproductive age are taking a folic acid-containing supplement [110]. This discrepancy suggests that there is a large segment of the population (ex. among younger women of lower socioeconomic status, less education, and/or being born outside of Canada) who are at risk of inadequate folic acid intake [111]. Thus, further studies are necessary to determine an accurate representation of the dietary folate intake and prevalence of folic acid-containing supplement use among Canadian women, as well as to target populations most at risk for low folate intakes.

Nonetheless, though measuring micronutrient consumption is one method of determining folate intake adequacy, red blood cell (RBC) folate functions as a measurement of long-term tissue stores of folate. Using the 2007-2009 CCHS, 78% of women of reproductive age demonstrate red blood cell (RBC) folate levels above that considered optimal for maximum NTD risk prevention (>906 nmol/L) [112]. Furthermore, 40% of women of childbearing age (15-45 years) had high RBC folate concentrations (>1360 nmol/L) [112]. This suggests that dietary folate intake may be sufficient for the majority of women in Canada and recommendations for supplementation may require reassessment.

In addition to these high intake levels, women who are at increased risk of NTDs and some other health risk factors are recommended to take up to 5.0 mg/day of folic acid three months pre- and post-conception [35, 113]. Data from the Québec Pregnancy Registry has suggested that though high dose periconceptional folic acid (5 mg) use has increased from 0.17% to 0.80% ($p < 0.05$) over the last decade, the prevalence of congenital malformations also increased by 15% (3.35% to 3.87%, $p < 0.05$) [114]. Currently, there is a folic acid clinical trial (FACT study) undertaken at the Ottawa Hospital Research Institute (OHRI) to determine whether a high dose (4.0 mg/day) of folic acid supplementation will reduce the incidence of preeclampsia, the most common complication associated with pregnancy [115]. Though benefits to folic acid supplementation during pregnancy are evident, more research is required to explore the adverse effects of folic acid and other micronutrient intake levels in order to determine an appropriate and safe range of supplementation.

1.4 Adverse Effects of Excessive Folic Acid Consumption

With growing interest in the use of folic acid supplementation to improve birth outcomes, it is essential that a comprehensive investigation into the safe therapeutic window is also undertaken. Currently, there are several animal and human studies providing evidence of adverse pregnancy outcomes resulting from exposure to excessive micronutrient intake. However, the adverse effects of high folic acid levels during pregnancy, including short-term and long-term effects on fetal metabolism and development, have only been explored in a limited number of studies. In this section, a brief synopsis of the literature available will be provided.

1.4.1 Adverse Effects of Excessive Micronutrient Consumption

Several human and animal studies indicate adverse effects of high micronutrient intake levels on their offspring. In comparison to pregnant rats on a standard diet, pregnant rats who consumed 10x their regular daily vitamin content had male offspring that displayed increased food intake after weaning, components of the metabolic syndrome (ex. Increased levels of fasting glucose, insulin, ghrelin), an 8% increase in body weight, a 27% increase in fat pad mass as well as offspring with altered short-term and long-term tissue fatty acid concentration [116, 117]. Specific micronutrients have also been linked to adverse embryonic development and fetal outcome. For example, exposure to high levels of a vitamin A-derivative (i.e. retinoic acid) during embryonic development can negatively affect the development of kidneys in hamster and mice models [118, 119]. Though necessary

during pregnancy, high levels of iron intake have also been found to be teratogenic to the brain, particularly during day 8 and day 9 of embryonic development [120]. Excessive iron consumption has also been linked to increased risk for SGA babies [121], decreased zinc absorption [122], oxidative stress [123, 124], and gastrointestinal symptoms [125]. Furthermore, excessive iodine intake during pregnancy has been associated with hyperthyrotropinemia in newborns and their mothers [126], as well as adverse effects during postnatal neurodevelopment in rat offspring [127]. Recent studies have also demonstrated that excessive maternal sodium intake during pregnancy may negatively impact postnatal brain development in rat offspring by altering protein expression in the neonatal cerebral cortex and hippocampus [128], as well as metabolism in the mother and offspring [129]. These studies demonstrate that consumption of high levels of essential micronutrients during pregnancy can be harmful.

Periconceptional nutrition has also been shown to affect fetal and postnatal development through epigenetic modifications of the genome by altering methylation patterns [130]. Without causing any genetic change, these epigenetic changes can result in long lasting effects on health by affecting gene expression. During early embryogenesis and fetal development, nutrients that function as methyl donors and cofactors are required in order to allow for the establishment of proper DNA methylation patterns [131, 132]. These nutrients include, but are not limited to, choline, vitamin B₆, folic acid, vitamin B₁₂, and methionine [131]. Improper establishment of the fetal epigenome, through aberrant nutrient availability throughout gestation, can impact gene expression patterns in the offspring in both the short and long term, and has been implicated in establishment of adult onset diseases in the offspring [133–135].

For example, a pivotal study performed on black Agouti mice demonstrated that a diet with high intakes of methyl donors, such as folate, vitamin B₁₂, betaine, and choline during pregnancy led to an alteration of gene expression and subsequently, an altered phenotype in the offspring [136]. This study was able to demonstrate that vitamin intake during pregnancy has direct repercussions in the metabolism and development of offspring. In another study, dams that were fed a high multivitamin diet showed evidence of having offspring with epigenetic hypermethylation on T cells, resulting in a decrease in T cell chemokine receptor expression [137]. Ultimately, this indicates that high micronutrient levels during pregnancy would result in a decreased response to pro-inflammatory signals in the mice offspring [137].

In a randomised placebo-controlled trial with 2404 pregnant women, it was found that high-doses of antioxidants, vitamin C and E, were correlated to an increased rate of low birth weight babies [138]. Additionally, high levels of vitamin C and E were shown to alter placental function and affect placental immunity using an *in vitro* culture model [139]. This evidence suggests that excessive intake of some vitamins may induce placental stress response pathways. However, little to no work has been done to study the relationship of high intake levels of other micronutrients on the placental health and function.

1.4.2 Adverse Effects of Excessive Folic Acid Consumption

Specific to folic acid, animal studies have shown that excessive consumption during pregnancy may be harmful during embryonic development in mice [140], leading to increased risk of mammary tumorigenesis, altered synaptic transmission, and increased seizure susceptibility in rat offspring [141, 142]. Studies on newborn piglets exposed to excessive folic acid *in utero* also demonstrated abnormal expression of several hepatic proteins involved in metabolic regulation, oxidative stress responses, and cell migration [143]. In human studies, excessive folic acid consumption during pregnancy has been associated with increased risk of respiratory disease [144], asthma, atopic dermatitis, and bronchiolitis in childhood for the offspring [145], though controversial data also suggests no association between folic acid and asthma [146]. Nonetheless, maternal folic acid supplementation among a genetically vulnerable subset of the population of pregnant women is associated with increased risk of early childhood retinoblastoma in the offspring [147]. It is possible that many of these effects are linked to epigenetic modifications that occurred during maternal folic acid intake during pregnancy.

Methylation of CpG islands in a gene's promoter region silences gene expression [15]. As a result, increased or decreased intake of methyl-donors (i.e. folate, choline, and methionine) may result in hypo- or hypermethylation of DNA and cause alterations in gene expression [136, 148–152]. Decreased folate intake has been shown to be linked to DNA hypomethylation of the p53 gene of rats [152], lymphocytes in postmenopausal women [153], and cervical tissue in adult women [154]. Decreased plasma folate, and subsequent DNA hypomethylation, has also been linked with gastric carcinogenesis in humans [155]. Similarly, increased maternal folate intake has been associated with altered global DNA methylation in the brain of offspring using a mouse model, including significantly different alterations in DNA methylation on several genes between male and female pups [156]. Increased maternal folate intake has also affected DNA methylation on genes associated with

neural growth and development [157], spinal cord regeneration [158], and lymphoblastoid cells [159]. As a result, there is growing concern in determining the appropriate window and dose of folic acid supplementation for proper fetal development and short- and long-term health outcomes for the offspring.

1.5 The Placenta

Maternal nutrition is a crucial factor that affects fetal development, growth, and health. In contrast to the postnatal environment where individuals control their nutrient intake, fetal nutrient status is strongly influenced by maternal nutrition, metabolism, and hormonal regulation. However, placental health and function ultimately dictates how the fetus will develop and grow across gestation, as the placenta regulates all transport and exchange of gases, nutrients and metabolites between the mother and fetus [160], and its endocrine function helps to promote appropriate maternal adaptation to pregnancy [161].

Structurally, the basal plate of the human placenta faces the maternal uterine endometrium; whereas the placenta's chorionic plate faces the fetus (Figure 3). In between these two plates lies the intervillous space, the site of nutritional exchange, where maternal blood is deposited. Floating within the intervillous space are placental villous trees that encase fetal capillary beds. Maternal blood in the intervillous space bathes the villous trees, thereby allowing the exchange of nutrients and molecules to occur between the mother and fetus [160].

The placenta is entirely fetal in origin and the primary cell type of the placenta is the trophoblast cell. During placental development, the trophoblast progenitor cells differentiate into two functionally distinct cell types: 1. The villous cytotrophoblast (CT) cells, or 2. The extravillous trophoblast (EVT) cells [162]. The CT cells continue to proliferate throughout gestation and terminally differentiate into an overlying multinucleated syncytiotrophoblast layer, which is in direct contact with maternal circulation. This important cellular layer is directly responsible for mediating nutrient, gas, and waste exchange between the maternal and fetal circulation [160]. The EVT cells are responsible for invading the uterine wall, thereby anchoring the placenta, and play an active role in remodelling the uterine vasculature, establishing an optimal utero-placental blood flow throughout gestation [160].

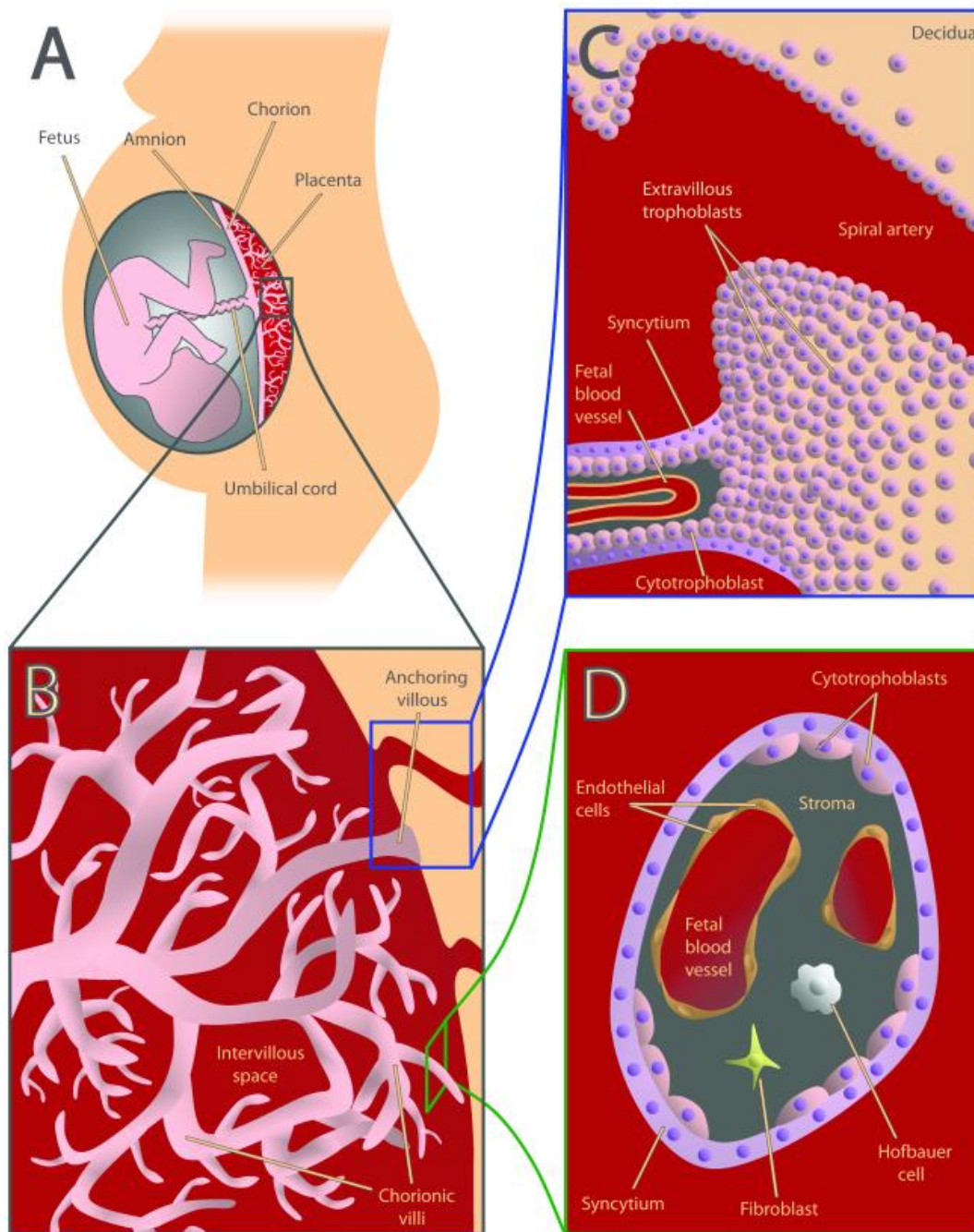


Figure 3. Key structures of the Placenta. (A) A representative rendering of the fetus and placenta during pregnancy. (B) The branched chorionic villous tree (pink) encasing the fetal capillaries and bathed in maternal blood found in the intervillous space. (C) The extravillous trophoblasts (EVTs) of the anchoring villi invade into the maternal uterine wall (decidua) to remodel the uterine spiral arteries. (D) A cross-section of the chorionic villous tree, demonstrating the underlying proliferative villous cytotrophoblast cells (CTs) and the overlying multinucleated syncytium (SynT) in direct contact with the maternal blood in the intervillous space. **Source:** Figure reproduced with permission from Dr. Julien Yokell-Lelièvre at The Ottawa Hospital Research Institute (OHRI).

1.5.1 Placental Folate Transporters

The placenta acts as a selectively permeable barrier that prevents direct interaction between the maternal and fetal blood supply, but facilitates the exchange of waste products, nutrients and other necessary molecules via passive diffusion and active transport [163]. Nutrients such as potassium, magnesium, calcium, and phosphate ions are actively transported across the placenta [163]; however, for other micronutrients, there are interspecies differences in placental transport. For example, movement of sodium and chloride ions occurs passively in sheep, but in rats, sodium ions are actively transported to the fetus and chloride ions are exchanged via a carrier and/or ion channel [163].

To maintain the transport of folate/folic acid across the placenta to promote fetal development and growth from early on in pregnancy, there are several transporters present within the human placenta (Figure 4): 1. Folate receptor α (FR α), 2. Proton-coupled folate transporter (PCFT), and 3. Reduced folate carrier (RFC) [164]. Each transporter has varying degrees of affinity and transport capacity for folates. The FR α and PCFT are co-localized on the microvillus plasma membrane (MVM) of the syncytiotrophoblast and actively move folate from the maternal circulation into the placenta via endocytosis [164]. In contrast, the RFC is located on both the basal membrane (BM) and MVM of the syncytiotrophoblast and has a high affinity for bidirectional transport of reduced folates. Multidrug resistance-associated proteins (MRPs), members of the ATP-binding cassette superfamily of transporters, are low affinity folate exporters found in intestinal and renal tissue [164, 165]. Though they are also distributed on the basal membrane of the placenta [166], their role in the placenta is currently unclear.

Overall, through the use of these transporters, the placenta actively shuttles folates from the mother to the fetus such that RBC and serum folates in the newborn are already 2-4 times that found in the mother, without any prenatal folic acid supplementation [41]. With high intake levels through supplementation, there is a need to investigate the impact supplementation may have on the placenta and the developing fetus. Furthermore, though folate is necessary for fetal development, the ideal range of supplementation must be determined for optimal health of both the mother and the offspring.

1.5.2 The Impact of Excessive Folic Acid on the Placenta

Despite increased folic acid consumption from fortified foods and prenatal supplementation, studies examining the effects of folic acid on the placenta are scarce. One

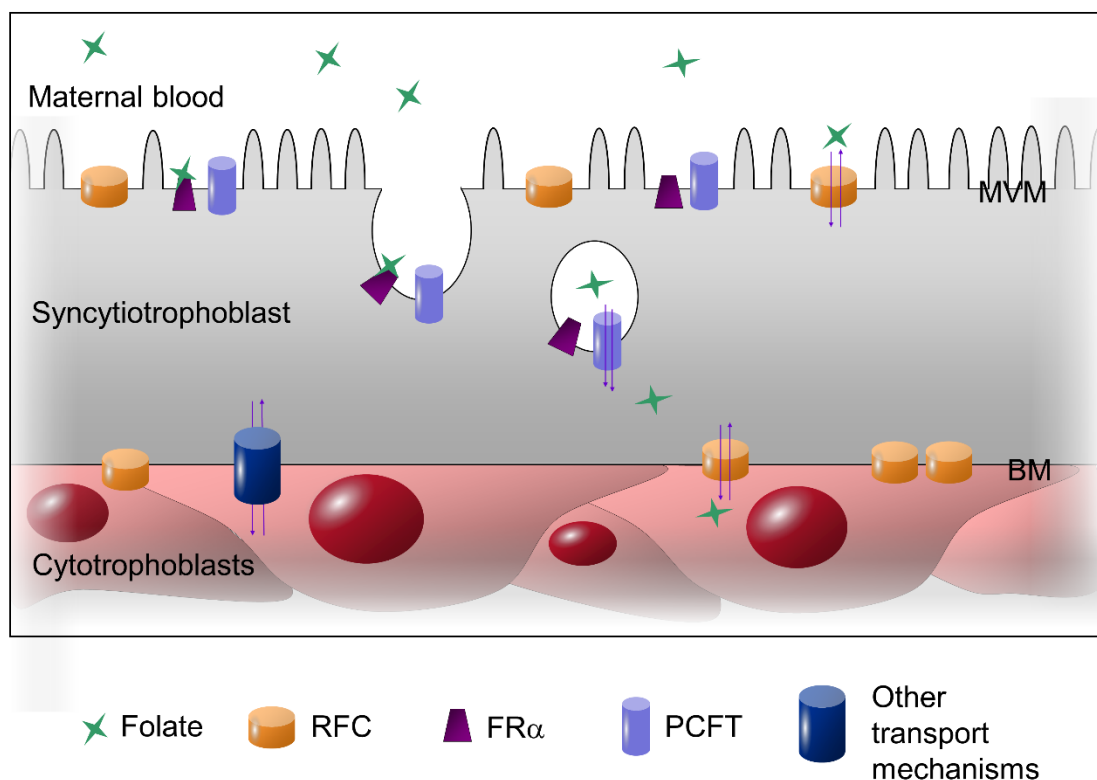


Figure 4. Folate Transporters. Model for folate transport across human placental syncytiotrophoblast. Once folate receptor α (FR α) binds folate (5-MTHF), co-localisation of FR α and the proton-coupled folate transporter (PCFT) to the microvillus plasma membrane (MVM) surface allows internalisation of both transporters as an endosome. Acidification of the endosome allows H $^+$ -coupled transport of folate by PCFT into the cytoplasm of the syncytiotrophoblast. Subsequently, FR α and PCFT are recycled back to the MVM surface. Favoured at physiological pH, reduced folate carriers (RFC) provide an alternate route for folate transport across the MVM. Folate transport across the basal membrane (BM) occurs via RFC. Other transport mechanisms may also allow folate transport across the BM. **Source:** Adapted from Solanky *et al.* [164].

study found that high concentrations of folic acid (100-fold increase from physiological adult serum folate levels) increased placental cell invasion, apoptosis, and vascular density, but decreased cell proliferation in first trimester placenta [167]. In contrast, a folic acid treatment approximating physiological levels of serum folate in adults (5.4-18 ng/mL) [101, 168], resulted in the most proliferation and least apoptosis of the folic acid concentrations tested on the placental tissue [167], thereby suggesting an impact of circulating folic acid concentrations on the health of this vital organ of pregnancy. Thus, more in depth research is needed to confirm these results and determine the implications of folic acid intake on placental health and function.

1.6 Study Rationale

The detrimental effects of various maternal nutrient deficiencies on fetal development and pregnancy outcome have led to the development and recommended use of multivitamin supplements to promote a healthy pregnancy [113]. Women are recommended by Health Canada to take multivitamin supplements containing 0.4 mg of folic acid prior to conception and during pregnancy, regardless of their nutritional status, to ensure adequate vitamin and mineral levels throughout gestation [29].

In industrialized countries, with the exception of iron, a large portion of middle- to upper-income pregnant women consume diets that already meet many nutrient requirements for a healthy pregnancy [169]. In addition to their regular diet, the use of prenatal multivitamin supplements has been shown to lead to excessive micronutrient intake levels [100]. The available literature indicates that high intake of vitamins and minerals are a reality for pregnant women due to recommended intake of multivitamin supplements by healthcare professionals. However, limited studies have assessed micronutrient intake from diet and supplementation among pregnant Canadian women. Thus, this will be the first large-scale study assessing micronutrient intake among pregnant Canadian women in a Québec population.

Among the studied nutrients, the risk of surpassing the UL is most likely the highest for folic acid based on recommended intake levels, food fortification, and supplementation. As a methyl donor, folic acid is known for its role in epigenetics and the risk of adverse effects at high and low intake levels. Evidence that high folic acid intake levels can trigger adverse effects in both animal and human models has been observed, and may have a severe impact on fetal health and development. Nevertheless, little work has been done to directly study the effects of high folic acid exposure on the vital organ of pregnancy – the placenta.

A healthy placenta is necessary for a healthy pregnancy. In fact, placental dysfunction has been directly linked to several adverse pregnancy outcomes, such as preeclampsia and fetal growth restriction [170]. At a time when clinical trials are in place to determine the efficacy of 4 mg of folic acid supplementation, as opposed to simply 0.4 mg to help prevent preeclampsia and congenital malformations [115, 171], the adverse effects of excessive folic acid levels needs to be further studied. If these excessive levels of folic acid adversely affect placental health and function, it is possible that fetal growth, development, and health may be compromised.

1.7 Hypothesis

The overarching hypothesis of the current research project is that excessive folic acid exposure throughout pregnancy, as a result of vitamin supplementation in addition to a balanced maternal diet, may result in compromised placental health and function.

Hypothesis 1: A balanced diet consisting solely of food sources is sufficient to provide pregnant Canadian women with adequate micronutrients necessary for a healthy pregnancy.

Hypothesis 2: Considering most women are consuming prenatal multivitamin supplements, intake levels for folic acid exceeds the tolerable upper intake level (UL) in a significant proportion of Canadian pregnant women.

Hypothesis 3: Exogenous exposure to increasing concentrations of folic acid adversely affects placental cell health and function *in vitro* in a dose-dependent manner.

1.8 Objectives

The overall objective of this research project is to assess micronutrient intake in a subsample of pregnant Canadian women to determine if they are meeting recommended intake levels, with a particular focus on folic acid; and to elucidate whether folic acid, obtained through the addition of multivitamin supplements to a nutritious diet during pregnancy, can negatively impact placental cell health and function.

Objective 1: Establish vitamin and mineral intake level from food sources and supplements for a Canadian (Québec) cohort of pregnant women.

Objective 2: Determine whether folic acid intake in the Canadian cohort of pregnant women exceeds the UL (in Objective 1), due to a daily prenatal multivitamin supplement.

Objective 3: Determine whether exogenous folic acid can impact placental cell health and function in a dose-dependent manner, using *in vitro* trophoblast cell culture and ex vivo placental explant culture models.

Chapter 2

Dietary Analysis of Micronutrient Intake during Pregnancy

2.1 Introduction

Adequate nutrition during pregnancy is a key ingredient for proper fetal development and positive pregnancy outcomes. As a public health measure to prevent neural tube defects (NTDs), Canada began fortifying all white flour, cornmeal, and enriched pasta with folic acid in 1998 [27]. Though this measure has resulted in a dramatic 46% decrease in NTDs in Canada [28], some women across the country still do not meet folate levels suggested to prevent NTDs [110, 112, 172]. In addition, only 3.5% of pregnant women in the Prenatal Health Project consumed recommended servings from all food groups recommended by the 2007 *Eating Well with Canada's Food Guide* [107], suggesting that supplementation is often necessary for a variety of micronutrients.

To combat low folate intakes, Health Canada and the Society of Obstetricians and Gynaecologists of Canada (SOGC) recommend women who are pregnant or planning to become pregnant to also take a daily multivitamin supplement containing 0.4 – 1 mg of folic acid [29, 30]. Results from the 2004 Canadian Health Measures Survey, a nationally representative assessment, and a study with young women in the province of Manitoba demonstrated that a low percentage (26-29%) of Canadian women of reproductive age are taking a folic acid-containing supplement [110, 173]. However, studies in the Canadian provinces of Alberta and Ontario have shown that the majority of pregnant women (80-97%) of higher socioeconomic status are compliant in taking a prenatal supplement containing folic acid [102, 107–109]. Using more recent data from the 2007 Canadian Health Measures Survey, approximately 40% of Canadians were identified as having high RBC folate concentrations (>1350 nmol/L) [112]. They also found that among Canadian women of child-bearing age, approximately 88% have above optimal levels of RBC folate to prevent NTDs (906 nmol/L) [112, 173]. Thus, due to food fortification and supplement usage, there is growing concern of increased folic acid intake during pregnancy. It is possible that a targeted approach to folic acid supplementation would be more effective to ensure folate adequacy among the pregnant population.

To better gauge micronutrient consumption among specific groups of pregnant Canadian women, the first aim of this study was to assess micronutrient intake from dietary sources in a subsample of pregnant women in the province of Québec. Of the folic acid-containing prenatal supplements on the market, *Materna*TM is one of the most common prenatal multivitamin taken by pregnant Canadians [102]. Based on the proportion of women who reported taking supplements in this cohort, the second aim was to estimate the shift in micronutrient intake status once women have taken a prenatal *Materna*TM supplement, and to determine the risk of exceeding the UL for folic acid intake.

2.2 Materials and Methods

2.2.1 Data Collection

Recruitment

The 3D Study (*Découvrir, Développer, Devenir*) was developed by the Integrated Research Network in Perinatology of Québec and Eastern Ontario (IRNPQEO). The IRNPQEO is a multidisciplinary group of researchers from five universities (McGill University, University of Ottawa, University of Montréal, Laval University, and the University of Sherbrooke) with the aim to better understand the effect of perinatal events on child development. Between May 2010 and September 2012, a total of 1612 detailed 3-day food records were collected from a large pregnancy cohort (N=2456) as part of the 3D Study.

Women included in this study were screened to participate during their first prenatal visit to one of ten participating clinics across Québec: the Centre Hospitalier Universitaire (CHU) Sainte-Justine, the CHU of Montréal - St. Luc Hospital, the Public Reproduction Centre of McGill University Health Centre, the Royal Victoria Hospital, the CHU of Sherbrooke, the CHU of Québec, Sacré-Coeur Hospital, Saint-Mary's Hospital Center, the Jewish General Hospital of Montréal, and the OVO Clinic.

To be eligible to participate, women were recruited during their 8-13th week of gestation, had a maternal age between 18-50 years, were fluent in either English or French, and planned to deliver at one of the study hospitals. Informed consent was obtained from women meeting the criteria and agreeing to participate in the study. Exclusion criteria included women who were planning to donate or bank her cord blood, intravenous drug users, HIV positive, or had renal disease with altered renal function, collagen vascular disease requiring active treatment, epilepsy, cardiovascular disease, serious pulmonary disease,

cancer, or a severe hematologic disorder. Multiple gestations were excluded from the 3D Study due to changes in nutrient requirements across gestation.

Food Record Collection

Data collection in the form of food records requires the participants to prospectively record what foods, and what quantity, were consumed throughout the day to avoid relying on memory. This method was selected over the 24-hour dietary recall method and food frequency questionnaires (FFQ) because the food record prevents perception and recall bias and can be used to estimate current usual intake of nutrients [174]. The food record method also allows participants the flexibility to include information on more ethnic and diverse foods than the FFQ.

Multiple days throughout the week or over different seasons should be used when analyzing nutrient intake using the food record method, as single day inputs are not representative of a complex diet. It has been shown that a 3-day food record, that includes at least one weekend day or holiday, has a high level of validity [175]. As a result, the nutrient intake of a large population with a variety of food choices and eating habits can be adequately considered.

Study personnel instructed each participant on how to complete a 3-day food record. Each participant was provided with a food record (Appendix A) to document food and beverage intake for 2 weekdays and 1 weekend day during their second trimester of pregnancy and were asked to complete and return the food record within 1 week. The food records were photocopied at each centre, and then the photocopies were sent to the main centre (CHU Ste-Justine), and then to the University of Ottawa for our analysis.

Demographics

Once enrolled in the study, participants completed a questionnaire regarding their demographic and socioeconomic characteristics. For example, regarding ethnicity, participants were asked in the questionnaire whether they believed they fell into the categories of: White, African American, Native African, South Asian, East Asian, Arab-West Asian, Latin American, Native-Aboriginal People, or Other (Table 3). If they selected “Other”, an open-ended question asked participants to specify.

The questionnaire was also used to calculate the participants’ body mass index (BMI = kg/m^2) by using their height and weight at their first prenatal visit as measured by study

staff (Table 5). Based on Health Canada classifications [176], participants were categorized as underweight (< 18.5 kg/m²), normal (18.5 - 24.9 kg/m²), overweight (24 – 29.9 kg/m²), or obese (≥ 30 kg/m²).

Furthermore, the questionnaire requested information regarding the participants' health history, drug and supplement intake, and personal habits (ex. Number of cigarettes per day) during each trimester of their pregnancy. Participants recorded whether they were taking vitamin/mineral or nutritional supplements 3 months prior to becoming pregnant, as well as during their second and third trimesters (Appendix B). Based on the self-reported individual answers in the questionnaires, smokers have been identified as light (1-14 cigarettes/day), moderate (15-24 cigarettes/day), heavy (25 or more cigarettes/day), and non-smokers [177, 178].

2.2.2 Sample Size Calculation

The sample size was based on the probability that women exceed the UL for at least one micronutrient (in this case, folic acid) when taking a prenatal supplement [179]. In a study performed in London (Ontario, Canada), it was found that 79.7% of pregnant women participants reported taking supplements containing folic acid during their pregnancy [102]. In another Canadian cohort of the Alberta Pregnancy Outcomes and Nutrition (APrON) study, between 91-97% of respondents reported taking multivitamin/mineral supplements in each trimester [108]. Using this information, a representative sample size of women was calculated using Cochrane's formula for dichotomous variables (above the UL or not):

$$\begin{aligned} n_o &= [(t)^2 * (p)(q)] / (d)^2 \\ &= [(1.96)^2 * (p)(q)] / (0.05)^2 \end{aligned}$$

where "t" is the value for the selected alpha level of 0.025 in each tail (t = 1.96); where "p" is the expected proportion, "q" is "1-p", "(p)(q)" is the estimate of the variance; and where "d" is the acceptable margin of error of 0.05. Since the calculated representative sample size (n_o) is greater than 5% of the population size (i.e. food records from 81 women, where N=1612), Cochran's (1977) correction formula was used to determine the minimum sample size (n₁):

$$n_1 = n_o / (1 + n_o / \text{Population})$$

Using these two formulas and expecting at least 80% of women to take a supplement, a minimum representative sample size of 213 women was deemed to be sufficient for the sub-analysis (Appendix C: Sample Size Calculation). To ensure sufficient data, a total of 216 completed 3-day food records were randomly selected. This sub-group analysis was approved by the University of Ottawa Health Science and Science Research Ethics Board (REB) as well as the REB at CHU Ste-Justine (Appendix E).

2.2.3 Food Record Analysis

The estimated food intake from the 3-day food records from each participant were entered into a nutrient analysis program, ESHA Food Processor™ (version 10.13.1.0, ESHA Research, Oregon), using foods primarily listed in the 2010 Canadian Nutrient File (CNF) Database. The CNF Database is compiled by the Nutrition Research Division at Health Canada and is the standard reference food composition database for the amount of nutrients in foods normally consumed in Canada [180]. This enabled increased accuracy in analysing vitamin and mineral intake levels in a Canadian population.

Food Record data entry into ESHA Food Processor™ was performed by two third year nutrition students (30% and 15% respectively), a research assistant with a PhD in nutrition (15%) and myself (40%). To minimize inconsistencies, each entered food record was reviewed in detail by two trained Registered Dietitians to maintain uniformity and validity among arbitrary decisions made.

Food and beverages listed by participants in their individual food records were first searched in ESHA Food Processor™ using items listed by the Canadian Nutrient File (CNF). If a brand was specified, but the item was not listed in the CNF, then the recipe and nutrition information was searched online and a recipe was created in ESHA Food Processor™. For brand items that were listed in CNF, but nutrient composition differed from what is published on the website, the CNF recipe was modified. Only seven items were selected from the United States Department of Agriculture (USDA) Nutrient Database: Greek yogurt (ESHA code: 16436), parmesan fondue (ESHA code: 43906), Goji berry (ESHA code: 18653), almond milk (ESHA code: 28100), Clif chocolate chip energy bar (ESHA code: 55176), focaccia bread (ESHA code: 38926), and calamari (ESHA code: 71702).

Portion sizes were entered according to what was stated by the participant. In the event that portion sizes were not mentioned (ex. Different soups, mixed dishes such as shepherd's pie, etc.), it was assumed that one serving was consumed according to the

“Quantity Not Specified” amount for the item in the USDA’s “What’s in the Foods You Eat?” database [181].

Daily Micronutrient Intake from Food Sources

Assuming a normal distribution of nutrient requirements, the EAR cut-point method is often used to estimate the risk of inadequate intake in a population [37]. Individuals in a population with intakes below the EAR would be considered at risk of inadequate intake, while those above the EAR would be considered to have met adequate intake requirements (Figure 2). Similarly, individuals who surpass the UL are deemed at risk of excessive intake.

Nutrient analysis of food and beverage intake was performed using the “EAR cut-point method” for micronutrients with an established EAR/RDA and when ESHA Food Processor™ listed micronutrient content information for more than 50% of foods available in the CNF database. Sufficient data (i.e. >50% of food items listed in CNF database have micronutrient content information) was available for vitamin A (95%), thiamine (vitamin B₁) (94%), riboflavin (vitamin B₂) (94%), niacin (vitamin B₃) (95%), vitamin B₆ (90%), folate (vitamin B₉) (89%), vitamin B₁₂ (94%), vitamin C (97%), vitamin D (90%), vitamin E (67%), calcium (99%), copper (91%), iron (99%), magnesium (92%), phosphorus (96%), selenium (81%), and zinc (92%). Manganese (84%), pantothenic acid (vitamin B₅) (83%), potassium (96%), and sodium (99%) did not have an EAR/RDA for pregnant women (AI only). Nonetheless, since ESHA Food Processor™ contained sufficient data (>50%) for these micronutrients, they were also included in order to determine intake levels.

Though iodine and molybdenum have an established EAR/RDA, they were excluded from the study due to lack of information on the percentage of foods with iodine and molybdenum composition data in the CNF. Only 2/216 participants were observed to have values for iodine intake and 1/216 participants for molybdenum. Micronutrients that only had an AI instead of an EAR/RDA for pregnant women and less than 50% of foods listed in the CNF had information on the micronutrient were excluded: vitamin K (49%) and choline (vitamin B₄) (42%). Other micronutrients that only had an AI for pregnant women, such as biotin (vitamin B₇), chromium, fluoride, and chloride were excluded as well due to lack of information on percentage of foods with composition data for these nutrients in the CNF.

The Shapiro-Wilk normality test was used to determine normality of distribution for each micronutrient. Normally distributed micronutrients included riboflavin, vitamin B₆, calcium, phosphorus, potassium, selenium, and zinc. The National Research Council (1986)

recommended transforming daily intakes so that the transformed values modelled a normal distribution [182]. Micronutrient intake became normally distributed after natural log transformation (vitamin A, thiamine, niacin, folate, vitamin E, copper, iron, magnesium, and manganese), square root transformation (vitamin C, vitamin D, and folic acid), and rank transformation (vitamin B₁₂). Micronutrient data that was transformed to achieve normality was compared to the same type of transformation for the DRI value. Transforming the data did not alter the results.

For normally distributed data, univariate outliers were detected using Z-scores with a standard deviation of 3 and multivariate outliers were detected using Mahalanobis distances (MD). For data without a normal distribution, univariate outliers were detected using the Tukey method and multivariate outliers were detected using the Minimum Covariance Determinant (MCD). Identified outliers were individually reviewed for data entry errors or assessed for unique dietary habits. If necessary, any errors in data entry were modified to ensure reliability and accuracy of data entry. The analysis was performed using SAS 9.3. All micronutrient intakes are presented as median values with 25th and 75th percentiles.

Using descriptive statistics, vitamin and mineral intakes during pregnancy were compared to established Dietary Reference Intake (DRI) values for pregnant women. Women with intakes below the established EAR for each micronutrient were identified as at risk of inadequacy [37]; whereas women with intake above the established UL were identified as at risk of excess consumption [38]. Pregnant women with micronutrient intake between the respective EAR and UL were identified as having adequate intake. In the case of vitamin A, inadequate and adequate consumption was determined based on retinol activity equivalents (RAE) of vitamin A. However, the UL for vitamin A is only for preformed vitamin A (retinol), given in retinol equivalents (RE) and so, percentage of women exceeding the UL for vitamin A was determined using vitamin A in RE. In the case of magnesium, separate EAR and RDA values have been determined for pregnant women aged 19-30 years and 31-50 years, though the UL remains the same for both groups. Levels of consumption at risk of exceeding the UL for folate are determined using the synthetic form of folate (i.e. folic acid). The percentage of women who surpass the UL for each identified vitamin and mineral, and the extent to which they surpass the UL, was calculated.

Like folate, the ULs for vitamin E and niacin apply only to the synthetic form which is obtained from supplements, fortified foods, or a combination of both. However, the extent

to which study participants surpassed the UL for these micronutrients could not be determined since ESHA Food Processor™ did not differentiate between the natural and synthetic forms.

Prenatal Supplementation

As discussed in Section 2.2.1, participants in the 3D study completed questionnaires regarding their supplement use before and during their pregnancy. Detailed information on supplement intake, including brand name and dosage, has been documented and are in the process of being entered for the entire cohort. This information will be made available in the future. Currently, only information as to whether or not participants were consuming a supplement (yes/no) prior to pregnancy and during each trimester (Appendix B: Supplement Intake Survey Question) has been received.

In a Canadian study, Roy *et al.* [102] discovered that of the 643 women who reported the name brand of their prenatal supplement in their study, Centrum® *Materna*™ (n = 592, 92%) was the most common. As a result, assuming all of the women who reported taking a prenatal multivitamin supplement were taking *Materna*™ in this cohort, hypothetical micronutrient intake from diet and supplementation was calculated (Table 11). In July 2013, Nestlé acquired Pfizer's infant nutrition products and therefore, Centrum® *Materna*™ has now become Nestlé® *Materna*™ [183, 184]. Nevertheless, the formulation for the *Materna*™ prenatal supplement has remained the same [185].

For Vitamin A, Nestlé® *Materna*™ contains 1500 µg of β-carotene as well as 300 µg of preformed vitamin A as retinyl acetate. Using the conversion factor (1 µg RAE = 1 µg preformed vitamin A = 1 µg retinol RE = 12 µg β-carotene) from the Institute of Medicine (IOM)'s Dietary Reference Intakes (DRIs), the hypothetical total intake of vitamin A is presented as 425 µg RAE (1500 µg β-carotene/12 + 300 µg preformed vitamin A) [53]. To determine the percentage of the population at risk of excess (intakes surpassing the UL), only preformed vitamin A (in RE) is used.

Like vitamin A, the risk of exceeding the UL for magnesium, folic acid, niacin, and vitamin E is based on the form of the micronutrient. The UL for magnesium only applies to intake from a pharmacological agent and does not include intake from food and water. For folic acid, niacin, and vitamin E, the UL only applies to the synthetic form which has been obtained from supplements, such as *Materna*™, and/or fortified food. However, ESHA Food Processor™ was unable to distinguish the form of niacin consumed through foods and so,

the percentage of women at risk of exceeding the UL for niacin was undetermined. Thus, in the analysis, the percentage of study participants at risk of exceeding the UL was determined for magnesium, folic acid, and vitamin E using the amount included in supplements and fortified food.

Nestlé® *Materna*TM also contains biotin, chromium, molybdenum, and iodine [185]; however these could not be analyzed for hypothetical intake due to lack of sufficient data in ESHA Food ProcessorTM. Furthermore, potassium, phosphorus, and sodium were excluded in this analysis because *Materna*TM did not contain these micronutrients.

To help healthcare professionals better target women who may need multivitamin supplementation, the chi-square test for independence was used to determine whether women with an adequate/inadequate micronutrient intake from diet alone differed from each other based on their demographic/socioeconomic status (Table 8) or likelihood for supplement consumption (Table 10). Adequacy for vitamin or mineral intake was coded as either *adequate* or *inadequate*. Vitamins analysed included vitamin A, thiamine, riboflavin, vitamin B₆, vitamin B₁₂, and vitamin C. Minerals analysed included calcium, copper, magnesium, and zinc. Folate, iron, vitamin D, and vitamin E were excluded from this analysis since the majority (>50%) of women were inadequate for these micronutrients. Thus, in Table 8 and Table 10, personal characteristics of women who were at risk of inadequacy were compared to women who had adequate micronutrient intake for all micronutrients except folate, iron, vitamin D, and vitamin E. The non-parametric chi-square test was performed to explore the differences between adequate vs. inadequate micronutrient intake and demographic and socioeconomic variables (as categorical variables). This test was deemed appropriate since non-parametric tests make no assumption about the probability distribution and was used with categorical variables only. Where significance was observed using chi-square analysis, the odds ratios (ORs) and 95% confidence intervals (CIs) were also estimated. All statistical analyses were done using SAS Version 9.3.

Of the demographic and socioeconomic factors examined, underweight (BMI < 18.5) women (N=7), single/divorced women (N=9), as well as moderate (N=0) and heavy smokers (N=1) were excluded from the chi-square analyses due to low frequencies (N<5) in each group of adequate vs. inadequate intake (vitamins or minerals or all micronutrients). Similarly, women who self-reported as African (American or Native), East/South Asian, Arab-West Asian, Latin American, or Other were reclassified as “non-white” because too many frequencies were less than 5. The effect of employment status on adequate and inadequate

micronutrient intake was not examined because some women reported as having multiple employment statuses (ex. Being a student and working part-time). Finally, women who did not report an answer to a survey question, were excluded only in the analysis for the respective category in which they were missing: BMI (N=5), Smoking (N=2), Education (N=2), Income (N=4), Second Trimester Supplement Usage (N=3), and Third Trimester Supplement Usage (N=11).

2.3 Results

2.3.1 Demographic and Socioeconomic Factors

Of the 2,456 eligible pregnant women who were recruited for the 3D study by the IRNPQEO, a total of 1,612 food records were returned to study personnel for a response rate of 65.6%.

Demographic characteristics (Table 3) of the subsample show that the majority of women are in their 30's (78%), in a relationship (96%), self-identified as white (80%), and have been pregnant before (58%). Of those women who selected "Other" for race, two women reported being from Haiti, one from Spain, one from Mauritius, one from Brazil, and one identified as an Ashkenazi and one as a Berber. Since Brazil is in Latin America, it was classified as such and only six participants were left in the "Other" category. Two women reported being ethnically white and Latin American, and so, they were placed into the "Other" category due to their mixed background. Though there were 5 women who self-reported to be single and 4 women who were divorced, there were no women who were separated or widowed.

Socioeconomic characteristics of the subsample of women (Table 4) demonstrate that most women were well-educated (93% attended/completed post-secondary school), had full-time jobs (74%), and were living in households with an annual income of \$80,000 or more (51%). Of the 8 women who reported as having "Other" employment, 5 women were on maternity leave, one was self-employed, one was a seasonal employee, and one was on sick leave.

Since women at increased risk of giving birth to a child with NTD are often recommended to take higher folic acid supplements (1 – 5 mg) [35], the prevalence of some risk factors of NTDs are highlighted in Table 5 and include first trimester BMI, diabetes, alcohol consumption, smoking, and a personal or family history of children with birth defects. The self-reported pre-pregnancy BMIs of the participants were not significantly different

from their BMI measured by study staff during their first trimester visit (paired t-test; $p=0.77$). Using the BMI measured by study staff and Health Canada's categories for BMI [176], 37% were overweight or obese during their first prenatal visit in their first trimester. The occurrence of diabetes was rare among study participants. Of the three women (1%) who reported having diabetes, one woman had type 1 diabetes and two women had type 2 diabetes. During their second trimester of pregnancy, 14% of the subsample of women reported drinking alcohol and only 5% reported smoking cigarettes weekly.

A total of 17 women (8%) reported having first degree relatives who had children with birth defects. Fourteen (6%) of these women reported having both a mother and father with affected children. Though six women (3%) reported having congenital anomalies in a previous pregnancy, only three study participants (1%) reported congenital anomalies during this pregnancy.

2.3.2 Daily Micronutrient Intake from Food Sources

Based on the 3-day food records of study participants, Table 6 highlights the median and the 25th and 75th percentiles (i.e. 1st and 3rd quartiles) of daily vitamin intake along with the current Dietary Reference Intakes for pregnant women. From food sources alone, most study participants seemed to be meeting recommended intake requirements for pregnant women (i.e. above the EAR) for vitamin A (80%), thiamine (88%), riboflavin (97%), niacin (100%), vitamin B₆ (65%), vitamin B₁₂ (95%), and vitamin C (90%). However, the majority (>50%) of women were at risk of inadequate vitamin consumption (i.e. below the EAR) for folate (67%), vitamin D (90%), and vitamin E (92%). Though adequate consumption could not be assessed for pantothenic acid due to unestablished EAR/RDA values, the data from the 3-day food records demonstrate that the median intake of pantothenic acid is slightly above the established AI (Table 6).

In Table 7, the study participants' daily mineral consumption levels from food alone is presented. Results show that most pregnant women appear to be consuming adequate levels (i.e. above the EAR) for calcium (82%), copper (99%), magnesium (73%), phosphorus (100%), selenium (100%), and zinc (75%). However, it seems that women are especially at risk of inadequacy (i.e. below the EAR) for iron (93%) from food sources. Manganese, potassium, and sodium did not have an EAR/RDA needed to assess adequacy; nonetheless, the median intakes were above the AI for manganese and sodium, but below the AI for potassium. Furthermore, 80% of women have daily sodium intakes that surpassed the UL.

When determining the differences in demographic and socioeconomic factors among women who are adequate vs. inadequate for vitamin consumption from diet alone, no significant difference was observed (Table 8). However, a significant difference between mineral adequacy vs. inadequacy for marital status ($p=0.002$), race ($p=0.003$), and income ($p=0.01$) was observed. Furthermore, the odds of being inadequate for minerals was significantly less for white women than for non-white women (OR = 0.37; 95% CI = 0.19-0.73), though the odds of being inadequate for minerals was higher for women with an average household income of \$40,000-\$59,000 than for those with an average household income greater than \$80,000 (3.33, 1.4-7.8). Interestingly, marital status significantly differed by race ($p<0.001$), income ($p<0.001$), as well as by age ($p=0.04$), but not by gravidity ($p=0.18$).

2.3.3 Prenatal Supplementation

The proportion of women who planned to become pregnant and took supplements prior to and while pregnant is shown in Table 9. Most women reported that they were planning to become pregnant (66%) and were taking a vitamin/mineral supplement prior to their current pregnancy (93%). Over the course of their pregnancy, around 10% of women subsequently stopped taking supplements in both second and third trimesters. In their second trimester, 83% of study participants reported taking a nutritional supplement and in third trimester, only 72% reported taking a nutritional supplement.

The odds of being inadequate for minerals from diet alone was observed to be approximately four-folds higher ($p<0.01$) in women who did not take a supplement 3 months prior to becoming pregnant compared to those who did take a supplement (OR = 3.9; 95% CI = 1.4–11.2) (Table 10). Indeed, among non-supplement users, 62.5% of women were identified for mineral inadequacy, while 37.5% of women were adequate for minerals. However, there was no relationship ($p>0.05$) between supplement usage in the second or third trimester of pregnancy and micronutrient adequacy from dietary sources.

Projected Micronutrient Intake after Prenatal Supplementation

Based on literature with evidence that over 90% of Canadian women are using *Materna*TM as their choice form of prenatal supplementation [102], Table 11 demonstrates hypothetical micronutrient consumption for our subsample ($n=216$) when the women who reported taking a supplement during their second trimester of pregnancy ($n=180$; 83%) are assumed to be taking Nestlé® *Materna*TM. Despite adequate intakes among the majority of women after supplementation for most nutrients, more than 10% of women would be at risk

of inadequacy for vitamin D (15%), vitamin E (15%), folate (11%), and magnesium (14%) due to inadequate diet combined with a lack of supplement usage.

Based on projected intakes after supplementation, it is expected that all of the women taking Nestlé® *Materna*TM (83%) will be at risk of exceeding the UL for folic acid (i.e. 1000 µg). The median intake of folic acid from diet and *Materna*TM supplementation (1099.6 µg) is projected to be 9.9% above the UL for folic acid, while the maximal intake of folic acid from food and supplementation in the subsample of women is projected to be 1282.66 µg. Additionally, it is expected that a portion of the subsample will also be at risk of excessive intake for iron (19%), calcium (1%), manganese (1%), and vitamin A (1%).

Since the UL for magnesium only applies to magnesium found in pharmacological agents and/or nutritional supplements (but not naturally-occurring magnesium in food), the magnesium found in *Materna*TM (50 mg) is not enough to surpass the UL. Thus, excessive intake of magnesium as a result of *Materna*TM supplementation is not a concern. Due to insufficient data differentiating between forms of niacin (natural or synthetic) found in each food item, the percentage of women at risk of exceeding the UL was undetermined for this micronutrient.

Due to a large percentage of the population taking prenatal supplements and their risk of exceeding the UL for folic acid, this micronutrient warrants further investigation. In Figure 5a, the distribution curve demonstrates that the majority of women do not meet recommended folate intake levels, as measured using dietary folate equivalents (DFE), for a healthy pregnancy from diet alone. However, once supplemented with the 1000 µg folic acid (equivalent to 1667 µg DFE) found in *Materna*TM supplements, the median folate (DFE) intake exceeds the recommended intake (600 µg DFE) by three-fold. Nevertheless, among the 17% of pregnant women who are not supplemented, 10.7% remain at risk of folate inadequacy with a median intake of 415.7 µg DFE.

The population distribution curve demonstrating folic acid intake among the subsample of women from diet alone and after 83% of the subsample have consumed a *Materna*TM supplement is shown in Figure 5b. The median intake of folic acid among supplement users (1118.3 µg folic acid) is projected to be nearly 12% above the established UL for folic acid.

Table 3. Demographic characteristics (n = 216 women)

Variables	Participants	
	Number (n)	Percentage (%)
Age (years)		
23-29	27	13
30-39	169	78
40-47	20	9
Marital Status		
Married	87	40
Common-Law	120	56
Single/Divorced	9	4
Race		
White	173	80
African (American or Native)	9	4
East/South Asian	10	5
Arab-West Asian	9	4
Latin American	7	3
Other ^a	8	4
Gravidity		
1	90	42
2	59	27
3	32	15
4	16	7
≥ 5	19	9

^a Includes mixed backgrounds and other ethnic groups.

Table 4. Socioeconomic characteristics (n = 216 women)

Variables	Participants	
	Number (n)	Percentage (%)
Education		
High School or Less	13	6
CEGEP/College/Technical Diploma ^{a, *}	60	28
Undergraduate Degree*	82	38
Post-graduate Degree (Masters/PhD)*	59	27
Missing Data	2	1
Employment Status^b		
Full-time	159	74
Part-time	21	10
Student	20	9
Unemployed	10	5
Housewife	6	3
Other	8	4
Annual Household Income		
< \$20,000	10	5
\$20,000 to \$39,000	21	10
\$40,000 to \$59,999	29	13
\$60,000 to 79,999	41	19
≥ \$80,000	111	51
Missing Data	4	2

^a CEGEP: *Collège d'enseignement général et professionnel* or General and Vocational College

^b 224 total answers given by 216 participants

* Attended/Completed diploma or degree

Table 5. Risk factors for NTDs (n = 216 women)

Variables		Participants	
		Number (n)	Percentage (%)
First Trimester Body Mass Index (kg/m²)			
Underweight	(< 18.5)	7	3
Normal	(18.5 – 24.9)	134	58
Overweight	(25 – 29.9)	53	24.5
Obese	(≥ 30)	27	12.5
Missing Data		4	2
Diabetes			
Yes		3	1
No		213	99
Alcohol Consumption during second trimester			
None		186	86
1 day/week		10	5
2 days/week		7	3
3 days/week		8	4
4 days/week		4	2
Smoking during second trimester (# of cigarettes/day)^c			
Non-Smoker		205	95
Light Smoker	(≤ 14)	10	4.5
Moderate Smoker	(15 – 24)	0	0
Heavy Smoker	(≥ 25)	1	0.5
Family history of relatives with children with birth defects^a			
Mother		15	7
Father		14	6
Sister		2	1
Brother		0	0
No Family History		199	92
Evidence for congenital anomalies: Previous pregnancy			
Yes		6	3
No		210	97
Evidence for congenital anomalies: Current pregnancy			
Yes		3	1
No		213	99

^a 230 answers given by 216 participants. Some women had multiple relatives with children with birth defects.

Table 6. Daily vitamin intake during pregnancy from food sources (n = 216 women)

Nutrient	EAR	RDA	UL	Median intake (25 th , 75 th percentile)	Risk of inadequacy (%)	Adequate intake (%)	Risk of excess (%)
Vitamin A (µg RAE)^a	550	770	--	798.6 (621.0, 1077.8)	19	80	--
Vitamin A (µg RE)	--	--	3000	442.6 (316.3, 573.7) ^e	--	--	1
Thiamine (mg)	1.2	1.4	--	1.9 (1.5, 2.2)	12	88	--
Riboflavin (mg)	1.2	1.4	--	2.2 (1.8, 2.7)	3	97	--
Niacin (mg NE)^b	14	18	35 ^d	36.6 (30.8, 42.8)	0	100	--
Vitamin B₆ (mg)	1.6	1.9	100	1.8 (1.5, 2.2)	35	65	0
Pantothenic Acid (mg)	--	6*	--	6.4 (5.3, 7.4)	--	--	--
Folate (µg DFE)	520	600	--	468.5 (379.1, 557.8)	67	33	--
Folic Acid (µg)	--	--	1000 ^d	120.3 (77.1, 165.6) ^f	--	--	0
Vitamin B₁₂ (µg)	2.2	2.6	--	4.5 (3.2, 6.0)	5	95	--
Vitamin C (mg)	70	85	2000	159.0 (102.1, 226.4)	10	90	0
Vitamin D (µg)	10	15	100	5.0 (3.0, 7.4)	90	10	0
Vitamin E (mg)^c	12	15	1000 ^d	7.2 (5.4, 9.5)	92	8	--

^a As retinol activity equivalents (RAEs). 1 RAE = 1 µg retinol, 12 µg β-carotene, 24 µg α-carotene, or 24 µg β-cryptoxanthin. The RAE for provitamin A carotenoids is 2x greater than retinol equivalents (RE), whereas the RAE for preformed vitamin A is the same as RE.

^b As niacin equivalents (NE); where 1 NE = 1 mg of niacin = 60 mg of tryptophan.

^c As α-tocopherol, which includes RRR-α-tocopherol, the only form of α-tocopherol that occurs naturally in foods, and the 2R-stereoisomeric forms of α-tocopherol (RRR-, RSR-, RRS-, and RSS-α-tocopherol) that occur in fortified foods and supplements. It does not include the 2S-stereoisomeric forms of α-tocopherol (SRR-SSR-SRS-, and SSS-α-tocopherol), which is also found in fortified foods and supplements.

^d The ULs for niacin, folic acid, and vitamin E apply to synthetic forms obtained from supplements, fortified foods, or a combination of both.

^e As preformed vitamin A, in retinol equivalents (RE), which is used to establish the UL.

^f Values for the synthetic form of folate (i.e. folic acid) from fortified foods.

* Adequate Intake (AI) values. RDAs have not been established for these micronutrients.

Sources: Dietary Reference Intakes (DRIs) established by the Institute of Medicine's Food and Nutrition Board. DRIs for vitamin D established in 1997; thiamine, riboflavin, niacin, pantothenic acid, vitamin B₆, folate, vitamin B₁₂ in 1998; vitamin C, vitamin E in 2000; vitamin A in 2001; and vitamin D DRIs were updated in 2011. Values listed are established for women between the ages of 19-50 years old during pregnancy.

Table 7. Daily mineral intake during pregnancy from food sources (n = 216 women)

Nutrients	EAR	RDA	UL	Median intake (25th, 75th percentile)	Risk of inadequacy (%)	Adequate intake (%)	Risk of excess (%)
Calcium (mg)	800	1000	2500	1182.3 (883.5, 1474.6)	18	82	0
Copper (mg)	0.8	1	10	1.6 (1.3, 1.9)	1	99	0
Iron (mg)	22	27	45	14.8 (12.7, 17.7)	93	7	0
Magnesium (mg)^a	290, 300	350, 360	350	356.2 (288.9, 434.7)	27	73	--
Manganese (mg)	--	2*	11	3.7 (2.9, 4.5)	--	--	0
Phosphorus (mg)	580	700	3500	1497.8 (1230.0, 1757.5)	0	100	0
Potassium (mg)	--	4700*	--	3287.4 (2766.3, 4036.3)	--	--	--
Selenium (ug)	49	60	400	112.9 (94.0, 133.7)	0	100	0
Sodium (mg)	--	1500*	2300	2949.6 (2427.0, 3500.3)	--	--	80
Zinc (mg)	9.5	11	40	11.6 (9.5, 13.9)	25	75	0

^a The EAR and RDA values listed for magnesium are from food sources for pregnant women ages 19-30 and 31-50 years old respectively. The UL for magnesium applies only to supplemented form found in medication and dietary supplements.

* Adequate Intake (AI) values. RDAs have not been established for these micronutrients.

Sources: Dietary Reference Intakes (DRIs) established by the Institute of Medicine's Food and Nutrition Board. DRIs for calcium, phosphorous, magnesium, established in 1997; selenium in 2000; copper, iron, manganese, and zinc in 2001; potassium in 2004; and calcium DRIs were updated in 2011. Values listed are established for women between the ages of 19-50 years old during pregnancy.

Table 8. Differences in demographic and socioeconomic factors for pregnant women (n=216) who are adequate vs. inadequate for vitamins or minerals from food sources

	Vitamin Adequacy ^e			Mineral Adequacy ^f		
	Adequate n (%)	Inadequate n (%)	Chi-square P-value	Adequate n (%)	Inadequate n (%)	Chi-square P-value
Total Women – n (%)	102 (47.2)	114 (52.7)	--	146 (67.6)	70 (32.4)	--
Age						
23 – 29	11 (10.8)	16 (14)	0.76	21 (14.4)	6 (8.6)	0.40
30 – 39	81 (79.4)	88 (77.2)		113 (77.4)	56 (80)	
40 – 47	10 (9.8)	10 (8.8)		12 (8.2)	8 (11.4)	
BMI (kg/m²)^a						
Underweight ^b (< 18.5)	--	--	--	--	--	--
Normal (18.5 – 24.9)	60 (60.6)	65 (61.9)	0.97	87 (62.6)	38 (58.5)	0.70
Overweight (25 – 29.9)	26 (26.3)	26 (24.8)		33 (23.7)	19 (29.2)	
Obese (≥ 30)	13 (13.1)	14 (13.3)		19 (13.7)	8 (12.3)	
Marital Status						
Married	38 (37.3)	49 (43)	0.26	49 (33.6)	38 (54.3)	0.002
Common Law	62 (60.8)	58 (50.9)		92 (63.0)	28 (40)	
Single/Divorced	--	--		--	--	
Race						
White	86 (84.3)	87 (76.3)	0.14	125 (85.6)	48 (68.6)	0.003
Non-White ^c	16 (15.7)	27 (84.3)		21 (14.4)	22 (31.4)	

Smoking (cigs./day in 2 nd trim.) ^a							
Non-smoker	(none)	97 (95.1)	107 (95.5)	0.88	139 (95.9)	65 (94.2)	0.59
Light Smoker	(≤ 14)	5 (4.9)	5 (4.5)		6 (4.1)	4 (5.8)	
Moderate Smoker ^d	(15 -24)	--	--	--	--	--	--
Heavy Smoker ^b	(≥ 25)	--	--	--	--	--	--
Education ^a							
High school or less		3 (3.0)	10 (8.9)	0.24	5 (3.5)	8 (11.6)	0.11
CEGEP/College/Diploma		26 (25.7)	33 (29.2)		41 (28.3)	18 (26.1)	
Undergraduate Degree		43 (42.6)	39 (34.5)		55 (37.9)	27 (39.1)	
Post-graduate Degree		29 (28.7)	31 (27.4)		44 (30.3)	16 (23.2)	
Income ^a							
< \$20,000		4 (4.0)	5 (4.5)	0.31	6 (4.2)	3 (4.4)	0.01
\$20,000-39,999		10 (9.9)	12 (10.8)		12 (8.4)	10 (14.5)	
\$40,000 – 59,999		9 (8.9)	19 (17.1)		12 (8.4)	16 (23.2)	
\$60,000 – 79,999		24 (23.8)	17 (15.3)		33 (23.1)	8 (11.6)	
> \$80,000		54 (53.5)	58 (52.3)		80 (55.9)	32 (46.4)	

^a Some women did not report answers regarding these variables: BMI (N=5), Smoking (N=2), Education (N=2), Income (N=4).

^b The chi-square analysis would not be a valid test due to low frequencies. Thus, the categories of Underweight women (N=7), Single/Divorced women (N=9) Moderate Smoker (N=0), and Heavy Smoker (N=1) were excluded from the analysis.

^c Includes women who self-reported as African (American or Native), East/South Asian, Arab-West Asian, Latin American, and Other.

^d There were no women in the category of Moderate Smoker.

^e Refers to vitamin A, thiamine, riboflavin, vitamin B₆, vitamin B₁₂, and vitamin C.

^f Refers to calcium, copper, magnesium, and zinc.

Table 9. Supplement consumption (n = 216 women)

Variables	Participants	
	Number (n)	Percentage (%)
Planned pregnancy		
Yes	142	66
No	5	2
No Data ^a	69	32
Vitamin/mineral supplementation 3 months prior to pregnancy		
Yes	200	93
No	16	7
No Data	0	0
Second Trimester Supplementation^b		
Yes	180	83
No	34	16
No Data	2	1
Third Trimester Supplementation^b		
Yes	156	72
No	49	23
No Data	11	5

^a Based on answer options in the questionnaire, 3 women reported that they did not know, 1 refused to answer, 11 selected “not applicable”, and there was no data for 54 women.

^b Participants were asked if they consume a medical/nutritional supplement (Appendix B).

Table 10. Differences in supplementation among pregnant women (n=216) who are adequate vs. inadequate for vitamins or minerals from food sources

	Vitamin Adequacy^b			Mineral Adequacy^c		
	Adequate n (%)	Inadequate n (%)	Chi-square P-value	Adequate n (%)	Inadequate n (%)	Chi-square P-value
Total Women – n (%)	102 (47.2)	114 (52.7)	--	146 (67.6)	70 (32.4)	--
Nutritional Supplementation						
3 months before pregnancy	97 (48.5)	103 (51.5)	0.18	140 (70)	60 (30)	0.008
Second Trimester ^a	84 (46.7)	96 (53.3)	0.40	122 (67.8)	58 (32.2)	0.83
Third Trimester ^a	72 (45.9)	85 (54.1)	0.61	107 (68.2)	50 (31.9)	0.85

^a There was no data for some women regarding Nutritional Supplementation in Second Trimester (N=3) and Third Trimester (N=11).

^b Refers to vitamin A, thiamine, riboflavin, vitamin B₆, vitamin B₁₂, and vitamin C.

^c Refers to calcium, copper, magnesium, and zinc.

Table 11. Daily micronutrient intake after hypothetical *Materna*TM consumption (n =216; where 180 women took supplements)

Nutrient	EAR	RDA	UL	Nestlé® <i>Materna</i> TM	Hypothetical Median Intake (25 th , 75 th percentile)	Risk of inadequacy (%)	Adequate intake (%)	Risk of excess (%)
Vitamin A (µg RAE) ^a	550	770	--	425	1172.5 (968.8, 1429.7)	3	96	--
Vitamin A (µg RE) ^b	--	--	3000	300	714.6 (576.7, 834.3)	--	--	1
Thiamine (mg)	1.2	1.4	--	1.4	3.1 (2.6, 3.5)	2	98	--
Riboflavin (mg)	1.2	1.4	--	1.4	3.4 (3.0, 4.0)	0	100	--
Niacin – NE (mg) ^c	14	18	35 ^e	18	53.3 (46.4, 59.7)	0	100	--
Pantothenic Acid (mg)	--	6*	--	6	12.0 (10.4, 13.3)	--	--	--
Vitamin B ₆ (mg)	1.6	1.9	100	1.9	3.6 (3.1, 3.9)	7	93	0
Vitamin B ₁₂ (µg)	2.2	2.6	--	2.6	6.8 (5.4, 8.4)	1	99	0
Vitamin C (mg)	70	85	2000	85	237.9 (175.2, 290.2)	2	98	0
Vitamin D (µg)	10	15	100	10	14.5 (12.0, 16.7)	15	85	0
Vitamin E (mg) ^d	12	15	1000 ^e	13.5	20.1 (18.0, 22.4)	15	85	0
Folate (ug DFE)	520	600	--	--	2737.2 (1800.1, 2785.3)	11	6	--
Folic Acid (µg)	--	--	1000 ^e	1000	1099.6 (1045.2, 1148.7)	--	--	83
Calcium (mg)	800	1000	2500	250	1401.8 (1099.3, 1629.5)	7	92	1
Copper (mg)	0.8	1	10	1	2.5 (2.2, 2.9)	0	100	0
Iron (mg)	22	27	45	27	40.7 (37.7, 44.1)	16	65	19
Magnesium (mg) ^f	290, 300	350, 360	350 ^e	50	402.5 (334.5, 476.1)	14	85	0
Manganese (mg)	--	2*	11	2	5.5 (4.5, 6.3)	--	--	1
Selenium (µg)	49	60	400	30	138.8 (120.2, 161.8)	0	100	0
Zinc (mg)	9.5	11	40	7.5	18.3 (15.5, 20.9)	4	96	0

^a As retinol activity equivalents (RAEs); where 1 µg RAE = 1 µg retinol RE = 12 µg β-carotene. The RAE for preformed vitamin A is the same as RE.

^b As retinol equivalents (RE); where 1 µg RE = 1 µg preformed vitamin A, which is used to establish the UL.

^c As niacin equivalents (NE); where 1 mg NE = 1 mg of niacin = 60 mg of tryptophan.

^d As α-tocopherol.

^e The ULs for niacin, folic acid, and vitamin E apply to synthetic forms obtained from supplements, fortified foods, or a combination of both.

^f The EAR and RDA values listed for magnesium are from food sources for pregnant women ages 19-30 and 31-50 years old respectively. The UL for magnesium applies only to the supplemented form found in medication (pharmacological agents) and dietary supplements

* Adequate Intake (AI) values. RDAs have not been established for these micronutrients.

Sources: Dietary Reference Intakes (DRIs) established by the Institute of Medicine's Food and Nutrition Board. DRIs for calcium, phosphorous, magnesium, vitamin D established in 1997; thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12 in 1998; vitamin C, vitamin E, selenium in 2000; vitamin A, copper, iron, and zinc in 2001; and calcium and vitamin D DRIs were updated in 2011. Values listed are established for women between the ages of 19-50 years old during pregnancy.

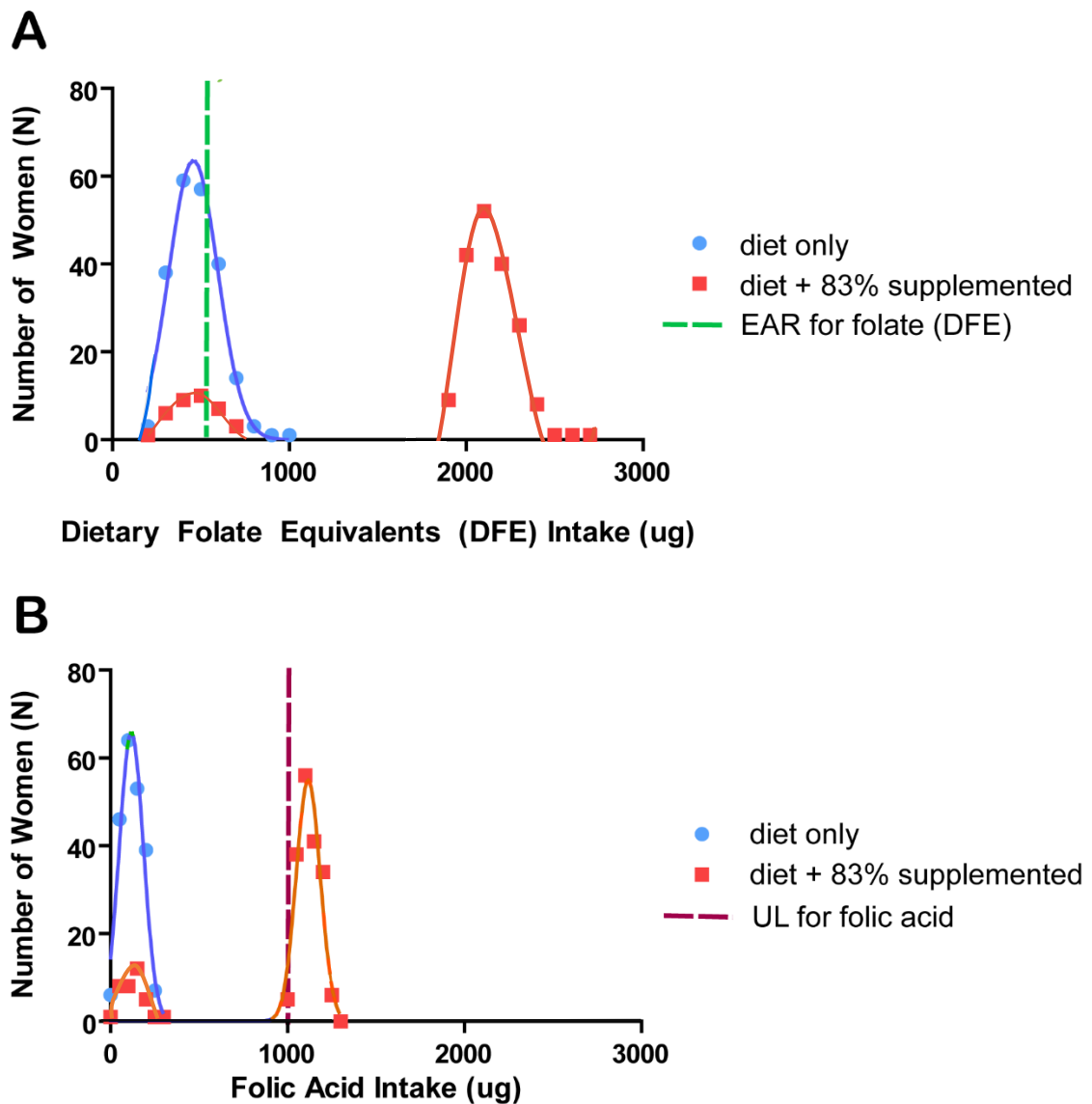


Figure 5. Folic acid consumption before and after 83% of women take *Materna*TM (N=216). Women who reported taking supplements (83%) were assumed to be taking *Materna*TM based on literature [102]. **(A)** Frequency distribution curve for number of women consuming folate (DFE) from diet only (blue) and after supplementation from diet and *Materna*TM (orange) together. Women below the Estimated Average Requirement (EAR) of 520 μg of folate (DFE) are deemed at risk of inadequacy and those above the EAR are assumed to have adequate intake. **(B)** Frequency distribution curve for number of women consuming folic acid from diet only (blue) and after 83% of women consume *Materna*TM in addition to their diet (orange). Women above the Tolerable Upper Intake Level (UL) of 1000 μg of folic acid are deemed to be at risk of excess and those below UL, but above the EAR, are assumed to have adequate intake.

2.4 Discussion

The benefits of adequate maternal nutrition, to support a healthy pregnancy and proper fetal development, are well established. At a time when fortification is mandatory for some food staples, and supplements are unanimously recommended for all Canadian women who are pregnant or planning to become pregnant, it is important to monitor micronutrient intake among the target population in case modifications to current policies are necessary. Thus, the aim of this study was to investigate dietary micronutrient intake among a Québec cohort of pregnant Canadian women. Overall, our findings confirm that food sources alone are insufficient to provide the majority of pregnant women with adequate intakes for vitamin D, vitamin E, folate, and iron and supports the need for supplementation during pregnancy. Though the majority of women reported supplementation during pregnancy, it is expected that supplement users are at risk of exceeding the UL for folic acid.

2.4.1 Demographic and Socioeconomic Factors

Epidemiological studies have shown that socioeconomic status can affect nutritional knowledge and diet quality [186]. Lower socioeconomic status and some ethnicities have been established to be risk factors for nutritional inadequacy as well as decreased use of prenatal healthcare [187–189]. Likewise, our study identified a relationship between mineral adequacy and the income of participants, suggesting that women with lower annual family incomes between \$40,000–59,000 were more likely to have inadequate intake for minerals compared to those with incomes above \$80,000. It is possible that no association was found for lower income strata due to small sample size ($n \leq 12$ per group). However, no associations were observed in our present study between vitamin status and any indicators of socioeconomic status. Previous findings have reported that younger and less educated mothers, smokers, and women with higher BMI are more likely to have poorer nutritional status and/or adverse pregnancy outcomes [190–195]. However, in the current project, these associations were not observed in our cohort, perhaps due to our skewed sample consisting of a majority of educated white women of higher socioeconomic status and in their mid-30's.

Interestingly, there appears to be a relationship between mineral adequacy and the marital status. Compared to the rest of Canada, Québec has a proportionally higher rate of couples living in common-law arrangements as opposed to being married [196, 197]. Reflective of this Québec culture, the majority of our study participants reported common-

law cohabitation. Though other researchers have found that marriage supports positive nutritional status [198], here we found that women in common law relationships were more likely to be adequate for micronutrient intake than married women. We also found that these women in common-law relationships were more likely to be white and have higher incomes than married women, suggesting that socioeconomic factors as well as racial background may account for differences in micronutrient adequacy among women in Québec. Therefore, health professionals should assess the need for supplements among women who may be at increased risk of micronutrient deficiencies and require supplementation.

2.4.2 Daily Micronutrient Intake from Food Sources

For the majority of women in this study, micronutrient intake from food sources was sufficient to meet the requirements for pregnancy for all micronutrients studied except for folate, iron, vitamin D, and vitamin E. Despite fortification of foods with folic acid, 67% of study participants remained at risk of folate inadequacy from diet alone. However, using the 2004 Canadian Community Health Survey 2.2, Shakur *et al.* [199] found that only 25% of non-supplement users and 33% of supplement-users were at risk of folate inadequacy from diet alone. Furthermore, other nationwide studies demonstrate that approximately one in five non-supplemented Canadian women of child-bearing age do not meet RBC folate concentrations deemed optimal for maximal neural tube defect reduction [112, 173]. The decreased risk of folate inadequacy is understandable since the EAR for non-pregnant populations is lower than it is for the pregnant population. As a result, it is understandable that certain populations, such as Québec women, who do not achieve recommended folate intake levels, may require increased attention to ensure sufficient folic acid intake through supplementation. With regards to iron, 93% of women in our study were at risk of inadequacy from food sources alone. In line with our findings, other North American studies agree that the risk of iron inadequacy among women of child-bearing age is a concern [169, 200–202]. Thus, the need for folate and iron supplementation is expected and supports Health Canada's recommendation for women to take multivitamin supplements containing at least 0.4 mg of folic acid and 16-20 mg of iron prior to conception and during pregnancy [29].

Vitamin D is a fat-soluble vitamin required for adequate calcium absorption and has a role in bone metabolism, anti-inflammatory processes, and supporting proper immune

functions [203]. Low serum vitamin D status has been identified as a marker of ill health [204]. Specific to pregnancy, low maternal vitamin D is believed to result in increased risk for adverse pregnancy outcomes such as preeclampsia, gestational diabetes mellitus (GDM), preterm birth, and small-for-gestational-age (SGA) [205]. Still, requirements during pregnancy remain the same as non-pregnant requirements since vitamin D can be synthesized by the body following exposure to adequate sunlight. Generally, women with darker skin pigmentation and during winter or living in areas where sunlight is limited, have reduced endogenous vitamin D synthesis [206–208]. Though 90% of the women in our study appear to have inadequate intakes of vitamin D from dietary sources, vitamin D produced by the body following sunlight exposure was unaccounted for. Furthermore, prenatal supplements, such as Materna™, contain the complete EAR and may solely suffice in providing at least half of the population with recommended levels of vitamin D [102]. Additionally, inadequacy is slightly overestimated because the developers of ESHA Food Processor™ acknowledge that 10% of foods in the database do not have information on vitamin D. Since most of the women in our study were white and reported regular supplement intake, it is likely that pregnant Canadian women have higher vitamin D serum levels despite low intake from food sources. After supplementation and sunlight exposure, studies using the Canadian Health Measures Survey did not suggest wide-spread vitamin D deficiency in the Canadian population [209, 210].

Vitamin E is a fat-soluble antioxidant that protects cell membranes due to its antioxidant activity, and has been shown to prevent platelet aggregation [211]. Like vitamin D, vitamin E requirements are the same for both pregnant and non-pregnant women, though instead, it must be obtained exclusively from the diet. Though over 90% of women in our study were found to be at risk of deficiency for vitamin E, this is an overestimation due to lack of a complete Canadian Nutrient File (CNF) database. Currently, the α -tocopherol (vitamin E) content of foods is only complete for 67% of foods listed in the CNF database [212]. Thus, it is possible that the women in this cohort have higher vitamin E intakes than portrayed due to overestimation of inadequacy and prenatal supplementation. Lower dietary vitamin E intake during the second trimester of pregnancy has been shown to be associated with third trimester insulin resistance and hyperglycemia in the mother [213]. As a result, it is important to ensure adequate intake which can be achieved by proper diet and supplementation as appropriate.

The majority of women in the current study were adequate for the other micronutrients studied, however 10-35% of the women were at risk of inadequacy for vitamin A, thiamine, vitamin B₆, vitamin C, calcium, magnesium, and zinc. For women with risk of inadequacy for numerous micronutrients, Ramakrishnan *et al.* [214] suggests that a prenatal multivitamin supplement may be more beneficial than simply a folate-iron supplement. Nonetheless, indiscriminately recommending a multivitamin may not be necessary for the entire pregnant population.

In addition to addressing inadequacies from diet alone within the pregnant population, risk of excess micronutrient consumption is also a concern, albeit to a lesser extent. From diet alone, only one woman exceeded the UL for vitamin A intake. This was due to consumption of 150 g of beef liver on one of the three days recorded in her food record. However, it is important to note that vitamin A has high within-person day-to-day variation and so, three days is not enough to accurately gauge vitamin intake [215, 216]. Vitamin A is also fat-soluble and can be stored by the body and used during periods of low intake. Thus, it is possible that if more days were analysed, both the 19% of women currently deemed to be at risk of inadequacy and the woman who exceeded the UL more would have an average vitamin A intake that falls within the recommended range.

The majority of women in this study were at risk of exceeding the UL for sodium, suggesting a high intake of salt-rich foods. Using data from the Canadian Community Health Survey [217], it is recognized that excessive sodium consumption is a concern for most Canadians [218]. Though sodium is often consumed in the form of table salt, a major contributor towards excess sodium consumption comes from processed foods as a flavor enhancer and preservative [219–221]. High daily sodium intake has been associated with increased risk of hypertension [222], as well as stroke and cardiovascular disease [223, 224]. With increased concern of the effects of maternal nutritional status during pregnancy and risk of adverse effects on fetal cardiac cells and brain development [128, 225], high sodium intake among the pregnant population should be addressed. Currently, Health Canada is working towards lowering the salt intake among Canadians from an average of 3400 mg/day of sodium to 2300 mg/day by 2016 by raising awareness, supporting research related to sodium reduction, and promoting lower sodium in processed foods [218]. With the exception of sodium, none of the other women in our subsample were at risk of excess consumption for any other micronutrient studied from their diet. Similarly, Shakur *et al.*

[199] found that Canadians above 14 years of age were not at risk of exceeding the UL from diet alone for vitamin A, vitamin C, vitamin D, niacin, folic acid, iron, zinc, and magnesium.

Overall, with the exception of sodium, excessive intake of micronutrients is not a reason for concern from food sources. However, inadequate intake of several micronutrients is still prevalent and may require supplementation to support a healthy pregnancy and proper fetal development.

2.4.3 Prenatal Supplementation

In line with other Canadian studies that suggest more than 80% of pregnant Canadians consume a prenatal supplement, where the majority are of higher socioeconomic status [102, 108], we found that 83% of women in our cohort took nutritional supplements in their second trimester. Like the women in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort, our results demonstrate that women were more likely to take supplements towards the beginning of their pregnancy, when risk of NTD occurrence is highest, and have decreased supplement consumption in second and third trimesters [108]. In a recent study, data on over 6,000 women who participated in the Public Health Agency of Canada's Maternity Experiences Survey was used to determine the relationship between awareness of benefits of periconceptional folic acid intake and the extent to which this awareness translated into supplement use [226]. Nelson *et al.* [226] found that 77.6% of the women who were surveyed were aware that taking folic acid periconceptionally may protect against NTDs, but only 68.8% of these women took folic-acid containing supplements. Thus, supplement usage in our study was above national average, but this is likely attributable to the demographic characteristics and high socioeconomic status of our study participants.

Using data collected by the National Birth Defects Prevent (NBDP) Study in the United States, it was found that education, employment, income, ethnicity, and maternal age were among the factors associated with preconceptional folic acid-containing supplement use [106]. High socioeconomic status is related to decreased risk of neural tube defects (NTDs) [227], suggesting a relationship between affluence and access to a healthier diet [186, 228]. Indeed, the majority of women in our study were of higher socioeconomic status based on their education level, employment status, and annual income. With the majority being in their mid-30's, the women in our study primarily self-identified as white, reported a planned pregnancy and commitment to supplement usage. Studies have shown that Caucasian women and non-Hispanic white adults are more likely to take folic acid supplements before

pregnancy than other ethnic groups [229, 230]. Furthermore, supplement usage increases among women who plan their pregnancy and have a higher level of nutritional knowledge [231, 232], while first time and younger mothers are often less-likely to consume a supplement [226, 229]. Interestingly, though over 40% of the women in our study were pregnant for the first time, more than 90% consumed a supplement. As Nelson *et al.* [226] suggests, high incidence of supplement use in our study is possibly due to a higher average age of 33.8 years, high socioeconomic status, and higher education level and cannot be generalized to other groups of women. Extra care and resources should still be provided to educate first time and younger mothers, as well as women of lower socioeconomic status to ensure nutritional adequacy.

Interestingly, only 1% of our sample had current pregnancies affected by congenital heart defects, despite reporting prenatal supplement consumption. Though folic acid fortification has been shown to dramatically reduce the prevalence of NTDs and reduce the risk of congenital heart defects [28, 233], Bedard *et al.* [234] found that folic acid fortification did not help prevent congenital heart defects in Alberta, Canada. This suggests that other risk factors, such as genetic predisposition and lifestyle factors play a role in the presentation of congenital anomalies and that folic acid supplementation alone does not eliminate the risk of NTDs [33, 235]. Thus, in addition to promoting adequate folic acid intake, increased effort is needed to raise awareness on the risk factors for congenital anomalies and develop preventive strategies to further preclude these defects. As a result, prior to indiscriminately subjecting women to increased folic acid supplementation, careful consideration of the risk-benefit of increased folic acid intake is needed.

Projected Micronutrient Intake after Prenatal Supplementation

At the time of our study, only data regarding whether or not the women were consuming a supplement was available. On the market, there are several non-prescription prenatal supplements that contain between 0.4 mg – 1 mg folic acid. Since Nestlé® *Materna*TM is the most common prenatal supplement that contains 13 essential vitamins and 10 essential minerals [102, 185], projected total micronutrient consumption was evaluated assuming supplement users consumed *Materna*TM. Using *Materna*TM, which contains 1 mg of folic acid, all supplement users are at risk of adverse effects associated with exceeding the UL for folic acid. Other North American research groups have also found that women of child-bearing age are at risk of higher circulating and intake and levels of folic acid after diet

and supplementation [101, 112, 236], suggesting the need to evaluate the positive and negative effects of high folic acid intake. Instead of Nestlé® *Materna*TM, using an alternative brand of prenatal supplements containing 0.4 mg of folic acid, as recommended by Health Canada, may be a viable option to keep intakes below the UL. Interestingly, Hursthouse *et al.* [237] found that women given a 0.14 or 0.4 mg/day folic acid supplement for more than 40 weeks did not have a difference in RBC folate levels between the two treatment groups and there was no difference in the odds of having low RBC folate levels (< 906 nmol/L) associated with increased risk of NTDs. This suggests that perhaps current recommendations of folic acid supplementation can be lowered to still achieve the desired effect of NTD reduction.

Nearly 20% of our subgroup may be at risk of excess consumption of iron after *Materna*TM supplementation. Though iron is necessary to prevent anemia during the increased demands of pregnancy, there is suggestion that elevated iron levels may be teratogenic during the period of embryonic development [120]. Excessive iron consumption has also been linked to increased risk for SGA babies [121], decreased zinc absorption [122], oxidative stress [123, 124], and gastrointestinal symptoms [125]. Thus, caution is warranted when taking supplements during this critical time period.

Only 1% of women in the study may be at risk of exceeding the UL for either calcium, manganese, or vitamin A after supplementation. Foods that contributed to higher than usual intake levels included extra servings of dairy products in the case of calcium, regular consumption of bran breakfast cereal in the case of manganese, and consumption of beef liver contributed significantly to boost vitamin A intake. Without long-term measurements, it is difficult to determine whether increased intake is due to unique within-person day-to-day variation in diet, increased appetite during pregnancy, or whether it is a concern over the long-term for these women. Thus, individual diet should be considered prior to multivitamin consumption.

The synthetic form of niacin is deemed potentially harmful. Exceeding the UL for niacin puts the individual at risk for skin rashes and flushing [12]. At extreme intake levels, there is potential for nausea, liver damage, increased blood glucose and peptic ulcers [12]. Though our results show that median intakes of niacin exceeded the established UL, ESHA Food ProcessorTM is currently unable to discriminate between the naturally-occurring and synthetic niacin. Thus, it was not possible to determine the risk of excess intake. Lagiou *et*

al. [100] also found that the average niacin intake from diet and supplements among a group of pregnant women also surpassed the UL, though they did not specify the source of niacin. In contrast, other studies have found that most women of child-bearing age in Canada and the United States are within the recommended range and meeting the EAR for niacin [169, 238]. Overall, it seems that adequate niacin intake is achieved through dietary sources and niacin supplementation may not be as necessary.

While developing Canada's Food Guide, Health Canada defined the threshold for low prevalence of inadequate intakes to be where 10% of the population have intakes below the EAR [239, 240]. Using this threshold, a low prevalence of inadequate intake is expected for vitamin A, thiamine, vitamin B₆, vitamin B₁₂, vitamin C, calcium, and zinc. The risk for inadequate intake (11-16%) for vitamin D, vitamin E, folate, iron, and magnesium remained near the low prevalence threshold. It is expected that those who remained at risk of inadequacy were among the 17% of women who reported no supplement intake. In another Canadian study, Roy *et al.* [102] found that around 20% of women, with a high socioeconomic status overall, were not taking prenatal supplements and a large portion had intakes below the RDA for iron, zinc, and folate levels. Thus, there is still room for nutritional improvement and more effort should be put into educating women of child-bearing age about the benefits of adequate micronutrient intake through diet and supplementation.

In general, it is evident that after supplementation with Nestlé® *Materna*TM, all supplement users may be at increased risk of excessive folic acid intake, while 1 in 5 women may be at risk of excessive iron intake. Using an alternative brand of prenatal supplements containing lower doses of folic acid or individually targeting women at risk of inadequacy may help keep intakes below the UL. Since a group of non-supplement users remain, educational programs are still recommended to help promote adequate micronutrient intake during pregnancy through diet and/or supplementation.

2.5 Limitations

2.5.1 Sources of Error in Nutritional Assessment Method

Assessments of diet within a given population allows for in-depth investigation into nutrient intake patterns across a population, identification of patient populations naturally at risk of nutrient deficiency or excess, as well as patient populations that may become at risk following a particular intervention and/or environmental change. Of the various methods used to analyse dietary intake in a population, the 3-day food record method was selected to assess nutrient intake within a large cohort of pregnant women. The 3-day food record is a prospective method that has been shown to be more reliable for habitual assessment of individuals than its retrospective counterparts: the 24-hour dietary recall method and food frequency questionnaires [241]. It allows the freedom to include more details and due to its prospective nature, diminishes much of the memory bias that occurs in retrospective methods. As suggested by Thompson *et al.* [242], by including two weekdays and one weekend day, variations in the diet during the week are accommodated. Though three days has been found to lead to optimum response rate and decreased participant burden [241], some micronutrients, such as vitamin A, have higher day-to-day variation and require at least 25-29 days for better representation of intake [215, 216]. Due to limited resources, high participant burden, and the potential for decreased response rate, such a long period of time is impractical.

Other limitations of this approach include decreased response rate, possible decreased motivation to complete and return the food record, respondent's error in estimating accurate portion sizes, reporting bias, and the possibility for alteration of habitual eating patterns due to self-monitoring [174]. Furthermore, this approach requires trained personnel and intensive data entry, leaving room for errors in transcription. As a result, interpretation of data must be conducted in light of these potential limitations.

2.5.2 Sources of Error in Food Composition Values

Despite having an updated food composition database and computer software, ESHA Food Processor™, there are still possible sources of error in assessing nutrient intake. Though relevant to the Canadian diet using the Canadian Nutrient File (CNF) database, a portion of the ESHA Food Processor™ relies on information from the USDA food composition database. For nutrients such as folate, both countries differ in their fortification policies and would affect calculated intakes. To minimize inaccuracies, only seven food items were

selected from the USDA database. Another limitation was the assumption that the nutrient content of specific foods remain constant. For example, the β -carotene content of a carrot may vary based on the size, growth, harvesting, processing, cooking, and storing conditions.

Though the CNF database contained micronutrient content information for the majority of food items, micronutrient inadequacy was overestimated for some micronutrients such as vitamin E since only 67.16% of the foods in the current Canadian Nutrient File database has been measured for their α -tocopherol content [212]. Additionally, for nutrients such as biotin, chloride, choline, chromium, fluoride, iodine, molybdenum, and vitamin K, we were unable to analyse nutrient intakes due to insufficient information in the CNF database.

2.5.3 Sources of Measurement Error in Dietary Assessment

Random and systematic measurement errors may occur while measuring food and nutrient intakes. These measurement errors include nonresponse bias, sampling bias, respondent biases, incorrect estimation of portion size, coding errors, and missing supplement usage information.

Among the pregnant women who were recruited for the IRNPQEO study, the response rate was 65.6%. This is close to the response rate achieved by another large cohort study using 3-day food records [243]. Nonetheless, a large portion of the women chose not to complete and return the food records, and may have resulted in a nonresponse bias. As a result, instead of having a random sampling of Québec women, the subjects who participated in urban centres may not be representative of pregnant Canadians in Québec. Our results contain mostly the affluent, educated, white pregnant population in Québec, Canada. However, it is recognized that culture, ethnicity, and environment play a role in nutrition and food choices in a group of people [244]. Based on the demographic and socioeconomic characteristics of the women in our study, these results are not generalizable to more ethnically diverse, socioeconomically poor, or Aboriginal groups of women in Canada. Other ethnic groups with varied diets and women with lower socioeconomic statuses and education levels may have altered different nutritional status and frequency of supplement use. Nonetheless, the pregnant population is a group that is often excluded from studies, so this study has the benefit of revealing current dietary intakes in a necessary subset of Canadians.

Furthermore, respondent bias, such as underreporting of food intake by approximately 20% in both men and women [245, 246], is often an issue in dietary assessment methods. Underreporting may occur due to errors in estimating portion sizes or in an effort to maintain socially desirable diets that includes fewer unhealthy food choices. Due to the lack of motivation or the inconvenience of having to record each item, individuals may also modify their food choices to healthier or simpler food for ease of recording and/or in apprehension of evaluation. Providing the participants with training on how to properly fill out the food records with an appropriate amount of detail and explaining the significance of the results and how their contribution plays a role in the study may help prevent reporting errors. Having a debriefing session by a nutritionist or study personnel has been shown to help prevent measurement error from food records [247], but due to limited resources, this was not possible. Instead, in order to improve accuracy and reliability, a brief training and an example of a completed food record was provided by study personnel prior to completion. Still, there were cases where participants were not detailed in their portion sizes and did not include recipes for some homemade food items. Though standard portion sizes and recipes were used instead, variations in micronutrient intake may have occurred.

Due to the volume of food records that needed to be analyzed to meet our sample size requirements, four people (myself and three others) initially participated in food record data entry. Coding errors may have occurred when converting household measures into grams or selecting the most accurate food item ESHA Food Processor™. To prevent intra-individual variability in data entry (ex. brand choices, food proportions, etc.) and ensure consistent decision-making on common foods, entered food records were systematically double-checked by two trained registered dietitians and extreme values were systemically double-checked for implausible entries. In the future, inter-individual variability can be prevented by limiting the number of people participating in data entry.

At the time of this study, only information regarding whether or not study participants used a nutritional supplement was available. Thus, only estimated intakes after supplementation could be determined. Based on the brand and composition of the supplement, micronutrient intake may vary between individuals. In the future, once the brand and composition of the supplements are released for study, a more accurate picture of micronutrient intake from dietary and supplementary sources can be determined.

In summary, the limitations in the assessment method, food composition data, and measurement errors must be considered when drawing conclusions from this study.

2.6 Future Directions

Dietary assessment methods allow researchers to determine a general representation of dietary inadequacies. Analysis of all completed 3-day food records (N=1612) collected by the 3D Study is almost finished. Once mother-medication logs specifying brand and dosage of prenatal supplements is made available, dietary intakes of micronutrients can be better defined. As part of the 3D study, IRNPQEO staff have collected follow-up growth and development indices for up to two years after birth. Thus, future studies may include investigating the relationship between the offspring of mothers, with adequate/inadequate diet in the presence/absence of prenatal supplementation, and their consequences on future health outcomes. For example, a nested case-control study can be done comparing the effects of high folic acid intake after supplementation (cases) with recommended intakes (controls) observed among mothers and their offspring. However, though this study was able to analyse the dietary intake of pregnant women who are mostly of higher socioeconomic status and living in an urban setting, further studies are required of pregnant women of lower socioeconomic status, diverse ethnic background, and/or in more rural areas in order to get a better idea of micronutrient adequacy in the Canadian pregnant population.

Due to aforementioned limitations of dietary assessment methods in accurately measuring micronutrient intake, laboratory methods could be used to supplement our findings and provide a more precise representation of bioavailable micronutrients. In addition to the 3-day food records of pregnant women, the IRNPQEO staff have also collected biological samples (ex. maternal and cord blood, placenta samples) of the resulting offspring as part of the 3D Study. In the future, laboratory methods that include biochemical tests (ex. using blood samples or measuring urinary excretion rate) may be a useful tool to determine metabolized levels of micronutrients and the level of micronutrients in reserve tissue and body fluids. This information can be used to compare to intake levels and to better assess dietary needs among the given population.

Finally, though the preventive effects of folic acid supplementation on adverse maternal and fetal outcomes have been researched [248], adverse outcome due to excessive levels have not been as thoroughly studied. The fetal programming hypothesis suggests that

events *in utero* can have long-term effects on the health of the offspring [249]. Functional biochemical tests (ex. changes in enzyme activity) may help elucidate the effects of increased or decreased levels of micronutrients on health and disease. Furthermore, given that the placenta is fetal tissue [250] and the gateway for nutrient transfer between the mother and fetus [251], studying the effects of different concentrations of micronutrients on placental tissue can also help reveal potential effects on the offspring.

2.7 Conclusion

In summary, using second trimester 3-day food records, dietary sources appear to provide the majority of women with adequate micronutrients for all micronutrients studied, except for folate, iron, vitamin D, and vitamin E. In contrast, the majority of the pregnant women in our study sample was found to be at risk of excessive sodium intake. Supplementation may be required for micronutrients found to be at risk of inadequacy for most pregnant women since food sources alone do not seem to provide the recommended amount for a healthy pregnancy. Though vitamin D can be synthesized through sun exposure, supplementation may also be required due to seasonal differences and varying sun exposure times. Furthermore, supplementation of other micronutrients may also be necessary for other subgroups of pregnant women in the Canadian population.

Adhering to national recommendations to take a prenatal supplement, 83% of women reported taking a nutritional supplement during their second trimester of pregnancy. Following supplementation, the majority of women may be at risk of excessive folic acid consumption, while nearly 20% of women may also be at risk of excessive iron consumption. At a time when fortification of food with folic acid is prevalent and recommendations for further prenatal folic acid supplementation is paramount, the benefits and adverse effects of excessive folic acid intake needs to be further investigated.

Chapter 3

Effects of Excessive Folic Acid on Placental Health & Function

3.1 Introduction

The detrimental effects of various maternal nutrient deficiencies on fetal development have led to the development and recommended use of multivitamin supplements to promote a healthy pregnancy [113]. Women are recommended by Health Canada to take multivitamin supplements containing 0.4 mg of folic acid prior to conception and during pregnancy, regardless of their nutritional status, to ensure adequate vitamin and mineral levels throughout gestation [29]. However, the available literature [100, 112, 199] and our findings in Chapter 2 suggest that a diet consisting of fortified foods and supplementation may result in excessive folic acid intake among women of childbearing age and pregnant women in Canada. Importantly, very little work has been done to determine whether exposure to these high levels of folic acid are in fact safe for the developing placenta and fetus.

The placenta is the vital organ of pregnancy, responsible for gas and nutrient exchange as well as hormone synthesis. Despite our recognition of the critical importance of this organ for a successful pregnancy, the effects of various environmental factors (i.e. nutrient supplements such as folic acid) on the health and functioning of the placenta are often overlooked. In light of the fetal programming hypothesis which suggests that events *in utero*, such as compromised placental health and/or function, may have long-term health effects on the offspring [249], the effect of feto-placental exposure to excessive folic acid *in utero* requires significant attention. The importance of studies aimed at determining safe therapeutic ranges of folic acid is particularly relevant in light of ongoing, large-scale randomized controlled trials determining the efficacy of high dose folic acid supplementation (4 mg/day) for the prevention of preeclampsia and congenital malformations [115, 171]. Currently, these trials are ongoing in the absence of any strong data indicating that this dose of folic acid is in fact safe for the feto-placental unit.

If these excessive levels of folic acid adversely affect placental health and function, it is possible that fetal growth, development, and health may be compromised. Evidence that

high folic acid intake levels can trigger adverse effects in both animal and human models have been observed [140–142, 144, 238], and may have a severe impact on fetal health and development. However, little work has been done to directly study the effects of high folic acid exposure on the placenta. Thus, the aim of the current study was to determine whether high doses of folic acid would negatively impact primary indicators of placental health and function, including trophoblast proliferation, viability, apoptosis, invasion, and/or β -hCG expression in an *in vitro* cell culture model and *ex vivo* placental explant culture model.

3.2 Materials and Methods

3.2.1 Placental Trophoblast Model Systems

Choosing the ideal *in vitro* model is critical for proper experimental research design. The most common methods include using immortalized human trophoblast cell lines, isolating primary cultures of human trophoblast cells and using *ex vivo* human placental explants from first trimester or term placenta [252, 253]. Animal models using rodents such as rats and mice are also common, but the main limitation in this approach lies in the difference in the process of placentation between humans and other organisms [253]. Though isolation and culture of primary cells allow for the study of non-transformed human placenta cells, the main limitations to this procedure are the altered cell physiology resulting from the extensive processing required in the cell isolation and purification protocol, the inability to maintain these cells in culture for more than a couple days and the inter-patient variability observed in each placenta [254]. Thus, immortalized human trophoblast cell lines (HTR-8-SVneo and BeWo) and *ex vivo* human placental explants were selected as representative models for this project.

Immortalized human trophoblastic cell lines have been developed to study both the extravillous and villous trophoblast cell lineages. Two trophoblast cell lines employed in the current research project are the widely used HTR-8/SVneo extravillous trophoblast cell line and the BeWo villous cytotrophoblast cell line. The HTR-8/SVneo cell line was established from first trimester extravillous trophoblasts transfected with the SV40 virus, and is often used to study the invasive properties of extravillous trophoblasts [253]. In contrast, the BeWo cell line was established from a malignant villous cytotrophoblast (choriocarcinoma) and is often used to study processes involved in villous cytotrophoblast proliferation and terminal differentiation into the multinucleated syncytiotrophoblast. The villous trophoblast cells, including the BeWo cell line, are responsible for hormone production, including human

chorionic gonadotropin (β -hCG) hormone production [253], which is often used as a marker of villous trophoblast cell health and function. Experimental designs using cell lines allow for tight regulation of experimental conditions and enable reproducibility.

Ex vivo human placenta villous explants are collected from fresh placentae, either from elective terminations in the first trimester or at the time of delivery in the third trimester. This *ex vivo* tissue model has the important advantage of maintaining the structural integrity of the tissue, allowing for the persistence of cell-cell interactions. Further, these tissues can be collected either from uncomplicated pregnancies or pregnancies associated with a clinical pathology, allowing for *in vitro* examination of physiological differences in placental function in these pathologies. Typically, the placental villous explant model is used to study the effects of external factors on tissue survival, functionality, and adaptation to exogenous exposures, by observing cellular processes such as proliferation, apoptosis, hormone production, and/or syncytial fusion.

A. *In vitro* Trophoblast Cell Culture Model

3.2.2 Cell Culture and Growth Conditions

The HTR-8/SVneo extravillous trophoblast cell line was generously provided by Dr. Charles Graham (Queen's University, Canada) and the BeWo villous cytotrophoblast cell line was obtained from the American Type Culture Collection (ATCC) bio-resource centre (Virginia, USA). The HTR-8/SVneo cell culture model was selected for study, as this model is representative of the invasive first trimester extravillous trophoblast cells of the placenta, which are involved in the invasion of the uterine wall and remodelling of the uterine vasculature [255]. The BeWo cell model was selected for study, as this model is representative of the villous trophoblast cells that are responsible for maternal-fetal exchange and the endocrine function of the placenta [253, 255, 256].

Both cell lines were cryopreserved and stored at -80°C . When thawed, both cell lines were maintained as monolayers in CORNING® 75 cm² Cell Culture Canted Flasks (Corning®; NY, USA) in a humidified incubator with 21% O₂, 5% CO₂ at 37°C. HTR-8/SVneo cells were grown in FA-depleted Gibco® 1X RPMI-1640 (Life Technologies™; NY, USA) supplemented with 20 ng/mL folic acid (FA) (Sigma-Aldrich; MO, USA), 5% fetal bovine serum (FBS), and 1%

100X Penicillin-Streptomycin (P/S) Solution (Mediatech, Inc.; VA, USA). The concentration of folic acid used for supplementation of the standard culture media (20 ng/mL) was selected to mimic concentrations of serum folate levels measured in pregnant women [101, 168] (Appendix D). BeWo cells were also grown in FA-depleted 1X RPMI-1640 supplemented with 20 ng/mL FA, 10% FBS, and 1% 100X P/S.

For maintenance of the cell lines, cells were passaged every 2-3 days (when they reached 75-80% confluence) and culture medium was changed every 2 days. The procedure for passaging the cells included washing the cells with 1X Dulbecco's Phosphate Buffered Saline (PBS; Corning® Cellgro®; VA, USA) and removal of cells by an enzymatic treatment using 1X Trypsin EDTA (Mediatech, Inc.; VA, USA) for 5 minutes. Cells were harvested, and subcultured in fresh flasks. To maintain consistency, experiments using HTR-8/SVneo and BeWo cells were restricted to passages below 30.

3.2.3 Folic Acid Treatment

Prior to any experimental protocol, HTR-8/SVneo and BeWo cells were serum-starved overnight in serum-free media (0% FBS). All dose-response experimental protocols, described below, involved the use of five folic acid experimental treatments (2, 20, 200, 2000, 4000 ng/mL), in which folic acid was dissolved into the FBS-supplemented cell culture media. These concentrations were selected to represent maternal folic acid deficiency (2 ng/mL), normal maternal physiological concentration (20 ng/mL), supraphysiological concentration (200 ng/mL) and toxicological concentrations (>2000 ng/mL). Unless otherwise specified, cells were exposed to the folic acid experimental treatments for 48 hours, at which point cells were harvested for analysis.

3.2.4 Microbiological Assay for Intracellular Folates

Trophoblast cells possess several folate transporters, responsible for shuttling folate both into and out of the cell [164]. In order to determine the degree of exogenous folic acid uptake into the HTR-8/SVneo and BeWo cell lines following folic acid treatments (Section 3.2.3), a microbiological assay was employed using glycerol-cryoprotected *Lactobacillus rhamnosus* [257–259], formerly known as a subspecies of *Lactobacillus casei* [260].

HTR-8/SVneo (N=3) and BeWo cells (N=3) were initially seeded into 6-well plates, at a density of 1.2×10^5 cells/well. Both cell lines were serum starved overnight and treated as outlined in Section 3.2.3. After 48 hours of treatment, cells were harvested for analysis of

intracellular folic acid content. The harvested cell pellet was suspended in 200 μ L of protein extraction buffer (2% w/v sodium ascorbate, 0.2 M β -mercaptoethanol, 0.05 M HEPES, 0.05 M CHES, ddH₂O), and protein concentration was quantified in each sample (25 μ L) using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories; CA, USA). The remaining sample (175 μ L) was boiled for 10 minutes at 100°C, cooled on ice for 10 minutes, centrifuged at 18,000 x g for 10 minutes at 4°C. Rat Conjugase Serum (1:4 dilution with supernatant) (Pel-Freez Biologicals, USA) was added to the supernatant and incubated at 37°C for 3 hours. The rat serum conjugase removes the polyglutamate residues from the folic acid found in tissue extracts and makes mono- and di-glutamyl folate available to analyse tissue folate content using the microbiological assay [261]. Samples were then boiled at 100°C, chilled on ice for 5 minutes, centrifuged at 18,000 x g for 5 minutes, and the final supernatant was collected and stored at -80°C pending analysis.

The treated HTR-8/SVneo and BeWo cell lysate samples were transported on dry ice to the Nutrition Research Division at the Sir Frederick G. Banting Research Centre (Health Canada, Ottawa, ON) for final determination of intracellular folate levels using the *L. rhamnosus* microbiological assay. Stock glycerol-cryoprotected *L. rhamnosus* (ATCC 7469) was prepared as described by Horne [257] and stored in 2 mL aliquots at -80°C. For each experiment, the *L. rhamnosus* inoculum was prepared by diluting stock *L. rhamnosus* (1:100) in sterile saline. As suggested by Horne [257], this dilution is experimentally selected for each stock after preparing a series of standard curves using a range of dilutions of the cryoprotected *L. rhamnosus* and determining which dilution results in the highest absorbance for all points on the standard curve.

Each HTR-8/SVneo and BeWo sample (10 μ L) was incubated with 20 μ L diluted *L. rhamnosus*, 1 μ L folate extraction buffer [2% w/v sodium ascorbate, 0.2 M β -mercaptoethanol, 0.05 M HEPES (pH 7.85), 0.05 M CHES (pH 7.85), sterile filtered water (ddH₂O)], 8 μ L working buffer [1 M potassium phosphate (pH 6.1), 16% w/v sodium ascorbate, ddH₂O], 150 μ L BD Difco Folic Acid Casei Medium (prepared according to manufacturer's instructions), and 111 μ L ddH₂O in 5 replicates in a 96-well microtiter plate. To maintain consistent air-to-plastic surface area in all wells [257], the outer wells (Row A and H, and columns 1 and 12) in the 96-well microtiter plate were not used in the assay and instead, were filled with ddH₂O blanks. The assay was calibrated using a folic acid (0-50 fmol) standard curve, in triplicate. The plates were incubated for 16 hours in a humidified chamber at 37°C.

Since the growth of *L. rhamnosus* is relative to the amount of total folate present in the medium, the total intracellular folate level can be assessed by measuring the turbidity of the inoculated medium at an absorbance of 595 nm using a spectrophotometer (Multiskan Spectrum v1.2, Thermo Fisher Scientific, USA). To minimize pipetting errors, the lowest and highest absorbance values were discarded in order to leave 3 out of 5 replicates for analysis. From the resulting data and the standard curve, the normalized average folate concentration for each sample (fmol/ μ g) was calculated.

3.2.5 Assessment of Trophoblast Health: Cellular Proliferation

An indicator of cell health is the ability of cells to proliferate into healthy daughter cells. 5-bromo-2'-deoxyuridine (BrdU) is a synthetic thymine analogue which becomes incorporated in *de novo* DNA in proliferating cells during the S-phase of the cell cycle [262, 263]. Thus, immunoreactivity to BrdU was used to identify the effect of exogenous folic acid on the proliferative capacity of HTR-8/SVneo and BeWo cells.

HTR-8/SVneo (N=3) and BeWo cells (N=3) were seeded at 2.5×10^4 cells/well in 12-well plates and serum starved overnight. They were then treated with different experimental doses of folic acid (2, 20, 200, 2000, 4000 ng/mL folic acid) as outlined in Section 3.2.3. After 24 hours of treatment, cells were transferred to Millicell EZ chamber slides (EMD Millipore, USA) at a density of 1.5×10^4 cells/well and incubated at 37°C for an additional 18 hours in their respective experimental folic acid treatments. Thereafter, 10 μ M 5-Bromo-2'-deoxyuridine (BrdU) was made up in the corresponding folic acid experimental treatments and was added to each well of the chamber slide and incubated for an additional 6 hours (total experimental treatment exposure of 48 hours).

At the time of fixation, without removing the culture media, cells were incubated in 150 μ l cooled acid ethanol fixative (90% ethanol, 5% acetic acid, 5% water) at room temperature for 5 minutes. The culture media and initial acid ethanol fixative was aspirated from the wells and 200 μ l of acid ethanol was added for 30 minutes at 4°C. Antigen retrieval was performed using 2M hydrochloric acid (HCl) for 20 minutes at room temperature, followed by neutralization with 200 μ l of 0.1M NaB₄O₇ for 10 minutes. Cells were washed 3 times in 1X PBS and then blocked with 1% Bovine Serum Albumin (BSA; Fisher Scientific, USA) in 1X PBS at room temperature for 30 minutes.

The primary monoclonal mouse anti-BrdU antibody (1:500 dilution in 1% BSA; Sigma-Aldrich, USA) was added to cells and incubated overnight at 4°C. After a second wash with 1X PBS, cells were incubated in red fluorescent-labelled rabbit-anti-mouse IgG secondary antibody (1:500 dilution in 1% BSA; Alexa Fluor® 594, Life Technologies, USA) for 1 hour at room temperature. After a third wash with 1X PBS, the cells were counterstained with blue fluorescent-labelled Hoechst stain (1:4000, diluted in PBS; DAPI, Life Technologies, USA) for 15 minutes at room temperature. After a final wash in 1X PBS, the slides were air-dried and mounted using PermaFluor™ Mountant (Thermo Scientific, USA). For each experiment, negative controls were run in parallel with omission of the primary antibody.

Two randomly selected fields of view were imaged for each experimental treatment per experiment, using Zeiss Axioskop 2 MOT *Plus* Microscope (Carl Zeiss Microscopy, USA) at 10X magnification. Mean proliferating cell counts (red immunofluorescence) and total cell counts (blue immunofluorescence) were performed using automated Image J Software v1.47 (National Institutes of Health, USA). Results were calculated as the number of proliferating cells divided by the total number of cells, expressed as a percentage, for each experimental treatment group.

3.2.6 Assessment of Trophoblast Health: Cellular Viability

Cellular viability is a measure of the number of living cells in a sample. The trypan blue exclusion assay is a common method used to determine cellular viability [264]. Viable cells, characterised by an intact cell membrane, are impenetrable by the trypan blue dye; whereas dead cells have a compromised cell membrane that allows trypan blue uptake [264].

HTR-8/SVneo cells (N=3) and Bewo cells (N=3) were seeded at a density of 5×10^4 cells/well into 12-well plates. After serum-starvation overnight, both cell lines were treated with experimental folic acid treatments (2-4,000 ng/mL) for 48 hours (Section 3.2.3). A positive control stress treatment of 1 μ M staurosporine (Sigma-Aldrich, USA) was included for each experiment. Staurosporine is a protein kinase inhibitor and is known to activate cellular stress-response and apoptosis pathways [265, 266]. Following experimental treatment incubation, the cells were harvested along with their overlying culture media, and analyzed using the automated Vi-CELL Cell Viability Analyzer (Beckman Coulter, Canada). Cellular viability was calculated as the number of viable cells (trypan blue negative) divided by the total number of cells (both viable and non-viable) expressed as a percentage.

3.2.7 Assessment of Trophoblast Health: Apoptosis Assay

Activated caspase-3 immuno-detection, a reliable method to observe apoptosis (programmed cell death) [267], was used to further test the effects of increasing concentrations of exogenous folic acid on trophoblast health.

HTR-8/SVneo (N=3) and BeWo cells (N=3) were seeded at a density of 2.5×10^4 cells/well in 12-well plates and serum starved overnight. The following day they were treated with different doses of folic acid (2 – 4000 ng/mL folic acid) for 24 hrs. Subsequently, the cells were washed in 1X PBS, trypsinized, and re-suspended in their respective folic acid treatments and transferred to Millicell EZ chamber slides at a density of 1.5×10^4 cells/well for an additional 24 hours at 37°C (total experimental treatment exposure of 48 hours). The cells were then washed with 1X PBS and fixed using 10% formalin. Antigen retrieval was performed using 100% cooled methanol for 20 minutes at 4°C, washed with 1X PBS, and blocked with 1% BSA (in 1X PBS) for 30 minutes.

Immunocytochemical detection of activated-caspase-3, as a marker of programmed cell death (apoptosis) was then performed on the cells. Briefly, cells were incubated with a polyclonal rabbit anti-human activated caspase-3 primary antibody (1:100 dilution in 1% BSA; Abcam, USA) overnight at 4°C. Cells were washed with 1X PBS and subsequently incubated with green fluorescent-labeled goat-anti-rabbit IgG secondary antibody (1:500 dilution in 1% BSA; Alexa Fluor® 488, Life Technologies, USA) for 1 hour at room temperature. Cells were again washed with 1X PBS and counterstained with blue fluorescent-labelled Hoechst stain (1:4000 dilution in 1X PBS) for 15 minutes at room temperature. After a final wash in 1X PBS, the slides were air-dried and mounted using PermaFluor™ Mountant. For each experiment, a positive control stress treatment of 1 μ M staurosporine was included. Negative controls were run in parallel for each experiment with the omission of the primary antibody.

Two randomly selected fields of view were imaged for each experimental treatment per experiment, using Zeiss Axioskop 2 MOT *Plus* Microscope at 10X magnification. Mean apoptotic cell counts (green immunofluorescence) and total cell counts (blue + green immunofluorescence) were performed using automated Image J Software v1.47. Results were calculated as the number of apoptotic cells divided by the total number of cells, expressed as a percentage, for each experimental treatment group.

3.2.8 Assessment of Trophoblast Function: Invasion Assay

A vital function of the extravillous trophoblast cell is the ability to actively invade the uterine wall and remodel the uterine vasculature [160]. As such, the effects of increasing concentrations of exogenous folic acid on this important function of the HTR-8/SVneo extravillous trophoblast cell line was examined using a Boyden chamber invasion assay (N=4).

HTR-8/SVneo cells were seeded at a density of 5.0×10^4 cells/well into a 12-well plate and treated in duplicate with 2, 20, 200, or 2000 ng/mL FA for 24 hours. Afterwards, the cells were serum-starved for 6 hours in their respective treatments (2-2000 ng/mL FA in FA-depleted 1X RPMI-1640 media, 1% 100X P/S). Then, cells were washed in 1X PBS, trypsinized, re-suspended into their respective treatments, and seeded into the top of a matrigel coated transwell insert (6.4 mm diameter, 8.0 micron pore; BD Biosciences, Canada) at a density of 2.5×10^4 cells/insert. In the bottom chamber of the invasion assay well, the respective treatment media (2-2000 ng/mL FA, 1X RPMI-1640, 1% 100X P/S) was added and supplemented with 5% FBS in order to act as a chemo-attractant to promote HTR-8/SVneo invasion through the matrigel-coated transwell insert. Cells were left to invade for 48 hours.

After the invasion period, the transwell membranes were fixed using 10% formalin for 2 minutes and permeabilized using 100% cold methanol for 20 minutes. Non-invaded cells were removed from the upper chamber of the transwell insert using a cotton swab and discarded. Successfully invaded cells that adhered to the bottom of the transwell insert were stained with mercury-free Harris Modified Hematoxylin with acetic acid (Fisher Scientific; NJ, USA) for 10 minutes, and quickly rinsed with tap water. The porous membranes of the insert were excised out and mounted onto slides using Shandon Immu-Mount (Thermo Scientific, USA). For each well, four randomly selected fields of view were taken using EVOS cell imaging system at 40X magnification. Invaded cells (blue stain) were counted using Image J Software v1.47.

3.2.9 Assessment of Trophoblast Function: β -hCG ELISA Assay

A vital function of the villous trophoblast cell, represented by the BeWo cell model, is the ability to produce and secrete hormones into the maternal circulation which promote appropriate maternal adaptation to pregnancy [160, 253]. One such hormone is human chorionic gonadotropin (β -hCG), which plays an important role in uterine decidualization and maternal immune tolerance to the developing fetus [268, 269]. As such, the effects of

increasing concentrations of exogenous folic acid on β -HCG hormone secretion by the BeWo villous trophoblast cell line were examined (N=3).

Briefly, BeWo cells were plated at a density of 5×10^4 cells/well and serum starved overnight in culture media supplemented with 20 ng/mL folic acid. The BeWo cells were then treated for 48 hours with different folic acid experimental treatments (2-4000 ng/mL). At the end of the treatment period, the media was collected, clarified by low-speed centrifugation at 1200 RCF ($\times g$) for 5 minutes using Heraeus™ Labofuge™ 400 (Thermo Fisher Scientific, USA), and stored at -80°C until β -hCG quantification. Concentrations of secreted β -hCG hormone within the overlying culture media was analyzed using a solid phase enzyme-linked immunosorbent assay (ELISA), as per manufacturer's instructions (IBL International, Germany) and read at 450 nm using Thermo Labsystems Multiskan Ascent spectrophotometer and analyzed using Ascent Software v.2.6. Each sample was run in duplicate. Using the included standard curve, the concentration of β -hCG (mIU/mL) was calculated. Data is expressed as relative β -hCG hormone secretion in comparison to the β -hCG hormone released at the 20 ng/mL folic acid treatment.

3.2.10 Statistical Analyses

All results are presented as mean $\pm$ SD from at least three independent experiments. All experimental FA dose-response data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc analysis using Prism software, version 3.03 (GraphPad, San Diego, CA). The difference between experimental treatments were considered statistically significant if $p < 0.05$.

B. In vitro Human Placental Explant Model

3.2.11 Placental Explant Collection and Preparation

Human placentae (N=6) were collected following elective Caesarean section from healthy term pregnancies at the Ottawa General Hospital with approval from the Ottawa Health Science Network Research Ethics Board (Protocol # 2011217-01H) and the University of Ottawa Health Sciences and Science Research Ethics Board (File #: H04-13-12; Appendix E). All patients recruited were at term (39th – 40th week's gestation) and had a BMI between

18 – 25 kg/m². Elective C-sections were the result of fetal breech presentation or repeat C-sections.

Placentae were collected within 30 minutes of delivery. Initially, five 1-inch³ pieces of tissue were dissected from the placenta, ensuring sampling from across the entire organ. The chorionic plate and basal plate were removed and the tissue samples were placed in 1X PBS and transported to the laboratory on ice. Within the laboratory, the placental samples were rinsed several times in 1X PBS to remove residual blood and small tissue explants (2-5 mg each) of terminal chorionic villi were dissected. The dissected explants were rinsed twice in 1X PBS and once in DMEM culture media. From each placenta ~75-100 explants were dissected. Explants were cultured on 15mm Costar® Netwell™ Inserts (Corning®, MA, USA) at the gas-liquid interface and sustained under normoxic culture conditions of 8% O₂ (physiological oxygen concentration [270]), 5% CO₂, balance N₂ at 37°C.

3.2.12 Folic Acid Treatments

Placental explants were cultured in FA-depleted 1X DMEM culture media (diluted D2424-10X DMEM media with added 3.7 g/L NaHCO₃, 0.584 g/L L-glutamine, 3500 mg/L at pH 7.0; Sigma-Aldrich; USA) supplemented with 5% FBS, and 1% 100X P/S. A series of folic acid experimental treatments were generated using this culture media (2, 20, 200, 2000, 4000 ng/mL folic acid). For each experiment (N=6; 1 experiment per placenta), approximately 7-9 individual explants per treatment group were placed on a netwell support and incubated in the corresponding experimental FA treatment for 48 hrs.

Afterwards, all explants from each treatment group were collected, weighed and processed for immunohistochemistry (Sections 3.2.13 and 3.2.15). The treatment media was also collected immediately and assessed for lactate dehydrogenase (Section 3.2.14), as a measure of placental viability, and stored at -80°C for future analysis of β-hCG (Section 3.2.16), as a measure of placental endocrine function.

3.2.13 Assessment of Placental Health: Cellular Proliferation

The syncytiotrophoblast cellular layer of the villous exchange region of the placenta is replenished throughout pregnancy through the proliferation and terminal differentiation of the underlying cytotrophoblast cells [160]. The effects of increasing concentrations of exogenous folic acid on cytotrophoblast cell proliferation were examined through immunohistochemical analysis of Ki67, a nuclear marker of cellular proliferation [271, 272].

Following the 48-hour experimental FA treatment exposure (Section 3.2.12), explants were fixed, embedded in paraffin blocks and processed for immunohistochemistry according to standardized protocols.

Sample Processing

Briefly, explants were fixed in 10% buffered formalin phosphate (Fisher Scientific; ON, Canada) for 24 hours at room temperature and subsequently transferred into 70% ethanol for storage. Prior to processing, explants were embedded into 2% w/v Agar (BioShop®; ON, Canada), made up in 1X PBS. Agar-embedded explants were serially dehydrated using the Fully Enclosed Tissue Processor Leica ASP300 S (Leica Biosystems, USA), and paraffin-embedded (TissuePrep® Fisher Scientific; NJ, USA) using the Leica EG 1160 Embedding Center, Dispenser + Hot Plate (Leica Biosystems, USA). For each sample, 5 µm sections were cut and mounted on slides (Superfrost®/Plus, Fisher Scientific, USA).

A slide from each FA treatment, for each experiment (N=6), was deparaffinised in histological grade xylenes and endogenous peroxidase activity was quenched using 3% H₂O₂ (diluted in methanol) for 10 minutes at room temperature. Following standardized rehydration protocols (5 minutes in 95%, 80, 70%, 50% ethanol and H₂O respectively), antigen retrieval was performed using 0.01 M sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, ddH₂O; pH 6.0) in a Decloaking Chamber™ (Biocare Medical; CA, USA) pressure cooker for 35 minutes, followed by a rinse in running hot tap water and 1X PBS. Nonspecific staining was blocked using Dako LSAB Protein Block Solution (Dako, USA) and incubated for 30 minutes in humidity chamber at room temperature.

Immunohistochemistry

Immunoreactivity to Ki67, a marker of cellular proliferation, was determined following incubation of each slide with a monoclonal mouse anti-human Ki-67 antibody (1:100 dilution in 1% BSA; Dako, USA) overnight at 4°C in a humidity chamber. After washing slides in 1X PBS (thrice, 5 minutes each), slides were incubated with a biotinylated goat-anti mouse IgG secondary antibody for 1 hour at room temperature followed by an incubation with peroxidase-conjugated streptavidin (DAKO LSAB2 System-HRP (Dako; CA, USA). Following a wash in 1X PBS (10 minutes), staining was developed with a 30 second exposure to 3,3'-Diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, MO, USA). Slides were rinsed in 1X PBS (thrice, 5 minutes each), and transferred to ddH₂O. Subsequently, slides were counterstained with hematoxylin for 60 seconds, rinsed in tap water, rapidly dipped in

acid alcohol (0.5% HCl in 70% alcohol) to promote color differentiation, and then rapidly dipped in 0.2% ammonium hydroxide (in ddH₂O) as a bluing agent. After a final rinse in tap water, sections were mounted using Shandon Immu-Mount™. For each experiment, a negative control was run in parallel with the omission of the primary antibody.

Analysis

The Leica Aperio ScanScope® slide scanner and the ScanScope Console v102.0.0.33 Controller V.102.0.0272 was used to take high quality images of each slide at 20x magnification. For computer-assisted image analysis, four random areas were analyzed from each image using Aperio ImageScope v11.2.0.780. Immunoreactivity to Ki67 was identified through DAB positive staining (red-brown) and counted manually using the Cell Counter Plugin in Image J Software v1.47. Data was expressed as the mean number of proliferating cells (Ki67 positive) per mean area of placental tissue imaged.

3.2.14 Assessment of Placental Health: LDH Cytotoxicity Assay

Lactate dehydrogenase (LDH) measurement in cell culture media is a widely used marker of cellular viability [264]. LDH is a cytoplasmic protein of high abundance, which is released when the integrity of a cell membrane becomes compromised through the process of necrosis – unregulated cell death [273]. Placental cell necrosis, following exposure to increasing concentrations of folic acid, was assessed through measurement of released LDH in the culture media following the 48-hour treatment (Section 3.2.12).

Overlying culture media from each of the experimental FA treatment groups was collected from each experiment (N=6), and LDH concentration was measured using the LDH Cytotoxicity Assay Kit II (abcam®; UK) according to manufacturer's instructions. The principle of this assay is an enzymatic coupling reaction. LDH released from damaged cells oxidizes lactate to produce NADH, which then reduces a water soluble tetrazolium salt (WST-1) to produce formazan, resulting in a yellow color [274, 275]. Thus, the intensity of the color correlates with the number of lysed cells. Colorimetric detection of LDH activity was measured at an absorbance of 450 nm using Thermo Labsystems Multiskan Ascent and Ascent Software v.2.6.

For each placenta, an extra set of 7-9 individual control explants were collected immediately following delivery, weighed, and flash frozen in liquid nitrogen and stored in -80°C. At the time of the LDH Cytotoxicity Assay, these control explants were thawed on ice

and homogenized in 1 mL 1X DMEM to release all cytoplasmic LDH. Cell debris was removed from the tissue homogenate using the Beckman Coulter™ Microfuge® 22R centrifuge for 2 minutes at 6,000 RPM and supernatant was collected. As this supernatant contained all of the LDH present in the equivalent number of explants placed in each experimental treatment well, this sample was deemed a 100% LDH control. LDH measurement results obtained from each experimental sample was normalized to the weight of corresponding tissue and expressed as a percentage of the 100% LDH control. Additionally, explants treated with staurosporine (1 μ M) were included as a positive control for cellular stress.

3.2.15 Assessment of Placental Health: Apoptosis

Cytokeratin 18 (CK18), an abundant protein in placental trophoblast cells, is cleaved by activated caspases during the apoptotic cascade revealing an epitope recognized by monoclonal antibody M30 [276]. To measure apoptosis in human placentae, the monoclonal antibody M30 has been established as an effective antibody to recognize this epitope [277]. Thus, in addition to necrosis, the effect of increasing concentrations of folic acid on placental programmed cell death (apoptosis) was determined through M30 immunoreactivity.

Sample Processing and Immunohistochemistry

Immunohistochemical processing of slides (5 μ m) generated from each experimental FA treatment (N=6 experiments) was conducted as outlined above (Section 3.2.13). In this instance, however, slides were incubated with the monoclonal mouse anti-human M30 antibody (1:100 dilution in 1% BSA; CytoDEATH, Roche Diagnostics, USA) overnight at 4°C in a humidity chamber and staining was developed with a 70 second exposure to DAB.

Analysis

The Leica Aperio ScanScope® slide scanner and the ScanScope Console v102.0.0.33 Controller V.102.0.0272 was used to take high quality images of the slides at 20x magnification. For computer-assisted image analysis, four random areas were analyzed from each image using Aperio ImageScope v11.2.0.780. Immunoreactivity to M30 was identified through DAB positive staining (red-brown) and quantified using Image J Software v1.47. The segmentation process resulted in generation of binary images from which the percentage area of positive cells compared to total area could be determined. For each experiment a negative control was run in parallel with the omission of the primary antibody. Additionally,

explants treated with staurosporine (2 μ M) were included as a positive control for cellular stress.

3.2.16 Assessment of Placental Function: β -hCG ELISA Assay

As detailed above (Section 3.2.9) β -hCG hormone production and secretion is a vital function of the placenta during pregnancy [160, 278]. As such, the effects of increasing concentrations of exogenous folic acid on β -hCG hormone secretion by placental explants were examined.

For each experiment (N=6), the media overlying the placental explants was collected following the 48-hour experimental FA treatment exposure (Section 3.2.12) and kept at -80°C until β -hCG quantification. As described in the cell culture experiments above (Section 3.2.9), β -hCG hormone secretion into the overlying culture media was analyzed using a solid phase enzyme-linked immunosorbent assay (ELISA) Kit, according to manufacturer's instructions. Each sample was run in duplicate. Using the included standard curve, the concentration of β -hCG was calculated, normalized to the mass of each sample of placental explants, and expressed as mIU/mL*mg. Data is expressed as relative β -hCG hormone secretion in comparison to the β -hCG hormone released at the 20 ng/mL folic acid treatment.

3.2.17 Statistical Data Analysis

All results are presented as mean $\pm$ SD from six independent experiments (one placenta per experiment). All experimental FA dose-response data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc analysis using Prism software, version 3.03 (GraphPad, San Diego, CA). Difference between experimental treatments were considered statistically significant if $p < 0.05$.

3.3 Results

A. In vitro Trophoblast Cell Culture Model

3.3.1 Intracellular Folate in Trophoblast Cells Following in vitro Exposure to FA

Intracellular folate was measured in the HTR-8/SVneo and BeWo trophoblast cell lines following *in vitro* exposure to increasing concentrations of folic acid (FA) over 48 hours (Figure 6). In both trophoblast cell lines, intracellular folate increased with higher doses of exogenous FA, indicating an increase in FA uptake as more exogenous FA becomes available.

Intracellular folate levels were significantly higher following treatment with 4000 ng/mL FA treatment when compared to treatments of 2, 20, and 200 ng/mL of FA for both HTR-8/SVneo ($p<0.0001$) and BeWo ($p<0.01$) cell lines. However, there was no significant difference observed between the 2000 ng/mL and 4000 ng/mL FA treatments. Interestingly, the BeWo cell line demonstrated a more pronounced increase in intracellular folate concentrations, compared to the HTR-8/SVneo cell line, at the same FA experimental treatment concentrations.

3.3.2 Trophoblast health: Effect of *in vitro* Exposure to FA on Cellular Proliferation

Cellular proliferation was measured in the HTR-8/SVneo and BeWo trophoblast cell lines following *in vitro* exposure to increasing concentrations of folic acid over 48 hours (Figure 7). Under basal conditions, BeWo trophoblast cells proliferated approximately twice as much as HTR-8/SVneo cells. Nevertheless, there was no significant change in the number of proliferative cells following exposure to increasing folic acid treatments for either HTR-8/SVneo or BeWo trophoblast cell lines.

3.3.3 Trophoblast health: Effect of *in vitro* Exposure to FA on Cellular Viability

The trypan blue exclusion test was used to assess cellular viability in the HTR-8/SVneo and BeWo trophoblast cell lines following *in vitro* exposure to increasing concentrations of folic acid (FA) over 48 hours (Figure 8). Compared to the baseline FA treatment of 20 ng/mL FA for the HTR-8/SVneo trophoblast cell line, elevated concentrations of FA (200, 2000, 4000 ng/mL) had no significant effect on cell viability, though low concentrations of FA (2 ng/mL) resulted in a significant reduction in viability ($p<0.01$). In contrast, elevated concentrations of FA (200 and 2000 ng/mL) resulted in a significant effect on BeWo cell viability when compared to baseline FA treatment of 20 ng/mL FA ($p<0.05$), though 2 ng/mL FA and 4000 ng/mL FA had no significant effect. The positive control stress treatment of 1 μ M staurosporine also caused a significant decrease in HTR-8/SVneo and BeWo trophoblast cell viability when compared to the 20 ng/mL FA treatment in both cell lines ($p<0.001$).

3.3.4 Trophoblast health: Effect of *in vitro* Exposure to FA on Apoptosis

The trypan blue exclusion assay (Section 3.3.3) identifies dead cells, however it is not capable of identifying cells that are in the process of undergoing apoptosis (i.e. programmed cell death). As such, initiation of apoptosis was also investigated in the HTR-8/SVneo and BeWo trophoblast cell lines following *in vitro* exposure to increasing concentrations of folic

acid over 48 hours (Figure 9). In both cell lines, for all experimental treatments tested, the presence of apoptotic cells (immunoreactivity to activated caspase-3) were sparse (1.3% – 7.0%). Increasing concentrations of folic acid had no significant effect on initiation of apoptosis in either the HTR-8/SVneo or BeWo trophoblast cell lines (Figure 9, C-D). However, the positive control stress treatment of 1 μ M staurosporine, resulted in a significant increase in the number of apoptotic cells in both cell lines ($p < 0.001$).

3.3.5 Trophoblast function: Effect of *in vitro* Exposure to FA on Cellular Invasion

The effects of increasing concentrations of folic acid were examined on the *in vitro* invasive function of HTR-8/SVneo trophoblast cells. Optimal trophoblast invasion was observed following treatment with 20 ng/mL FA. While there appeared to be a trend of decreasing trophoblast invasion at higher concentrations of FA (200, 2000 and 4000 ng/mL) this finding was not significant. However, treatment with low concentrations of FA (2 ng/mL), equivalent to FA deficiency, resulted in a significant decrease in HTR-8/SVneo invasion ($p < 0.01$) compared to the physiological 20 ng/mL FA treatment (Figure 10, A-C).

3.3.6 Trophoblast function: Effect of *in vitro* Exposure to FA on β -hCG Production

The effects of increasing concentrations of folic acid were examined on the *in vitro* endocrine function of BeWo trophoblast cells, through measurement of β -hCG hormone secretion into the culture media. There was no difference in β -hCG secretion across all experimental FA treatments (2-4000 ng/mL) following a 48-hour incubation (Figure 11). Overall, approximately 857 mIU/mL of β -hCG hormone was released into the media when 5×10^4 cells/well were initially seeded.

B. *In Vitro* Human Placental Explant Model

3.3.7 Placental health: Effect of *in vitro* Exposure to FA on Cytotrophoblast Proliferation

The effects of increasing concentrations of folic acid treatment on cytotrophoblast cell proliferation was additionally examined using the human placental explant culture model. Cellular proliferation remained unchanged across all folic acid treatments (2-4000 ng/mL) tested (Figure 12). For all experimental treatments, an average of 19 cytotrophoblast

cells were undergoing proliferation (total average cell count of 46 cytotrophoblast cells), as indicated by Ki67 immunoreactivity, across two random fields of view (20X magnification).

3.3.8 Placental health: Effect of *in vitro* Exposure to FA on LDH Release

LDH release into the culture media was measured as an assessment of tissue viability, as necrotic cells demonstrate loss of membrane integrity and subsequent release of cytoplasmic proteins. There was no change in LDH release by placenta explants across all experimental FA treatments tested (Figure 13). For all experimental treatments, approximately 45.5% of the intracellular LDH was released into the culture media per gram of placental explants. Furthermore, following a 48-hour incubation with the positive stress control (2 μ M staurosporine), there was no effect on placental cell viability, as measured by LDH release.

3.3.9 Placental health: Effect of *in vitro* Exposure to FA on Trophoblast Apoptosis

Initiation of apoptosis in placental explants was also examined following treatments with increasing concentrations of FA (2-4000 ng/mL). In all samples, apoptosis was restricted primarily to the syncytiotrophoblast layer (Figure 14, A-B). Due to the multinucleated nature of the syncytial layer, the area of M30 immunoreactivity, reflected as a percentage of total tissue areas, was calculated from 2 random fields of view (20X magnification) for each experimental FA treatment. There was no significant difference in apoptotic area across all experimental FA treatments. For all experimental treatments, approximately 1% of total area was identified as apoptotic area using M30 immunoreactivity. However, a 48-hour incubation with 2 μ M staurosporine resulted in an increase in M30 immunoreactivity to approximately 3.7% of total area, indicative of increased apoptosis.

3.3.10 Placental function: Effect of *in vitro* Exposure to FA on β -hCG Hormone Production

The effects of increasing concentrations of folic acid were examined on the *in vitro* endocrine function of human placental explants, through measurement of β -hCG secretion into the culture media. The concentration of β -hCG released by the explants was similar between all experimental FA experimental treatments (Figure 15). The mean concentration of β -hCG, normalized to placenta explant weight, was 3.1 mIU/mL*mg across all experimental treatments.

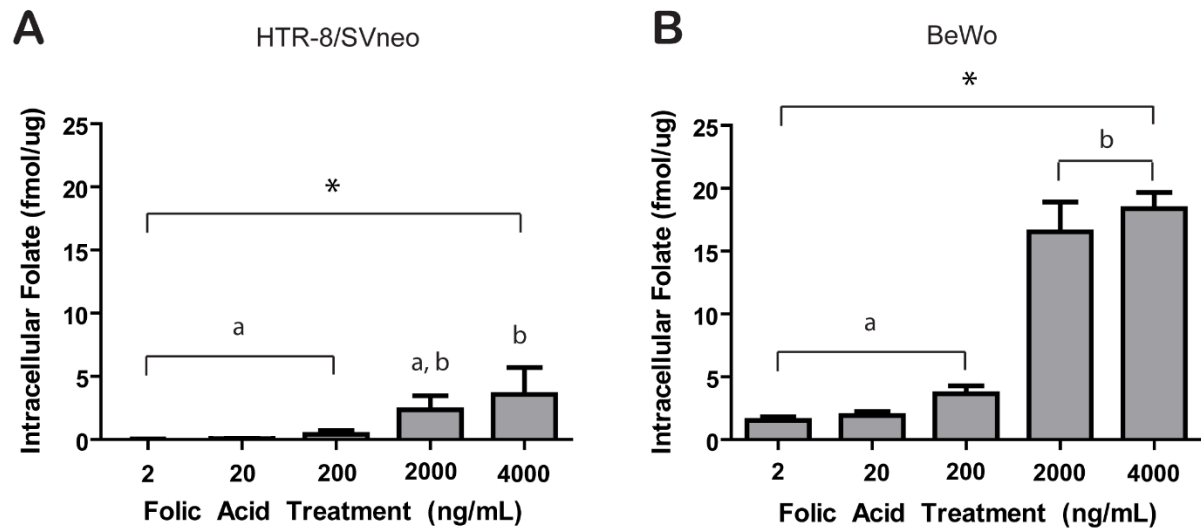


Figure 6. Intracellular folate in trophoblast cell lines following exogenous folic acid treatment. Quantity of intracellular folate in **(A)** HTR-8/SVneo and **(B)** BeWo placental trophoblast cell lines following a 48-hour treatment with increasing concentrations of folic acid (2-4000 ng/mL). Intracellular folate levels were measured using the *Lactobacillus rhamnosus* microbiological assay. Data is presented as mean mean $\pm$ SEM (N=3). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis ($p < 0.05$) and is marked with an asterisk (*). Data columns labelled with different letters (a, b) are significantly different from each other ($p < 0.05$).

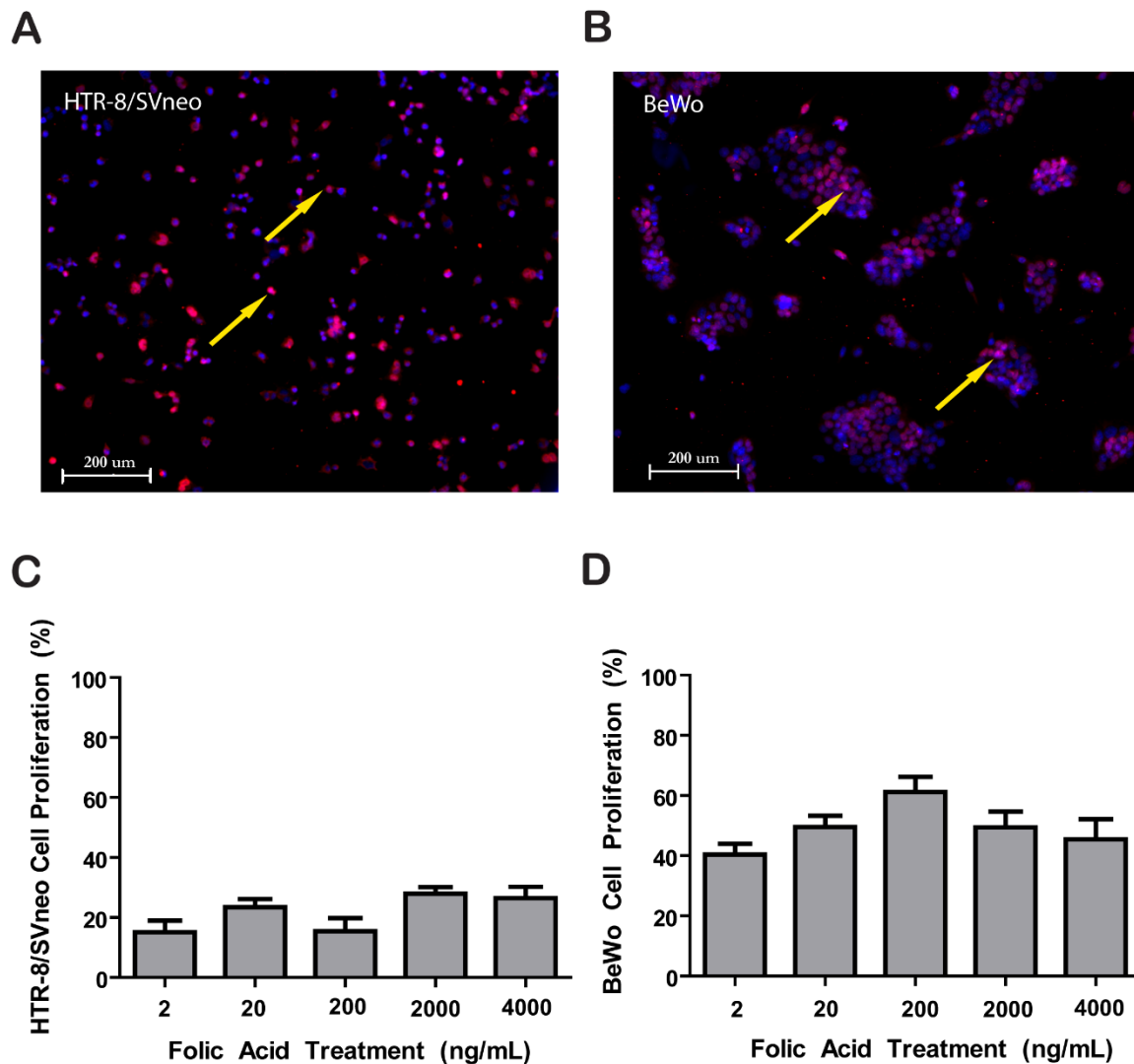


Figure 7. Effect of exogenous folic acid treatment on cellular proliferation in trophoblast cell lines. (A-B) Representative images demonstrating immunoreactivity to BrdU (red), identifying a proliferating cell, in HTR-8/SVneo (A) and BeWo (B) cell lines after a 48-hour treatment with 20 ng/mL folic acid. Yellow arrows highlight proliferating cells in red. All cellular nuclei were counted using a DAPI (blue) counterstain. (C-D) Quantification of the percentage of proliferating (C) HTR-8/SVneo and (D) BeWo trophoblast cells following 48-hour exogenous folic acid treatments (2-4000 ng/mL). Results were calculated as the number of proliferating cells expressed as a percentage of total cells. Data is presented as mean % $\pm$ SEM (N=3). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

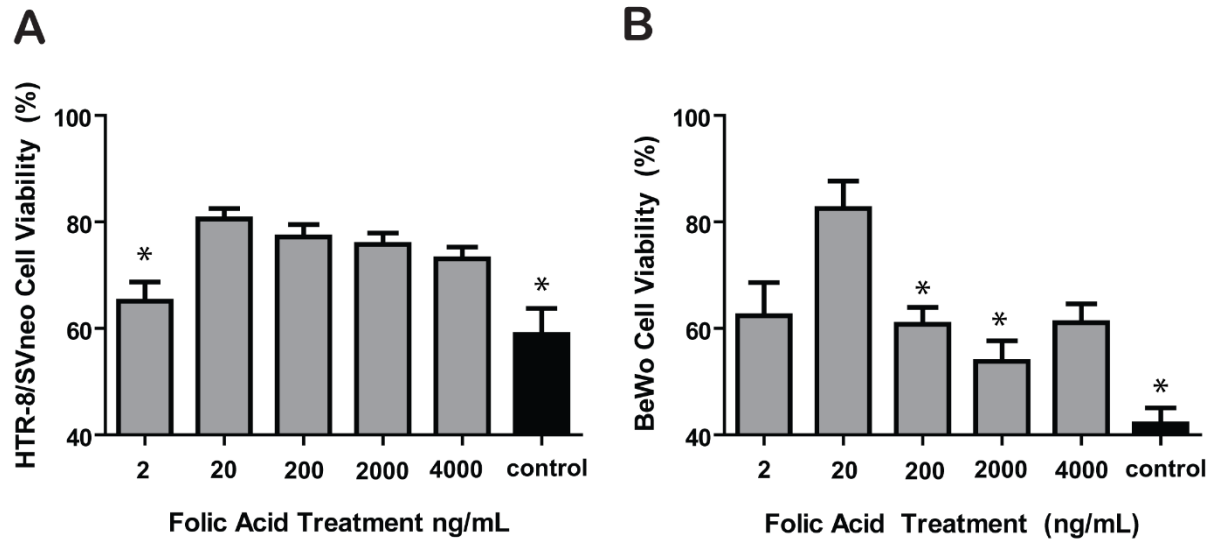


Figure 8. Effect of exogenous folic acid treatment on cellular viability in trophoblast cell lines. Percentage of live **(A)** HTR-8/SVneo and **(B)** BeWo trophoblast cells per total cell count following a 48-hour treatment with increasing folic acid (2-4000 ng/mL). As a positive stress control (black bar), cells were treated with 1 μ M staurosporine. Data is presented as mean % $\pm$ SEM (N=3). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05). Data columns that are significantly different from the 20 ng/mL FA treatment are marked with an asterisk (*).

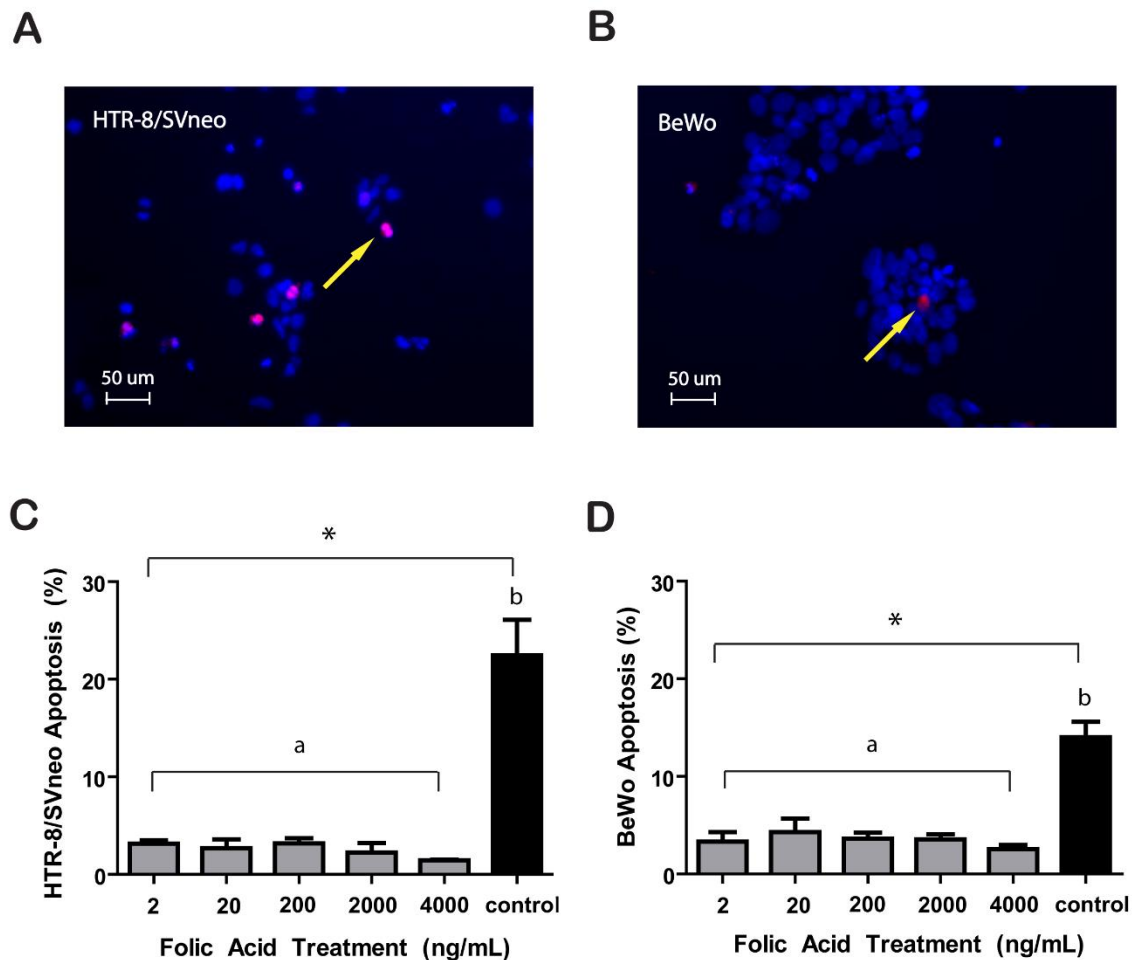


Figure 9. Effect of exogenous folic acid treatments on initiation of apoptosis in trophoblast cell lines. (A-B) Representative images demonstrating apoptotic cells (red) in (A) HTR-8/SVneo and (B) BeWo cell lines after 48-hour treatment with 20 ng/mL folic acid using immunoreactivity to activated caspase-3. Yellow arrows highlight apoptotic cells in red. All cellular nuclei were counted using a DAPI (blue) counterstain. (C-D) Quantification of the percentage of apoptotic (C) HTR-8/SVneo and (D) BeWo cells following 48-hour exogenous folic acid treatments (2-4000 ng/mL). As a positive stress control (black bar), cells were treated with 1 μ M staurosporine. The mean number of apoptotic cells calculated as percentage of a total cell count is presented as mean % $\pm$ SEM (N=3). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05). Data columns labelled with different letters (a, b) are significantly different from each other (p <0.05).

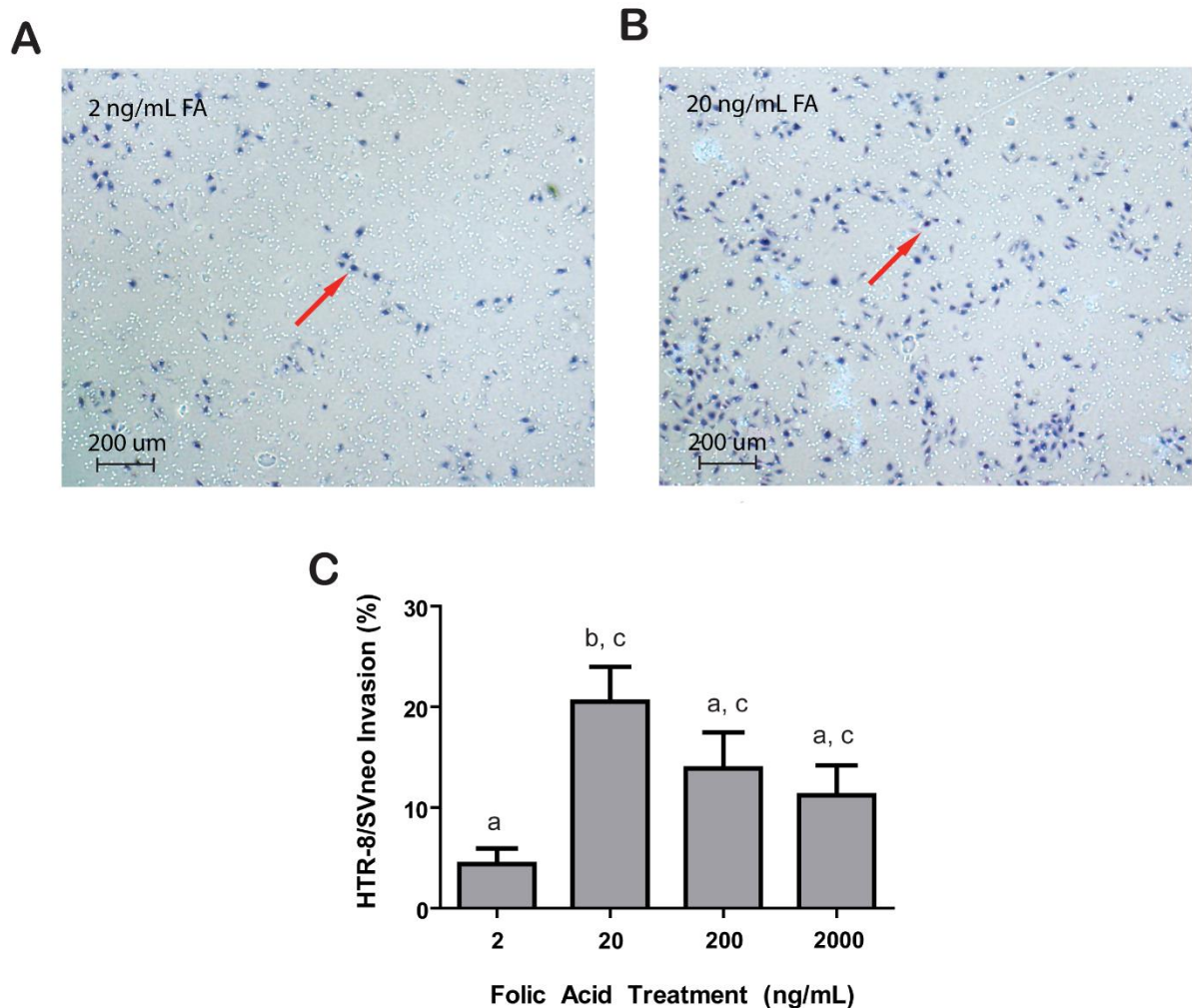


Figure 10. Effect of exogenous folic acid on HTR-8/SVneo cell invasion.

(A-B) Representative images highlighting invaded HTR-8/SVneo cells (blue), indicated by red arrows, after 72-hour treatment with (A) 2 ng/mL and (B) 20 ng/mL folic acid treatment. **(C)** Quantification of HTR-8/SVneo cell invasion through a matrigel coated boyden chamber following treatment with increasing concentrations of folic acid (2-2000 ng/mL). The mean number of invaded cells calculated as percentage of the total number of invaded cells in each individual experiment is presented mean % $\pm$ SEM (N=3). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05). Data columns labelled with different letters (a, b, c) are significantly different from each other (p <0.05).

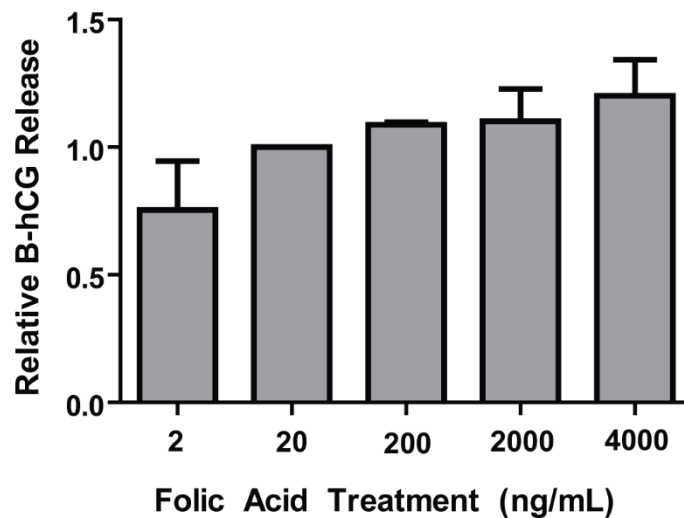


Figure 11. Effect of exogenous folic acid on β -hCG hormone secretion from BeWo trophoblast cells. Concentration of β -hCG released following 48-hour folic acid treatment (2-4000 ng/mL) using an enzyme-linked immunosorbent assay (ELISA). Data is presented as relative concentration (mIU/mL) of β -hCG hormone secretion mean $\pm$ SEM (N=3) in comparison to 20 ng/mL FA treatment. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

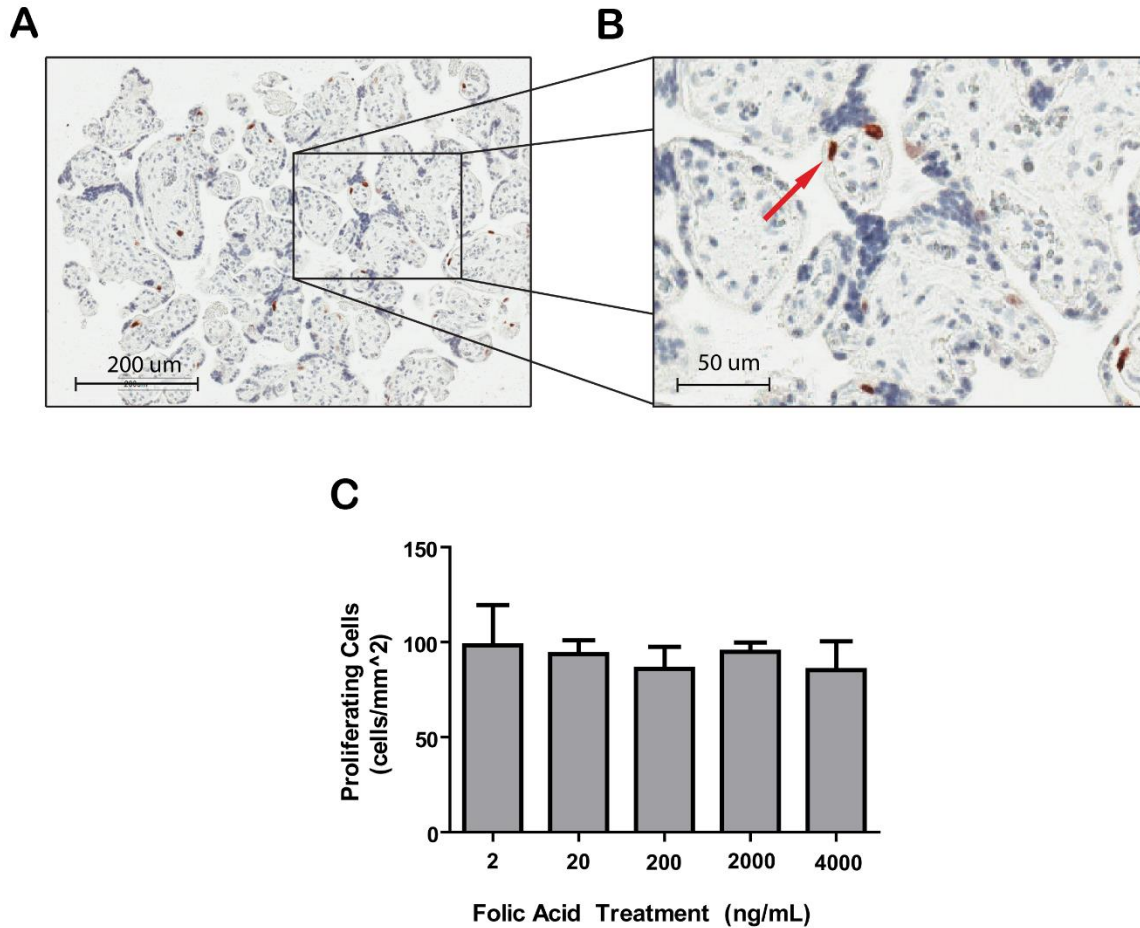


Figure 12. Effect of exogenous folic acid on cytotrophoblast proliferation in human placental explants. (A) Representative image demonstrating cytotrophoblast proliferation after exposure to 20 ng/mL of folic acid, as indicated by Ki67 immunoreactivity (red-brown nuclei highlighted with a red arrow) (B) Quantification of data calculated as mean number of proliferating cytotrophoblast cells (ki-67 positive) per total area of tissue in 2 random fields of view (20X magnification) per experimental FA treatment. Data is presented as mean $\pm$ SEM (N=6). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

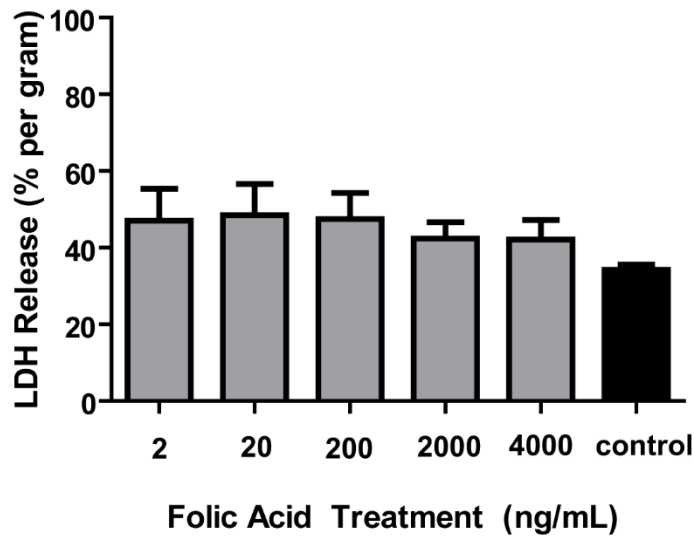


Figure 13. Effect of exogenous folic acid on lactate dehydrogenase (LDH) release by human placental explants. No effect on LDH release by placental explants following 48-hour treatments with increasing concentrations of FA (2-4000 ng/mL) indicates no change in tissue viability following increasing FA exposure. Measured LDH release for each treatment sample was normalized to the mass of the corresponding placental explants and expressed as a percentage of a 100% LDH positive control. The 100% LDH control represents the amount of total cytoplasmic LDH present in an equivalent mass of placental explants. As a positive control (black bar), cells were treated with 2 μ M staurosporine. Data is presented as mean $\pm$ SEM (N=6). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

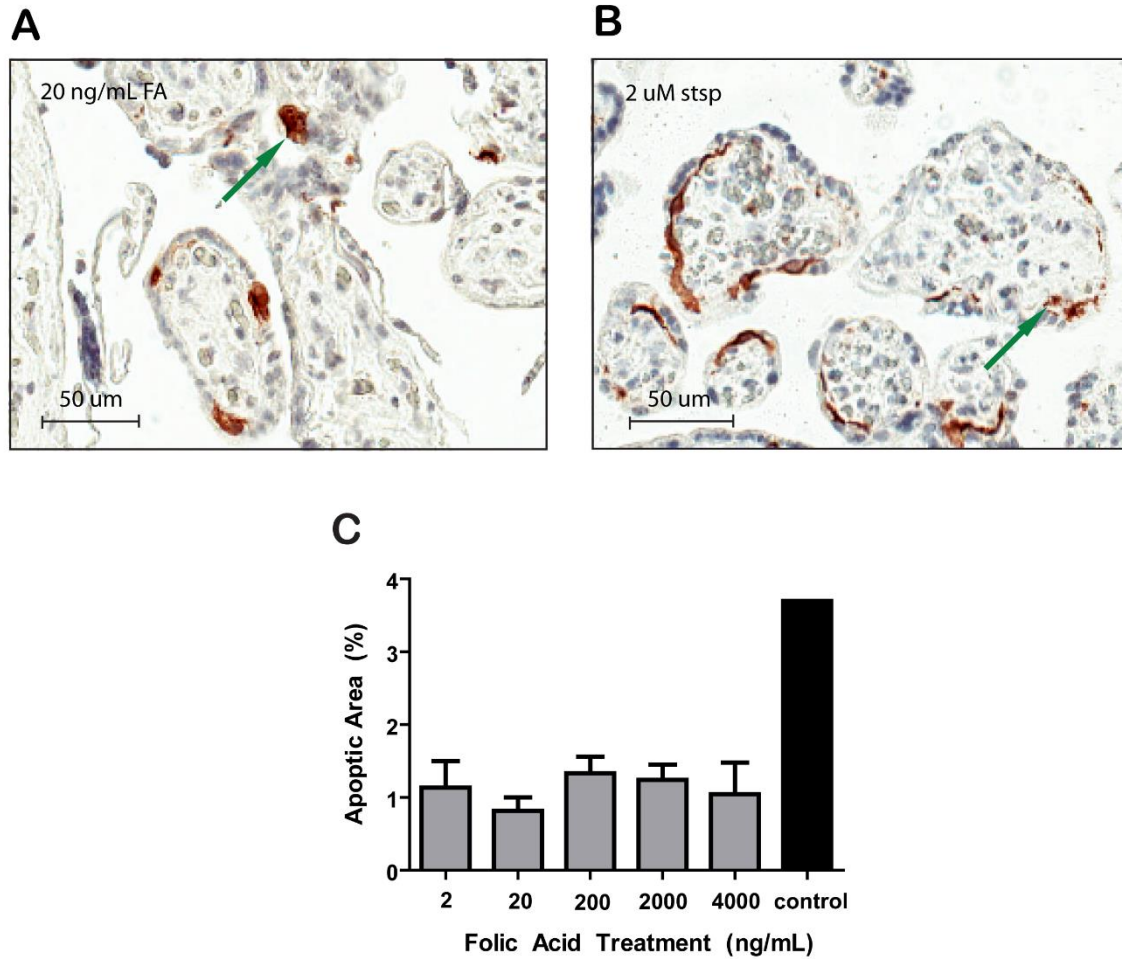


Figure 14. Effect of exogenous folic acid on induction of apoptosis in human placental explants. (A-B) Representative images demonstrating apoptotic cells (red-brown staining, highlighted by green arrow) after 48-hour exposure to (A) 20 ng/mL folic acid and (B) 2 μ M staurosporine (stsp). **(C)** Quantification of apoptotic area, calculated as mean total area of M30 immunoreactivity (red-brown) relative to mean total tissue area (expressed as a %) in 4 random fields of view at 20X magnification. As a positive stress treatment control (N=1), cells were treated with 2 μ M staurosporine (black) for 48 hours. Data is presented as mean $\pm$ SEM (N=6). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

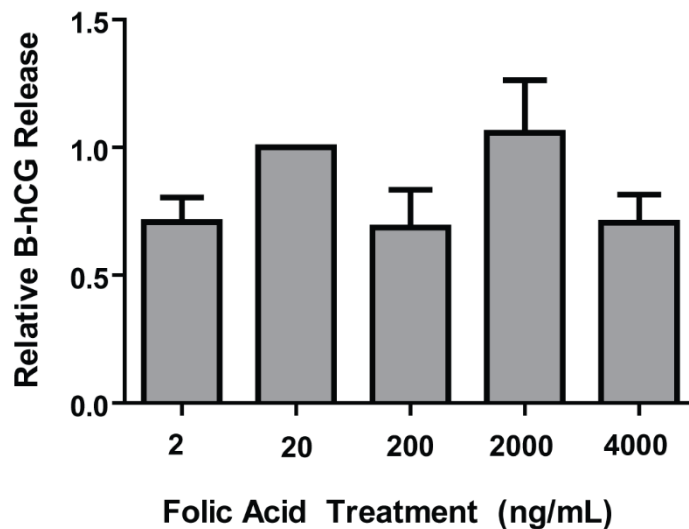


Figure 15. Effect of exogenous folic acid on β -hCG hormone secretion by human placental explants. The concentration of β -hCG released following 48-hour exposure to increasing concentrations of folic acid (2-4000 ng/mL) was determined using an enzyme-linked immunosorbent assay (ELISA), and was normalized to the mass of each sample of placental explants. Data is presented as the mean relative concentration (mIU/mL*mg) of β -hCG hormone secretion $\pm$ SEM (N=6) in comparison to 20 ng/mL FA treatment. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* p <0.05).

3.4 Discussion

Folate, the generic term for the B₉-vitamin, is globally acknowledged to play a critical role during pregnancy and fetal development. Natural forms of folate found in foods is not harmful to the body; however, folic acid, the synthetic form of folate, has been found to lead to adverse effects at high intake levels and has led to the establishment of the UL of 1 mg by the Institute of Medicine [3, 5, 38]. Recently, several studies have raised concerns that pregnant North American women are consuming excessive folic acid levels [100, 101, 112]. This is mainly due to current recommendations to take a folic acid-containing supplement (which may contain 0.4 - 5 mg of FA) prior to and during pregnancy, in addition to a diet containing folic acid-fortified foods [27, 29]. Vital to a healthy pregnancy, the placenta is responsible for mediating the transfer of nutrients and waste between the mother and the fetus. Placental damage and dysfunction are recognized to be the cause of several adverse pregnancy outcomes, including pre-term birth, fetal growth restriction and preeclampsia. When safety determinations are made for any form of maternal exposures during pregnancy, including dietary supplementation, it is imperative that the effects of these exposures on this critical organ of pregnancy are considered. Nonetheless, limited studies have explored the effects of excessive folic acid on placental health and function. Thus, the effect of excessive exogenous folic acid on placental health and function was investigated using both *in vitro* cell culture and *ex vivo* explant models.

3.4.1 Intracellular Folate Assay

The human placenta, and the corresponding human placenta cell lines, demonstrate a series of folate transporters on their cell membrane [164, 279]. These transporters allow for the transport of folic acid into the cell, but additionally are capable of shuttling folic acid back out of the cell [164, 279]. In one study, Tam *et al.* [280] determined that supplementation with 1.1 mg or 5 mg of folic acid did not significantly alter serum/plasma levels of unmetabolized folic acid, suggesting that the human body is capable of adapting to high levels of folic acid. This function has been postulated to minimize toxicity; however this has not been previously tested in the placenta. In order to determine if exogenous exposure to increasing folic acid concentrations was paralleled by a corresponding increase in intracellular folate concentration in trophoblast cell lines, the *L. rhamnosus* microbiological assay was employed. Using this assay, it was verified that not only does exogenous folic acid enter both HTR-8/SVneo and BeWo cells, but at high doses of exogenous folic acid treatment

(2,000-4,000 ng/ml), significantly more folic acid enters the cells. These results indicate that the intracellular milieu of a trophoblast cell is strongly influenced by exogenous folic acid exposure, and that increased folic acid ingestion by the mother may certainly result in increased intracellular trophoblast folate concentrations.

Though higher concentrations of exogenous folic acid led to increased intracellular folate, a dose-dependent increase was not observed in either cell line, between treatments with low (0-200 ng/mL) or high (2000 ng/mL or 4000 ng/mL) concentrations of folic acid. This is possibly due to a lack of sensitivity of the assay to detect changes in the lower range of folic acid treatment. The lack of significant difference may also be due to the presence of transporters working to shuttle folic acid back into maternal circulation [164, 281, 282], to maintain a relatively constant level of intracellular folates when the range fluctuates within physiologically relevant concentrations. At higher concentrations of folic acid treatments, the lack of difference is possibly due to saturation of these folate transporters, such as FR α , PCFT, and RFC, which are believed to support the movement of folates across the microvillous membrane of syncytiotrophoblasts [283]. Nevertheless, it is important to note that though significant increases were observed at the higher concentrations (2000 ng/mL and 4000 ng/mL) of folic acid, both of these concentrations immensely exceed what is likely to be found in humans and were used to establish a range for risk of adverse effects to placental cells.

Intriguingly, BeWo cells were observed to have a higher rate of folic acid uptake than HTR-8/SVneo cells. This difference is likely due to the presence of transporters in the BeWo cell membrane [284, 285], as well as the cell type and function of BeWo cells. The BeWo cell line is a model of cytotrophoblast cells that can differentiate into syncytiotrophoblast cells, the trophoblast cell lineage that is in direct contact with maternal blood and responsible for maternal-fetal nutrient exchange [253]. Interestingly, the intervillous space in the placenta has been shown to concentrate folic acid [41, 286], resulting in a 2-4 fold increase in folic acid in the fetal circulation in comparison to the maternal circulation [41]. This indicates a preferential fetal gradient of folic acid across the placenta. In line with this observation, in the current study, BeWo cells were observed to have increased folate uptake approximately 2-4 times higher than HTR-8/SVneo cells, which do not play a role in trans-placental nutrient transfer. Thus, it is understandable that BeWo cells, as the gate keepers of nutrient exchange, would be capable of optimal folic acid transfer.

Overall, these experiments demonstrated increased folic acid uptake with increased exogenous folic acid exposure in both the HTR-8/SVneo and BeWo cell culture models. Accordingly, these immortalized cell lines were accepted to be suitable for the *in vitro* study of the effects of excessive folic acid exposure on trophoblast health and function.

3.4.2 Assessment of Trophoblast Health

Folate derivatives play an important role in one-carbon metabolism to support both DNA and RNA synthesis, as well as DNA methylation [10]. These processes are necessary for rapidly-dividing cells such as those of the placenta and the developing offspring. With the availability of excess folic acid feeding into the one-carbon metabolism pathway, it was hypothesized that the increased availability of folate metabolites would translate into increased DNA and RNA synthesis, as measured by an increase in placental cellular proliferation.

Surprisingly, in both trophoblast cell lines (HTR-8/SVneo and BeWo), and in the *ex vivo* term placental explant culture model, we saw no significant increase in cellular proliferation with increasing folic acid concentrations. These findings are in line with a study by Klingler *et al.* [287], in which supplementation with 5-methyltetrahydrofolate (5-MTHF), the metabolically active form of folate normally found in circulation, had no effect on human placental cell proliferation or apoptosis if taken after the 20th week of gestation. The lack of an observed increase in trophoblast proliferation is possibly due to the unchanged levels of important cofactors, such as vitamin B₆, which is required by the enzyme serine hydroxymethyltransferase to promote DNA synthesis. Furthermore, standard cell culture media normally used to maintain and sub-culture the immortalized cell lines contains exorbitant concentrations of folic acid (10⁵ times more than physiologically relevant concentrations). In the current experimental design, both cell lines were sub-cultured in a specially formulated, FA-reduced cell culture media for two weeks prior to any experimentation. This media contained physiologically relevant concentrations of folic acid (20 ng/mL). This was done in an attempt to minimize the potential lingering effects of the high doses of folic acid these cells would have been exposed to in their earlier passages. However, it is possible that two weeks of sub-culturing in this FA-reduced media was insufficient to alter the previously established cell biology/physiology (i.e. DNA replication machinery), and therefore no effect was observed. Similarly, it is possible that experimental

concentrations had no effect on placental explants since all term placentae that were collected for analysis were exposed to folic acid supplementation *in utero*.

In contrast, studies using first trimester placentae demonstrated increased sensitivity to folate concentrations. In an *in vivo* human study, methotrexate, a folic acid analog often used as an abortifacient, was shown to significantly decrease trophoblast proliferation [288], emphasizing the need for folate during cellular proliferation. However, a study by Williams *et al.* [167] demonstrated that exogenous folic acid, at folic acid concentrations 100X higher than physiological levels observed in circulation (10^{-6} M) [289], decreased proliferation in first trimester placental explants. Taken together, there is likely a temporal influence of folic acid on placental proliferation, possibly with significant influence of folic acid on trophoblast proliferation early in gestation and less of an effect in later pregnancy. Further investigation of this temporal relationship is certainly warranted, in order to best understand when pregnant women require folic acid supplementation and to establish a safe therapeutic window.

In addition to cellular proliferation, an assessment of cell death is also required in the determination of cellular health following excessive folic acid exposure. In a study by Williams *et al.* [167], a significant increase in apoptosis was observed following treatment of first trimester human placental explants with folic acid at 10^{-6} M (100X the concentrations found in human circulation). We therefore hypothesized that exogenous exposure to high concentrations of folic acid would result in decreased trophoblast viability, via increased activation of apoptotic cascades, in trophoblast cell lines and in an *ex vivo* third trimester placenta explant model. However, our results demonstrate that there was no significant difference in apoptosis following increasing folic acid exposure in either cell lines or term placental explants. This discrepancy may be attributed to the culture media used by Williams *et al.* [167], which was not originally folic acid-depleted media and was unaccounted for in their findings. Furthermore, the differences in the treatment period (48 hours in the current study vs. 6 days in the Williams study) may have impacted the results. In an animal study by Marsillach *et al.* [290], it was found that excessive folic acid intake over several weeks was required to alter the expression of genes involved in apoptosis. And finally, like the observations discussed related to cellular proliferation, it is possible that there is a temporal relationship in which the early (first trimester) developing placenta is more vulnerable to excessive folic acid exposure than the fully developed term placenta. Further investigation specifically addressing this hypothesis is warranted.

Nonetheless, our findings suggest approximately 1% of trophoblast tissue in term human placenta explants were undergoing apoptosis under basal conditions. This is higher than other studies, using healthy term placentae, that found the rate of apoptosis to be less than 1% [277, 291, 292]. It is important to note that in their studies, apoptotic nuclei were calculated as a proportion of all trophoblast nuclei; whereas in our study, we measured apoptosis as a percentage of apoptotic surface area which could certainly explain the difference. In our study, as observed by Austgulen *et al.* [277] in placental explants, apoptosis was localized to the syncytiotrophoblasts which are in direct contact with the folic acid-treated culture media. Accordingly, BeWo cells, representing the syncytiotrophoblasts, were found to have decreased viability at higher concentrations than the control, suggesting increased cell death, likely through necrotic cell death pathways. This suggests that excessive folic acid may negatively impact trophoblast health, though it remains unclear. In the case of HTR-8/SVneo cells, though excessive folic acid did not significantly affect cellular viability, treatment with 2 ng/mL of folic acid significantly decreased cellular viability. In circulation, this concentration is associated with folate deficiency, and emphasizes the need for folate to support trophoblast health.

3.4.3 Assessment of Trophoblast Function

A fully functioning placenta is vital to the progression of a healthy pregnancy and delivery of a healthy child. As such, not only do trophoblast cells need to be viable and proliferating appropriately, but they must also be able to perform necessary functions throughout gestation. Two important functions performed by the placenta include: 1) invasion of the uterine wall and establishment of the utero-placental circulation, optimizing blood flow to the developing fetus [160]; and 2) hormone production, including the production of human chorionic gonadotropin (β -hCG) which promotes placental angiogenesis and vasculogenesis [293], trophoblast differentiation [294, 295], placental growth [296, 297] and suppression of the maternal immune response [298–300].

Trophoblast Invasion

The extravillous trophoblast cell plays an important role in the anchoring of the embryo to the endometrium and subsequent invasion into the uterine wall [160]. It was hypothesized in the current project that exposure to excessive concentrations of folic acid would increase trophoblast invasion using the in vitro HTR-8/SVneo cell culture model. However, no change in trophoblast invasion was observed between physiologically relevant

concentrations of folic acid (20 ng/mL) and high concentrations of folic acid (200-2000 ng/mL). These findings are in contrast to findings by Petersen *et al.* [301], who observed increased invasion at elevated physiological levels of folic acid in cancer cell lines. Similarly, Williams *et al.* [167], demonstrated a 30% increase in trophoblast invasion for every 100-fold increase in exogenous folic acid (10^{-10} – 10^{-6} M), using placental explants from first trimester placentae. However, it is important to re-iterate that in their study, standard tissue culture media was used and the excessive folic acid exposure due to inherent levels in the media itself was unaccounted for. Further, the model employed in the Williams study was a first trimester explant sprouting assay, and not the Boyden chamber invasion assay using the immortalized HTR-8/SVneo EVT cell line employed in the current study. Future experiments using isolated primary cell cultures from first trimester placentae or using *in vivo* animal models may help further clarify the effect of increasing doses of folic acid on trophoblast invasion.

Interestingly, while we did not find evidence of compromised trophoblast invasion with excessive folic acid exposure, we did observe the inhibition of trophoblast invasion following treatment with very low concentrations of folic acid (2 ng/mL), which mimics folic acid deficiency in a human population [302]. In support of this finding, Floridon *et al.* [303] observed diminished invasion in ectopic pregnancies when folic acid was in short supply due to exposure to methotrexate. It is possible that the reduced invasion observed at low folic acid levels is due to reduced cell number as a result of increased cell death. Additionally, poor dietary and low circulating folate concentrations has been associated with increased pregnancy complications such as spontaneous abortion [304], preterm delivery [305, 306], decreased birth weight [307, 308], and preeclampsia [309, 310]. These findings have important health implications, indicating that FA deficiency not only puts the developing fetus at risk of NTD, but may also explain the increased risk of placenta-mediated disorders of pregnancy.

β-hCG Hormone Production

As previously mentioned, β-hCG is required to maintain a successful pregnancy. Expressed by syncytiotrophoblast cells early in pregnancy and onwards, the effect of exposure to varying concentrations of folic acid was assessed in both the BeWo cell line and human placental explants. However, in both models no significant effects of exogenous folic acid treatment on β-hCG hormone production and secretion were observed. After pregnant

women were treated with methotrexate, a folic acid analog, no effect on β -hCG release was also observed [288]. Taken together, this suggests that folic acid, either at very low or very high concentrations, does not independently interrupt the endocrine function of trophoblast cells. Interestingly, β -hCG secretion is decreased when the placenta is exposed to high concentrations of formic acid, as observed in alcohol-abusing mothers [311], but this effect is mitigated when the placenta is simultaneously exposed to high concentrations of folic acid (10^{-6} M) and suggests that interactions with other compounds *in vivo* may be involved in enabling folic acid to have an effect.

3.5 Limitations

There were several strengths of the presented experimental design. At the forefront, the use of both *in vitro* cell culture models (HTR-8/SVneo and BeWo cell lines), as well as an *ex vivo* term human placenta explant model allowed us to examine a variety of placental health and function parameters. Further, the analysis of range of exogenous folic acid treatments, spanning concentrations that would be equivalent to maternal folic acid deficiency (2 ng/mL), normal maternal physiological concentration (20 ng/mL), supraphysiological concentration (200 ng/mL) and toxicological concentrations (>2000 ng/mL). Our experimental design also took into account and corrected for the very high concentrations of folic acid found in standard tissue culture media, a step that has been overlooked in most publications on this topic to date. However, there remains some limitations to this study that should be addressed in future research projects.

First, it is important to note that models are imperfect by nature, and are used to approximate the events that occur *in vivo*. For example, when conducting experiments using term human placental explants, it is impossible to control for environmental stressors throughout the pregnancy that may have affected the placenta. Important to the current project, this would include maternal folic acid intake and/or supplementation at different points across the gestation. Furthermore, once the placenta has been extracted following delivery and is exposed to atmospheric oxygen levels, considered hyperoxic for placenta tissue [312], placental health and function can be compromised. To minimize adverse effects, explants were collected as efficiently as possible and placed within an oxygen regulated incubator at 8% oxygen (physiologically relevant [313]) within one hour of delivery. Further, all results were normalized through the use of an untreated control collected from the same placenta and processed in the same fashion. With regards to *in vitro* experiments using cell

lines, results cannot be directly extrapolated due to the mutant condition inherent to any cell line. Nonetheless, it is a method that allows researchers to control for external variables that may otherwise affect results. Therefore, though imperfect, both models have been used to complement each other, but further studies using alternative models (ex. Isolated primary cultures) can be used to confirm results.

Second, though folic acid-free cell culture was used in all experiments, it is unknown to what extent folates were present in the FBS used to supplement the media. In an attempt to address this limitation, we did conduct a series of additional experiments, in which intracellular folic acid was compared in cells supplemented with normal FBS vs. cells supplemented with charcoal treated FBS, a treatment which removes endogenous folates from the FBS. We observed no difference in intracellular folic acid between these two treatments (Appendix D), and as such felt confident that the concentration of folates/folic acid that may be present within the FBS was negligible within the context of our study design. In addition, it is possible that the cell lines used were accustomed to high experimental folic acid concentrations due to exorbitant amount of folic acid in standard cell culture media, in which they would have been originally isolated, transformed and sub-cultured prior to these experiments. We attempted to address this concern by sub-culturing all cells in FA-depleted culture media for two weeks prior to experimentation, however this may not have been a long enough time to allow for significant changes in cellular phenotype. Also, our experimental protocol involved a 48-hour experimental treatment incubation. *In vivo*, however, supplements containing folic acid are consumed over a much longer period of time, and as such, it is possible that a longer experimental incubation time would be required to see any significant effects on placenta cell health and/or function.

Finally, the lack of folic-acid mediated differences in cell health and function may possibly be due to unchanged concentrations of available cofactors in the culture system, such as vitamin B₆ and vitamin B₁₂, both required for folic-acid dependant one-carbon metabolism. Without paralleled increased in these and other cofactors, excess folate may not be fully metabolized and made available for increased production of purines and pyrimidines in DNA and RNA synthesis or function as a methyl donor. Culture media is formulated to contain many micronutrients and amino acids, including cofactors acting as methyl donors in one carbon metabolism. Though folic acid-free media was purchased to control for the effects of folic acid, controlling for the effects of other metabolites may also be required. As is the case *in vivo*, a combination of one-carbon metabolism cofactors may

need to be studied and adequately controlled in order to observe the true effects excessive folic acid intake may have on placental cells.

3.6 Future Directions

Further experiments are necessary to confirm our results and address some of the limitations in this study. The risk of neural tube defects occurs within the first trimester of pregnancy and the risk of excessive folic acid intake runs high during this time period due to recommendations for widespread maternal supplement usage. One avenue to study the effects of high concentrations of folic acid during the first trimester would be to use elective early-termination placentae from otherwise healthy pregnancies. In this way, the effects of excessive folic acid concentrations can be observed on human placental tissue during this sensitive time period and there will be decreased exposure to environmental stressors that may confound or create variability in results. Furthermore, the experimental folic acid incubation period could be increased to better characterize adverse effects that occur due to chronic exposure to excessive folic acid concentrations, as in the case of many pregnant women.

Moreover, fetal programming and the environment *in utero* is gaining more significance as the spotlight shifts from developmental and other short-term effects, to possible epigenetic changes and the long-term effect based on maternal diet. As a result, though findings in this study did not point to any significant alterations in placental health and function as a result of exposure to increased levels of exogenous folic acid, it is possible that folate's role as a methyl donor may result in epigenetic changes in the placenta and offspring. By modifying DNA and protein methylation patterns, altered gene expression may ensue and cause adverse health effects in the short- and long-term and/or result in adult onset diseases in the offspring [130, 133–135]. Maternal folic acid intake during pregnancy has already been associated with epigenetic changes on several fetal genes [314–316]. However further studies are required to gather a comprehensive understanding of the epigenetic effects and its implications on fetal health and future development. As the placenta is fetal tissue, future experiments studying any epigenetic alterations in gene expression in the placenta may help elucidate potential effects on fetal development and the offspring.

Finally, diet and nutrition does not depend solely on the variability of one nutrient, but on the combination of nutrient-nutrient interactions and compensatory changes from

other nutrients as well [317]. As previously mentioned, several cofactors are required to support one-carbon metabolism. Prenatal supplements, such as *Materna*TM, take this into consideration and include these cofactors (ex. Vitamin B₁₂ and vitamin B₆, zinc) in its formulation [185]. To better represent potential effects that may occur as a result of excessive folic acid intake during pregnancy, the combined effect of increased metabolites involved in one-carbon metabolism should also be considered in future experiments.

3.7 Conclusion

Through this study, using both *in vitro* cell lines and *ex vivo* human placental explants, we have been able to demonstrate that exposure to excessive concentrations of folic acid (200-4000 ng/mL) does not significantly affect primary indicators of placental health and function as measured through cellular proliferation, apoptosis, invasion, and β -hCG hormone production. Cellular viability, at increased concentrations of folic acid (200-2000 ng/mL), was decreased in the BeWo cell line, though HTR-8/SVneo and placental explants remained unaffected. At low concentrations of folic acid (2 ng/mL), concentrations similar to a woman with folic acid deficiency, cellular viability and capacity for invasion seemed to be significantly compromised in the HTR-8/SVneo cell line. The results from this study can be used to assist the re-evaluation of current nutritional standards with regards to pregnancy and help determine an ideal therapeutic window of folic acid intake among pregnant women and women of child-bearing age. Despite known benefits in preventing NTDs, further studies are recommended to determine potential adverse effects of excessive folic acid intake on the pregnant population, the placenta, and the offspring.

Chapter 4

Conclusion

Using a representative sample of 3-day food records collected by the IRNPQEO, this study allowed detailed examination of the micronutrient intake among pregnant women in Canada. Moreover, at a time when folic acid-containing supplementation is recommended to all women who are planning or are currently pregnant, this study helped elucidate the effects of excessive folic acid on placental health and function by measuring the effects on trophoblast cell viability, proliferation, apoptosis, invasion, and hormone production. In this chapter, study findings are summarised and future directions are presented.

4.1 Summary of Findings

Overall, the initial hypothesis was refuted: food sources alone do not seem to provide adequate micronutrients recommended for a healthy pregnancy for women in Québec, Canada. The majority of study participants (>50%) were consuming adequate levels of vitamin A, thiamine, riboflavin, niacin, vitamin B₆, vitamin B₁₂, vitamin C, calcium, copper, magnesium, phosphorus, selenium, and zinc from their diet. However, the majority of women (>50%) may be at risk of inadequacy for vitamin D, vitamin E, folate, and iron from diet alone, supporting the need for supplementation of specific micronutrients during pregnancy. With the exception of sodium, excessive intake of micronutrients above the established tolerable upper intake level (UL) is not a reason for concern from food sources.

Consistent with the second hypothesis, the majority (83%) of participants reported consumption of daily nutritional supplements during the second-trimester of their pregnancy. Thus, the risk of exceeding the established UL for folic acid may be high for pregnant women of high socioeconomic status from Québec, Canada.

Finally, with respect to the third hypothesis, no significant differences in the effect of excessive exogenous folic acid exposure (200-4000 ng/mL) on trophoblast cellular proliferation, apoptosis, invasion, and β -hCG hormone production were observed *in vitro*, in either HTR-8/SVneo or BeWo trophoblast cell lines, or in human placental explants as measured in this study. However, decreased cellular viability was observed in the BeWo cell line at increased concentrations of folic acid (200-2000 ng/mL), though HTR-8/SVneo and placental explants remained unaffected. Furthermore, at low concentrations of folic acid (2 ng/mL) congruous with folate deficiency, cellular viability and the invasion capacity of HTR-8

/SVneo cells were significantly compromised.

Overall, strengths of this study include dietary assessment using a sample size randomly selected from a large cohort of pregnant women, assessing dietary intakes in a pregnant Canadian population, expected response rates for 3-day food records, and a robust experimental design using both *in vitro* cell culture and human placental explants. Limitations include the lack of a nationally representative pregnant population for the 3-day food records, unavailability of accurate intakes from supplements, short treatment period for *in vitro* experiments, and the use of term placentae. Nonetheless, our findings are relevant in the wake of increased folic-acid containing supplement recommendations and can be considered when establishing policies and endorsements in Canada.

4.2 Significance

Maternal nutrition is a modifiable public health risk factor that can be improved to positively impact future generations [188]. For this reason, it is imperative that pregnant women are recommended to consume an appropriate diet consisting of all necessary nutrients for the success of their pregnancy and future health of their offspring. Through the use of 3-day food records from pregnant women in Québec, Canada, this study has generated a better understanding of the micronutrient intake among a population of pregnant women where the majority is non-ethnic, educated, and affluent.

Since the majority of women in this group are taking a folic acid-containing supplement as recommended by Health Canada and SOGC [29, 30], this study draws attention to the potential for exceeding the currently established tolerable upper intake level (UL) for folic acid intake (1 mg/day) and examines potential adverse effects of excessive folic acid intake on placental health and function. Though further studies are required to characterize the potential for adverse effects due to excessive folic acid intake, results from this study suggest no significant effect on placental health and function. Additionally, clinical trials are ongoing to assess the potential benefits of up to 4 mg of folic acid on placenta-mediated pregnancy outcomes and to prevent NTDs [115, 171]. It is important to note that currently, the established value of 1 mg/day is the same for adult men, women, and pregnant women. It was also established prior to mandatory flour fortification policies in Canada and the United States based on dated studies concerned with the ability of excessive folic acid intake to mask vitamin B₁₂ deficiency. Thus, in combination with future research, the results from this study can be used to help revise the UL for folic acid in the pregnant population.

Appendix A: Sample Food Record

Attached, below, is a sample of the instructions (p.104-110), blank template for Day 1 out of 3 (p.111-114), and additional details collected in the food record (p.115-121) as part of the 3D Study by the IRNPQEO.



Day Month Year

IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre ID Monogram

Food Diary
Second trimester of pregnancy

IRNPQEO -3D Study
Questionnaire 2C, Version 3, June 14th 2011

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IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

Thank you for your continuing participation in this study!

We are interested in reviewing your diet during the second trimester of your pregnancy. This food diary will allow you to record all the foods and beverages you will consume during **three days (two weekdays, one weekend day)**, each day corresponding to a 24 hour period), over the course of the next week. Please record in this diary all foods and drinks consumed (including water and alcoholic beverages), both day and night. To avoid any missing information, we ask you to note everything about your meals and snacks **as you go through the day** instead of waiting for a specific time.

During these three days, it is **very important** that you don't change your current eating habits just because you are keeping this food record. Continue your diet as usual while simply taking note of what you eat and drink at different times of the day.

At the end of these three days of diary, you will find additional questions about your diet that we would like you to answer. Once completed, please return this food diary booklet to the research nurse in the provided pre-stamped and pre-addressed envelope.



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

Instructions for completing this Food Diary

- Each day, starting from and ending at midnight, is divided into 7 sections.
- In the corresponding section, in the column **Time**, indicate the precise time at which you ate or drank.
- In the column **Meal or Snack**, indicate if this was a meal or a snack.
- In the column **Place**, record the place where you ate your meal or snack, according to the following options:
 - H: Home;
 - R: Restaurant
 - C: Cafeteria
 - O: Other (Specify)
- In the column **Description of food and drinks**, record all food and beverages (including water) you consumed at this time. Take care to specify important details such as:
 - the name of the food or drink;
 - the type (e.g., minestrone soup, vegetarian pizza) and the brand of the product, if it is known;
 - the cooking method, if applicable (e.g., fried, broiled, oven-baked, steamed);
 - any extra (e.g., fats or oils, sugar/substitute, gravy, salt, pepper, ketchup and other condiments).
- In the column **Amount Consumed**, record the quantities you consumed, using common household measures [e.g., tablespoon (Tbsp), teaspoon (tsp), cup (c)], the weight or volume indicated on the food package (e.g., a 200 ml juice box, one quarter of a quiche of 550g), number of units (e.g., 1 average size apple, 1 chicken leg, 2 slices of bread) and/or the dimension of the food (e.g., a piece of banana, 10 cm long; 1 mini carrot). **N.B. Take care to subtract any quantity served that was not consumed.**
- If the food consumed is a **Recipe**, write down in the space provided, the ingredients, the quantities and the cooking method. If the food was prepared by someone else (e.g., a friend, at a restaurant), and you don't know the recipe, describe the dish as best as you can (e.g., a tomato sauce with mushrooms, spinach and celery).

Once these three days of diary are completed, do not forget to answer the additional questions about your diet, which you will find following the section for the 3rd day of the diary.

Resources

- On the following pages, you will find an **example** of how we would like you to record information in the food diary.
- At the end of the food diary, a **MEMORY AID** provides the relevant information for recording the various types of food you might consume.
- If you have any questions about how to fill in the food diary, please contact your site's research nurse.



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

DAY 1 Date: 24th September, 2008 (Wednesday)

EXAMPLE

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between midnight and 6:00				
04h00	Snack	Home (H)	Tap water	¼ cup
Between 6:00 and 9:00				
08h15	Meal	H	7 grain bread, Country Harvest, toasted (45 g / slice) With margarine, Becel with olive oil, hazelnut spread, Nutella and raw banana (average) Cereals, Mini Wheat (original) With 2% M.F. milk, Natrel, with omega 3 and dried cranberries Orange juice, Minute Maid, frozen concentrate, diluted with water (3.5 cans)	1 slice 1 tsp. 1 Tbsp ½ ½ cup ½ cup 1 Tbsp 200 ml
Between 9:00 and 12:00 (noon)				
10h00	Snack	R	Muffin, dates and nuts, Tim Horton Decaffeinated coffee With cream And sugar	½ muffin 1 medium size 2 creamers of 15 ml each 1 sachet

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IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre		ID		Monogram	

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between 12:00 and 14:00				
12h15	Meal	H	Chicken leg, oven-baked, with bone and skin, carrots and onions Served with St-Hubert BBQ sauce, made from dry mix (prepared according to directions) White rice, long grain, converted, Uncle's Ben (original), cooked according to directions with half water, half chicken stock and semi-salted butter Vegetable juice, V8	Meat + skin : 1 piece (3.5 in. X 2 in. X 1 in.) 2 Tbsp ¾ cup 1 can (156 ml)
Between 14:00 and 17:00				
15h20	Snack	O (friend)	Yoghurt, Activia, strawberry and kiwi	100 g
16h30	Snack	H	Crackers, Breton, 50% less salt Cheddar cheese, light, P'tit Québec (22% M.F.) Bottled water, Naya	3 crackers 1 piece: 1 in. X 3 in. X 2 in. ¾ of a 500 ml bottle



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between 17:00 and 20:00				
18h30	Meal	H	Macaroni and Cheese (see recipe) English cucumber, with peel, raw, salt and pepper Mini carrots, raw With dip, Philadelphia, Vegetable Ranch, Kraft Skim milk, Québec Applesauce, unsweetened, homemade	1 ½ cup 3 slices, 1 cm thick 5 1 Tbsp 200 ml ½ cup
Between 20:00 and 23:59				
20h30	Snack	H	Egg sandwich – for 1 sandwich: 2 slices of bread (same as breakfast) 1 hard boiled egg 1 Tbsp of salad dressing Miracle Whip, light 1 romaine lettuce leaf White cake, Duncan Hines (commercial mix prepared according to directions: added 1 egg and 2% milk) With a thin layer of ready-made chocolate frosting, Betty Crocker	¼ of the sandwich 1 piece = 6 cm X 4 cm X 3.5 cm

¹Place of Meal/Snack: H= Home; R = Restaurant; C= Cafeteria; O= Other (Specify)



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

RECIPE - PLEASE write down recipes or ingredients of combination dishes, if not already described.

Macaroni and cheese

2 cups – elbow macaroni, dry, Catelli (boiled in water with salt and oil)

Cheese sauce:

2 ½ cups – skim milk

3 Tbsp – all- purpose flour

3 Tbsp – semi-salted butter

¾ tsp – prepared yellow mustard

¾ tsp – Worcestershire sauce

2 ml – pepper

2 cups – Mozzarella cheese, partly skimmed (16 % M.F.), grated

Garnish:

1 cup – breadcrumbs

¼ cup – Parmesan cheese, grated



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre		ID		Monogram	

DAY 1 Date: _____

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between midnight and 6:00				
Between 6:00 and 9:00				
Between 9:00 and 12:00 (noon)				



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between 12:00 and 14:00				
Between 14:00 and 17:00				



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre		ID		Monogram	

Time	Meal or Snack	Place ¹	Description of food and drinks	Amount consumed
Between 17:00 and 20:00				
Between 20:00 and 23:59				

¹Place of Meal/Snack: H= Home; R = Restaurant; C= Cafeteria; O= Other (specify)



ID

Monogram

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RECIPE - PLEASE write down recipes or ingredients of combination dishes, if not already described.

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IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

Please answer the following questions which will allow specifying important details about your diet over the course of the last week.

1. Over the course of the last week, what type of milk did you consume most often?

- | | |
|--|--|
| ☐ Whole milk (3.25% M.F.) | ☐ Skimmed milk |
| ☐ 2% M.F. milk | ☐ Soy beverage |
| ☐ 1% M.F. milk | ☐ Other(s), specify : _____ |

2. Over the course of the last week, did you add sugar and milk or cream (or a substitute) in your coffee/ tea?

Coffee : (check all that apply)

- | | | | | | |
|---|--------------------------------|--------------------------------|---|---|-------------------------------|
| ☐ I did not have any coffee over the course of the last week | | | | | |
| ☐ Milk | ☐ Cream | ☐ Sugar | ☐ Artificial creamer | ☐ Artificial sweetener | ☐ None |

If yes, how much? _____

Tea: (check all that apply)

- | | | | | | |
|--|--------------------------------|--------------------------------|---|---|-------------------------------|
| ☐ I did not have any tea over the course of the last week | | | | | |
| ☐ Milk | ☐ Cream | ☐ Sugar | ☐ Artificial creamer | ☐ Artificial sweetener | ☐ None |

If yes, how much? _____

3. Over the course of the last week, what type of water did you drink most often?

- | | |
|---|--|
| ☐ Tap water | ☐ Bottled water |
| ☐ Filtered water | ☐ Well water |

4. Over the course of the last week, what type(s) of bread did you eat?

- | | | |
|---|--------------------------------------|--|
| ☐ White | ☐ Whole wheat | ☐ Other(s), specify : _____ |
| ☐ High fibre white | ☐ Multigrain | |

Specify the complete name of the product(s), the brand and, ideally, the weight per slice (available at the top of the *Nutrition Facts* table on the food label):



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QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

5. If you ate meat over the course of the last week, did you eat the visible fat?

- ☐ Yes ☐ No ☐ A little ☐ I did not eat any meat over the course of the last week

6. If you ate poultry over the course of the last week, did you eat the skin?

- ☐ Yes ☐ No ☐ A little ☐ I did not eat any poultry over the course of the last week

7. Over the course of the last week, what type(s) of fat did you use for cooking?

Specify the **complete name** of the product(s), the **brand** and **other relevant details** (see *Memory Aid for Fats and Oils*):

8. Over the course of the last week, what type(s) of fat did you use for spreading (e.g., on bread, toast, crackers)?

Specify the **complete name** of the product(s), the **brand** and **other relevant details** (see *Memory Aid for Fats and Oils*):

9. Over the course of the last week, what type(s) of vinaigrettes, mayonnaise and salad dressings did you use?

Specify the **complete name** of the product(s), the **brand** and **other relevant details** (see *Memory Aid for Fats and Oils*):

10. Over the course of the last week, did you add salt to your food at the table?

- ☐ Rarely ☐ Occasionally ☐ Very often ☐ Never ➤ Go to question 12



IRNPQEO-3D Study
QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre		ID		Monogram	

11. If you added salt at the table, what type of salt did you use?

- ☐ Ordinary salt
 ☐ Salt substitute
 ☐ Other(s), specify : _____

12. Over the course of the last week, did you add ordinary salt to your food during cooking?

- ☐ Rarely
 ☐ Occasionally
 ☐ Very often
 ☐ Never

13. Over the course of the last week, did you eat the skin on fruit and vegetables (including potatoes)?

List the fruits and vegetables you ate with the skin: _____

List the fruits and vegetables you ate without the skin: _____

14. Over the course of the last week, did you follow a special diet (e.g., vegetarian, low- cholesterol, weight reducing, etc.)?

- ☐ No
 ☐ Yes, specify: _____

PLEASE add any other information that you feel is important about your diet over the last week.

Thank you for your cooperation!



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QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre		ID		Monogram	

Memory Aid

BREAD, CEREALS, PASTA AND RICE	• Bread : Type of flour (e.g., whole wheat, white, rye), type of bread (e.g., sliced, Kaiser, hamburger bun, hot-dog bun), brand and other relevant information on the label (e.g., weight per slice) • Cereals: Type (complete name on the food package), brand, with or without milk • Pasta: Type (e.g., spaghetti, macaroni), variety (e.g., spinach pasta, high protein, whole wheat, egg pasta, vegetable pasta), brand, preparation and cooking method (e.g., salt and oil added in boiling water) • Rice: Type (e.g., white, brown, basmati, instant), brand, preparation and cooking method (e.g., according to directions or not, prepared with water, chicken or vegetable stock, added fat, added vegetables)
OTHER BAKED GOODS	• Complete name (e.g., chocolate chip cookies, angel food cake, carrot muffin, cinnamon bun), brand, form (e.g., frozen product, refrigerated dough, commercial mix, homemade recipe) and other characteristics (e.g., type of garnishes or frosting, nuts, dried fruits) • If homemade recipe or prepared using a commercial mix, list the ingredients that were used for preparation
FRUITS AND VEGETABLES	• Type (e.g., green grapes, apple, orange, spaghetti squash, red or green cabbage, green or yellow beans) • Form (e.g., fresh, dried, canned, frozen) • Preparation (e.g., sliced, diced, mashed, grated) • Eaten raw or cooked, with or without peel • Cooking method (e.g., reheated only, boiled, steamed, microwaved, oven-baked, fried), ingredients added while cooking (e.g., sugar, flour, cream, type of milk, butter, type of sauce) • Garnishes (e.g., type of sauce, cream, ice cream, sour cream, butter, etc.)
MILK AND MILK PRODUCTS	• Type (e.g., milk, cheese, cream, evaporated milk, sweetened condensed milk, ice cream, ice milk, yoghurt), brand, % milk fat (%M.F.) • Variety (e.g., Cheddar cheese, Gouda, Cottage; chocolate milk, vanilla ice cream, strawberry stirred yoghurt) and special characteristics (e.g., with omega-3, with added fibre, with added calcium)



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QUESTIONNAIRE 2C – Mother's Food Diary (between 20-24 6/7 weeks)

Centre	ID	Monogram

MEAT AND POULTRY	- Type (e.g., beef, pork, lamb, chicken, turkey, duck) - Meat and poultry cuts (e.g., eye of round steak, sirloin tip steak, cross rib roast, chicken breast, wings, leg or thigh) - Cooking method (e.g., braised, grilled, roasted, sauté, boiled, stewed, fried), with bone or boneless, with or without skin for poultry, added ingredients (e.g., gravy, marinades), type of fat used, if any (if the fat is consumed, specify the quantity) - Visible fat on meat, eaten or not; skin on poultry, eaten or not - If a weight is recorded, specify if this refers to weight before or after cooking
PROCESSED MEAT	- Type of meat (e.g., turkey Bologna, pork and beef mortadella, all beef hot dog) - Form (e.g., sliced, flaked) and other characteristics (e.g., regular, light, lean or low fat) - Cooking method, if applicable
FISH	- Fish species and form (e.g., fresh, canned, frozen, dried, salted or smoked) - Description (e.g., filet, slice, fish stick), breaded or battered - Cooking method (e.g., grilled, oven-baked, poached), type of fat used, if any (if the fat is consumed, specify the quantity) - If a weight is recorded, specify if this refers to weight before or after cooking
EGGS, LEGUMES, NUTS AND SEEDS	Eggs: special characteristics (e.g., with omega-3), cooking method (e.g., scrambled, fried, soft-boiled, hard-boiled, omelette), type of fat used and additional ingredients, if any (e.g., milk, vegetables, salt and pepper) Legumes: type (e.g., kidney beans, lentils, chick peas), form (e.g., dried, canned) Nuts and seeds: type (e.g., walnuts, pumpkin seeds, almonds), form (e.g., whole, chopped), processing (e.g., roasted, dried, salted)



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Centre	ID	Monogram

SOUPS AND BEVERAGES	• Carbonated beverages: brand, serving size, type (e.g., regular or diet, with or without caffeine), with or without ice (with ice, estimate the proportion of ice in the recorded quantity of beverage consumed) • Pure fruit juices and fruit flavoured drinks: type (e.g., apple juice, nectar, fruit punch, etc.), form (fresh, refrigerated, frozen concentrate, canned or bottled), brand, 100% pure juice or not, sweetened or unsweetened, with or without added vitamin C (see information on the label), recipe for dilution if the product is a frozen concentrate (e.g., 1 can of juice for 3 cans of water) and the type of water used (tap water, well water, filtered water, bottled water) • Chocolate flavored beverages: form (e.g., chocolate powder, cocoa, chocolate syrup), liquid used for preparation (water, milk and % M.F.), other added ingredients (e.g., sugar or substitute, marshmallows) • Soy or rice beverages: product name, brand, flavour, enriched or not with vitamins and minerals, (see information on the label) • Soup : type (e.g., homemade, canned ready-to-serve, condensed, dehydrated), variety (e.g., chicken noodle, cream of vegetable), brand, special characteristics (e.g., low sodium, low fat), liquid used for preparation, if any (e.g., water, milk or cream and % M.F.) as well as the recipe for dilution if it differs from directions • Water : type (e.g., tap water, well water, filtered water, bottled water, mineral water), brand
SNACK FOOD	• Type (e.g., potato chips, nachos, popcorn, chocolate bar, granola bar, crackers), brand, flavour (e.g., BBQ, salt and vinegar, cheese flavoured, caramel) and other characteristics (e.g., regular, light, low fat or low sodium) • Extras (e.g., seasonings, butter)
FATS AND OILS	• Type (e.g., margarine, butter, oil, salad dressing, shortening, lard), brand, characteristics (e.g., regular, light, low calorie, unsalted, semi-salted) • Relevant details for margarines: type of oil used in the product (e.g., soy, sunflower, colza, corn, mixed), hard or soft margarine • Relevant details for mayonnaises, salad dressings and vinaigrettes: variety (e.g., Ranch, Caesar, Italian, real mayonnaise, mayonnaise type dressing)



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Centre	ID	Monogram

PREPARED MEALS	- Commercial products (e.g., frozen or refrigerated meals, canned pasta, quiche, pizza) : type, product name, weight on the food package and brand - Homemade meals: ingredients in recipe - Restaurant meals: name of restaurant, meal description, water, bread, condiments and extras N.B. For pizza, specify: where it comes from (e.g., frozen product, restaurant); the type of crust (e.g., thin or thick; regular, whole wheat or stuffed); the type of topping (tomato sauce, type of meat, cheese, types of vegetables, extras); the size (small, medium, large, extra-large, or the diameter in inches) for pizza from restaurant; the weight on the food package for frozen products.
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Appendix B: Supplement Intake Survey Question

Women who took part in the 3D Study filled out a questionnaire during each trimester of their pregnancy. Since most questionnaires were conducted in French, the original French question and the English translation are provided in Table 12, below. The answers (yes or no) to these questions were analyzed to determine proportion of women taking supplements during pregnancy (Table 9).

Table 12. Survey Questions Answered by Study Participants

Trimester	Original Question	English Translation	Answer
1	Avez-vous déjà consommé des vitamines, minéraux ou suppléments diététiques dans les 3 mois précédant ou depuis le début de votre grossesse?	Have the expectant mothers previously consumed vitamins, minerals, or dietetic supplements in the 3 months preceding or before the start of their pregnancy?	Yes or No
2 and 3	Avez-vous consommé des médicaments ou suppléments nutritionnels depuis votre dernière visite ou en consommez-vous actuellement?	Have the expectant mothers consumed or are currently consuming any form of medication or nutritional supplements since their last visit?	Yes or No

Appendix C: Sample Size Calculation

Sample size calculation was based on the expected proportion of women taking supplements among the study participants. Based on Canadian studies, the expected proportion (p) was estimated to be between 80% [102] and 97% [108].

A representative sample size of women was calculated using Cochran's (1977) formula for dichotomous variables (above the UL or not) [179]. An example is provided for an expected supplement intake of 80%:

$$\begin{aligned}n_o &= [(t)^2 * (p)(q)] / (d)^2 \\&= [(1.96)^2 * (0.80)(0.20)] / (0.05)^2 \\&= 246\end{aligned}$$

where "t" is the value for the selected alpha level of 0.025 in each tail (t = 1.96); where "p" is the expected proportion, "q" is "1-p", "(p)(q)" is the estimate of variance; and where "d" is the acceptable margin of error of 0.05 [179]. In the sample size calculation, a value of 0.80 represents the proportion of women (p = 0.80 = 80%) who may be at risk of being above the UL for folic acid due to supplement intake; and 0.20 represents the proportion of pregnant women (q = 1-p = 1-0.80 = 0.20 = 20%) consuming levels of folic acid below the UL. Since the expected sample size of 246 food records is greater than 5% of the population size (i.e. 81 food records for N=1612), Cochran's (1977) correction formula was used to determine the minimum sample size required [318]:

$$\begin{aligned}n_1 &= n_o / (1 + n_o / \text{Population}) \\&= 246 / (1 + 246/1,612) \\&= 213\end{aligned}$$

Therefore, with a dietary food records database from 1,612 women and an expected proportion of supplement intake at 80%, the minimum sample size would be satisfied if food records were analyzed from 213 women. To ensure sufficient data, a total of 216 food records were randomly selected.

Table 13, below, demonstrates the scenarios for representative sample size calculation based on the percentage of women taking supplements among the study participants.

Table 13. Scenarios for Representative Sample Size Calculation

Variables	Option 1	Option 2	Option 3	Option 4	Option 5	Option 6
t	1.96	1.96	1.96	1.96	1.96	1.96
p	0.70	0.80	0.83	0.85	0.90	0.97
q	0.30	0.20	0.17	0.15	0.10	0.03
d	0.05	0.05	0.05	0.05	0.05	0.05
n_0	323	246	217	196	138	45*
N	1612	1612	1612	1612	1612	1612
n_1	269	213	191	175	127	--

*There is no correction applicable for Option 6 since $n_0=45$ is less than 5% of the population size (i.e. 81 food records where $N=1612$).

Appendix D: Folic Acid Content of Culture Media

D.1 Folic Acid Levels in Non-Pregnant and Pregnant Women

Serum (or plasma) folate levels and red blood cell (RBC) folate levels are two measures used to assess folate in the blood. Serum folate is sensitive to dietary fluctuations and reflects the short-term status of folate. RBC folate is a measurement of long-term folate stores or prolonged deficiency. As established by the World Health Organization in 2005, based on data from the National Health and Nutrition Examination Survey (NHANES III), low serum folate is defined as less than 4 ng/mL, while low RBC folate is defined as less than 151 ng/mL [319].

During pregnancy, there is increased demand for folate due to rapid fetal development. As such, the reference range for pregnant women is accepted to be higher during pregnancy. For adult non-pregnant and pregnant women, the reference ranges for serum and RBC folate are defined in Table 14.

Table 14. Normal Reference Ranges for Folate in Adult Women*

	Non-pregnant Women	Pregnant Women
Serum Folate (ng/mL)	5.4 – 18.0	0.8-24.0
RBC Folate (ng/mL)	150 – 450	109-828

*Abbassi-Ghanavati *et al.* [168]

To reflect these concentrations in the experimental design, the appropriate cell culture media was determined as outlined in Section D.2 and D.3.

D.2 Standard Cell Culture Media

Standard media used in cell culture contains exorbitant amounts of folic acid, 10^5 times more than physiologically relevant concentrations. Following a comparison between standard RPMI-1640 (#R8758; Sigma-Aldrich, USA) and standard DMEM (#10-013-CV; Corning Cellgro), folic acid-free RPMI-1640 (#R1145; Sigma-Aldrich, USA) and folic acid-free DMEM (#D249; Sigma-Aldrich, USA) cell culture media was purchased and independently supplemented with experimental folic acid concentrations in the laboratory (Table 15).

Table 15. Cell Culture Media: Nutrients Involved in One-Carbon Metabolism

Nutrients	Standard		Utilised for Experiments	
	RPMI R8758 [1X] g/L	DMEM 10-013-CV [1X] g/L	RPMI ^a R1145 [10X] g/L	DMEM ^b D249 [10X] g/L
Riboflavin (B ₂)	0.0002	0.0004	0.002	0.004
Vitamin B ₆	0.001	0.004	0.01	0.04
Folic Acid	0.001	0.004	--	--
Vitamin B ₁₂	0.000005	--	0.00005	--

^aRPMI-1640 media used for *in vitro* cell culture experiments using HTR-8/SVneo and BeWo cell lines.

^bDMEM media used for *ex vivo* human placental explant experiments.

D.4 Culture Media

Due to the excessive level of folic acid present in standard cell culture media, for the experiments described in this thesis, folic acid-free media was purchased and prepared in the laboratory to contain 20 ng/mL folic acid (baseline level representing physiologically relevant concentration). Subsequently, folic acid treatments were added accordingly to obtain a final concentration required for the experiment. Experimental culture media contained 2, 20, 200, 2000, 4000 ng/mL FA as outlined in Section 3.2.3.

Furthermore, each aliquot of fetal bovine serum (FBS) contains varying amounts of nutrients depending on the calf it was obtained from. To determine whether any folate that is present in regular FBS may affect intracellular folate concentrations in HTR-8/SVneo and/or BeWo cells, a series of experiments were conducted to test differences in intracellular folate using untreated vs. charcoal-treated FBS (provided by Dr. Amanda MacFarlane, Health Canada). An FBS supplement is normally used in cell culture models to provide an array of necessary growth factors which stimulate proliferation and normal cell function. By charcoal-treating the FBS, any folates present in the serum would be removed prior to experimentation. To determine the potential effect of folate metabolites present in standard FBS, intracellular folate was measured in both the HTR-8/SVneo and BeWo cell lines using the *L. rhamnosus* microbiological assay as outlined in Section 3.2.4 for both standard FBS-supplemented experimental media and charcoal-treated FBS-supplemented experimental media (Figure 16). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis ($p < 0.05$).

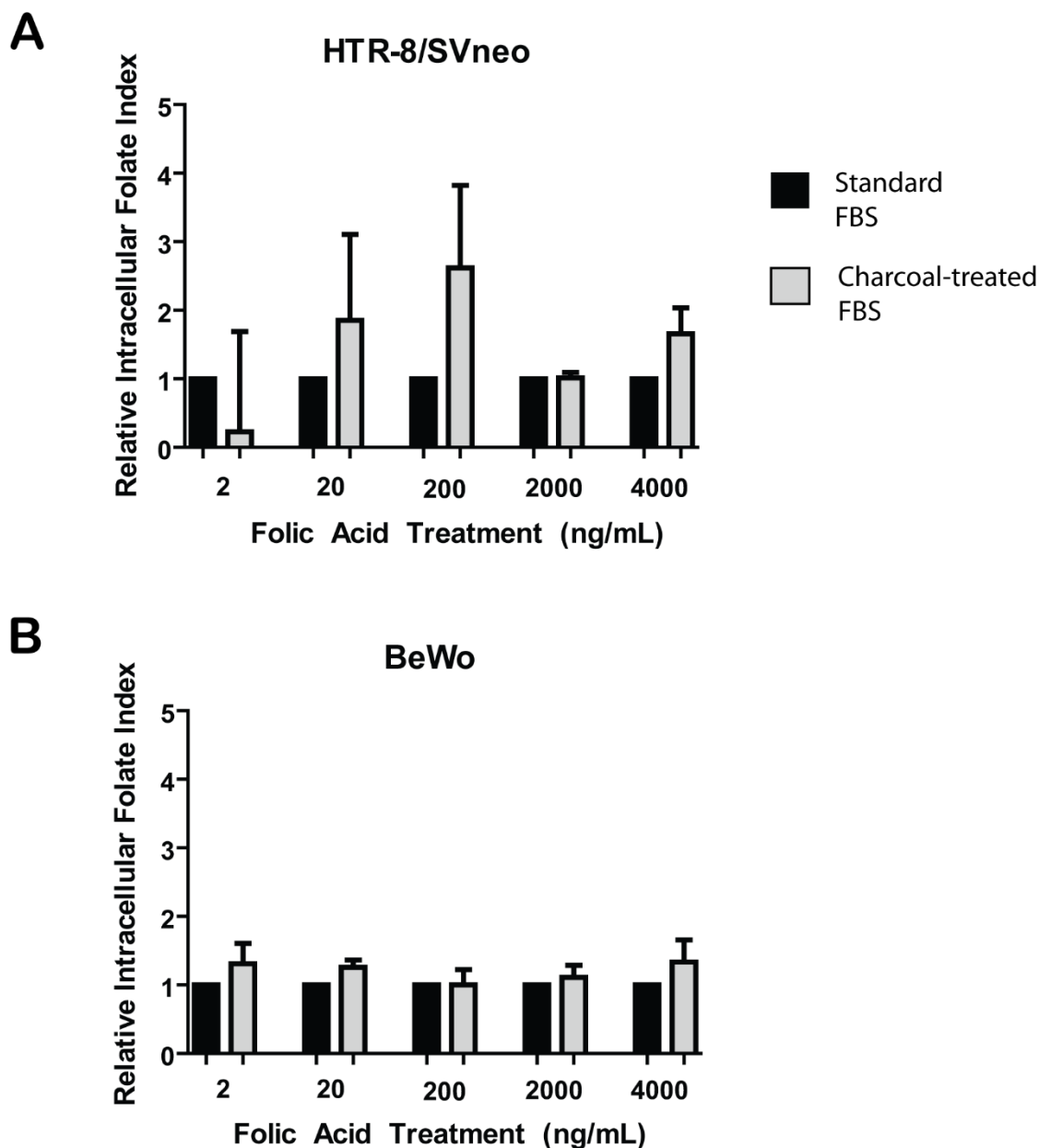


Figure 16. Relative Intracellular Folate. Relative intracellular folate found in **(A)** HTR-8/SVneo and **(B)** BeWo cells, as measured using the *L. rhamnosus* microbiological assay, following exposure to increasing concentrations of exogenous folic acid. Experiments conducted using exogenous FA concentrations of 2-4000 ng/mL in either standard FBS-supplemented media (black) or charcoal-treated FBS supplemented media (gray). Data is presented as mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (* $p < 0.05$).

The results of these validation experiments demonstrated no significant differences in intracellular folate metabolites with standard or charcoal-treated FBS supplementation, indicating no significant contribution of FBS-derived folate metabolites in our experimental protocol. Thus, the standard FBS supplementation was used for all further experiments presented within the current project.

Appendix E: Research Ethics Board (REB) Approvals & Permissions

For this study, the following Research Ethics Board (REB) Approvals are attached:

1. Research Ethics Board at CHU Ste-Justine

(Protocol # 3679)

Note: This approval was given to Dr. William Fraser, the Principal Investigator for the 3D Study, undertaken by the Integrated Research Network of Perinatology of Québec & Eastern Ontario (IRNPQEO).

2. The Ottawa Hospital Research Ethics Board

(Protocol # 2011217-01H)

Note: The approval for the Ethics Renewal was given to the Late Dr. Andrée Gruslin as the Principal Investigator. Dr. Shannon Bainbridge, my supervisor, was a Co-Investigator listed on this study, under which the placentae were collected.

3. The University of Ottawa - Health Sciences and Science Research Ethics Board

(File #: H04-13-12)

4. Permission from Health Canada

Given to reproduce and use the figure entitled, “Relationship of DRI values to risk of nutrient inadequacy and risk of adverse health,” in this thesis.

REMARQUES DU COMITÉ D'ÉTHIQUE RÉUNION DU 11 AVRIL 2013

TITRE DU PROJET: L'effet de la prise de suppléments de multivitamine prénatale sur la réponse placentaire du stress

RESPONSABLES DU PROJET: FRASER WILLIAM, M.D., chercheur responsable. Chercheuses principales : Bénédicte Fontaine-Bisson et Shannon Bainbridge, Université d'Ottawa. Collaboratrice: Lise Dubois

NO DE PROTOCOLE (notre référence): # 3679

LE PROJET EST ACCEPTÉ.

Comité administratif

Le projet a été évalué par le comité administratif et les commentaires ont été envoyés au chercheur.

Comité scientifique

Le projet est accepté.

Protocole

Le protocole est accepté.

Formulaire d'information et de consentement

Le consentement donné par les participantes pour la Banque de données et de prélèvements de l'Étude 3D permet la réutilisation des données pour la présente étude.

Formulaire de consentement de l'étude 3D (projet #2899) est expiré depuis le 23 mars 2013. SVP, veuillez renouveler ce projet.

Afin de faciliter la vérification des changements demandés par le CÉR, nous vous demandons de bien vouloir répondre point par point aux remarques du Comité en indiquant le numéro de la remarque.

De plus, concernant les changements devant être apportés au(x) formulaire(s) de consentement (si applicable), s'il vous plait nous fournir une copie où sont surlignés vos changements ainsi qu'une copie originale pour l'estampillage



Ottawa Hospital Research Ethics Boards / Conseils d'éthique en recherches

725 Parkdale Avenue, Box 411, Ottawa, Ontario K1Y 4E9 613-795-5555 ext. 14902 Fax: 613-761-4311
<http://www.ohri.ca/ohreb>

Wednesday, February 06, 2013

Dr. Andree Gruslin
Ottawa Hospital - General Campus
Department of Obstetrics/Gynecology/Newborn Care
501 Smyth Road
Ottawa, ON K1H 8L6

Dear Dr. Gruslin:

RE: Protocol# - 2011217-01H Improving Placental Health Through Education and Better Prevention
Renewal Expiry Date - Wednesday, February 05, 2014

I am pleased to inform you that your Annual Renewal Request (listed above) was reviewed by the Ottawa Hospital Research Ethics Board (OHREB) and is approved. No changes, amendments or addenda may be made in the protocol or the consent form without the OHREB's review and approval.

The Protocol Amendment Report dated February 1, 2013 extending the study end date to March 2014 is approved.

Dr. Felipe Moretti and Dr. Griffith Jones have been removed as co-investigators.

Renewal is valid for a period of one year. The validation date should be indicated on the bottom of all consent forms and information sheets (see attached copy). Approximately one month prior to that time, a single renewal form should be sent to the OHREB office.

The Tri-Council Policy Statement requires a greater involvement of the OHREB in studies over the course of their execution. As well, you must inform the Board of adverse events encountered during the study, here or elsewhere, or of significant new information which becomes available after the Board review, either of which may impinge on the ethics of continuing the study. The OHREB will review the new information to determine if the protocol should be modified, discontinued, or should continue as originally approved.

Yours sincerely,

Raphael Saginur

Raphael Saginur, M.D.
Chairman
Ottawa Hospital Research Ethics Board

/kd



Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

Ethics Approval Notice

Health Sciences and Science REB

Principal Investigator / Supervisor / Co-investigator(s) / Student(s)

<u>First Name</u>	<u>Last Name</u>	<u>Affiliation</u>	<u>Role</u>
Shannon	Bainbridge	Health Sciences / Others	Supervisor
Benedicte	Fontaine-Bisson	Health Sciences / Others	Co-Supervisor
Tasfia	Ahmed	Health Sciences / Others	Student Researcher

File Number: H04-13-12

Type of Project: Master's Thesis

Title: Effect of prenatal multivitamin supplementation on placental stress response

Approval Date (mm/dd/yyyy)	Expiry Date (mm/dd/yyyy)	Approval Type
06/10/2013	06/09/2014	Ia

(Ia: Approval, Ib: Approval for initial stage only)

Special Conditions / Comments:

N/A



Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

This is to confirm that the University of Ottawa Research Ethics Board identified above, which operates in accordance with the Tri-Council Policy Statement and other applicable laws and regulations in Ontario, has examined and approved the application for ethical approval for the above named research project as of the Ethics Approval Date indicated for the period above and subject to the conditions listed the section above entitled "Special Conditions / Comments".

During the course of the study the protocol may not be modified without prior written approval from the REB except when necessary to remove subjects from immediate endangerment or when the modification(s) pertain to only administrative or logistical components of the study (e.g. change of telephone number). Investigators must also promptly alert the REB of any changes which increase the risk to participant(s), any changes which considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project and safety of the participant(s). Modifications to the project, information/consent documentation, and/or recruitment documentation, should be submitted to this office for approval using the "Modification to research project" form available at: <http://www.research.uottawa.ca/ethics/forms.html>.

Please submit an annual status report to the Protocol Officer four weeks before the above-referenced expiry date to either close the file or request a renewal of ethics approval. This document can be found at: <http://www.research.uottawa.ca/ethics/forms.html>.

If you have any questions, please do not hesitate to contact the Ethics Office at extension 5387 or by e-mail at: ethics@uOttawa.ca.

Signature:

Riana Marcotte

Riana Marcotte
Protocol Officer for Ethics in Research
For Daniel Lagarec, Chair of the Sciences and Health Sciences REB

August 13th, 2014

Ref: HC2014-0435

Bénédicte Fontaine-Bisson, DtP/RD, PhD
Assistant Professor
Honours Bachelor of Nutrition Sciences
University of Ottawa
25 University
Ottawa, ON K1N 6N1

Using the Dietary Reference Intakes (DRIs)

Dear Dr. Fontaine-Bisson,

On behalf of Health Canada, I am pleased to grant to the **University of Ottawa/Bénédicte Fontaine-Bisson**, permission reproduce and use Health Canada's figure entitled: **Relationship of DRI values to risk of nutrient inadequacy and risk of adverse health effects** from Health Canada's web contents entitled: *Using the Dietary Reference Intakes (DRIs)*, for both its educational and non-commercial purposes.

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Should you have any questions, or if I can be of further assistance, please do not hesitate to contact me.

Yours sincerely,

Louise Sicard

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cc: John Holt, Corporate Communications, CPAD
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Appendix E: Research Ethics Board (REB) Approvals

Canada 

Appendix F: Scholastic Achievements

This thesis work led to the following scholastic recognitions:

F.1 Prizes and Awards

- 2014 *Conference Travel Grant*, Faculty of Graduate & Postdoctoral Studies
- 2014 *Conference Travel Grant*, REDIH
- 2013-14 *Ontario Graduate Scholarship (OGS)*
- 2013-14 *Reproduction, Early Development and the Impact on Health (REDIH) Scholarship*
Canadian Institute for Health Research (CIHR) - (declined in favor of OGS)
- 2013-14 *University of Ottawa Excellence Scholarship*
- 2013 *Graduate Student Travel Grant*, Faculty of Health Sciences
- 2013 *Research Travel Grant*, Faculty of Graduate & Postdoctoral Studies
- 2013 *Conference Fund Travel Grant*, Canadian Union of Public Employees
- 2013 *Academic Project Fund Conference Grant*, Graduate Students Association
- 2012-14 *University of Ottawa Graduate Admission Scholarship*

F.2 Abstracts & Refereed Conference Proceedings

- 2014 **Ahmed, T.;** Sirois, M.; Bainbridge, S.; Dubois, L.; Fontaine-Bisson, B. (2014) "Food sources do not provide pregnant Québec women with sufficient micronutrients as recommended by the Dietary Reference Intakes (DRIs)." *Applied Physiology Nutrition and Metabolism*. 39 (5): 613. Poster presentation at the Canadian Nutrition Society (CNS) Annual Meeting, St. John's, NL. (June 5-7).
- 2013 **Ahmed, T.;** Roberts-MacDonald, M.; Fontaine-Bisson, B.; Bainbridge, S. (2013). "Effects of niacin supplementation on placental stress response," *Placenta*. 34 (9): A36-A37. Poster presentation at the 2013 International Federation of Placenta Association (IFPA) Meeting, Whistler, BC, Canada. (September 11-15).

- 2013 **Ahmed, T.;** Fellus, I.; Fontaine-Bisson, B.; Bainbridge, S. (2013). "Effects of folic acid supplementation on placental health and function," *Placenta*. 34 (9): A82. Poster presentation at the 2013 International Federation of Placenta Association (IFPA) Meeting, Whistler, BC, Canada. (September 11-15).

F.3 Academic Conference Attendance

- 2014 Canadian Nutrition Society (CNS) Annual Meeting, **St. John's**, Newfoundland, Canada; June 5-7, 2014.
- 2014 CIHR Training Program in Reproduction, Early Development and the Impact on Health (REDIH) Semi-Annual Meeting, **Montreal**, Québec, Canada; June 4-5, 2014
- 2014 17th Annual Interdisciplinary Conference, University of Ottawa, **Ottawa**, Canada; March 6, 2014.
- 2013 CIHR Training Program in Reproduction, Early Development, and the Impact on Health (REDIH) Semi-Annual Meeting, **Ottawa**, ON, Canada; December 2-3, 2013.
- 2013 13th Annual Ottawa Hospital Research Institute (OHRI) Research Day, University of Ottawa, **Ottawa**, Canada; November 14, 2013.
- 2013 International Federation of Placenta Association (IFPA) Meeting, **Whistler**, British Columbia, Canada; September 11-14, 2013.
- 2013 8th Annual Human Placenta Workshop, Queens University, **Kingston**, ON, Canada; July 14-20, 2013.
- 2013 16th Annual Interdisciplinary Conference, University of Ottawa, **Ottawa**, Canada; March 22, 2013.
- 2012 36th Eastern Canadian Perinatal Investigators Meeting and Symposium; **Toronto**, ON, Canada; November 14-16, 2012.

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